Original Paper

Interleukin-1 β Promotes Epithelial-Derived Alveolar Elastogenesis via α v β 6 Integrin-Dependent TGF- β Activation

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Key Words

Bronchopulmonary dysplasia • IL-1 β • Elastin • EMT α v β 6

Abstract

Background/Aims: IL-1 β creates persistent pulmonary inflammation accompanied by elevated transforming growth factor β (TGF- β) levels and is associated with abnormal elastogenesis, which is observed in bronchopulmonary dysplasia (BPD). Although progress has been made in this field, the mechanisms underlying this process remain only partially understood. **Methods:** We assessed aberrant elastin localization-associated signaling in mouse pups exposed to 85% O₂ treated with either IL-1Ra or 1D11, using morphometric analyses, quantitative RT-PCR, immunostaining, and ELISA. We also evaluated the derivation of elastin-producing cells using dual marker tracking. The regulatory mechanisms of IL-1 β were investigated in vitro in lung epithelial and mesenchymal cells. **Results:** Elevated levels of IL-1 β , α v β 6 and TGF- β 1 were each associated with aberrant elastin production in O₂-exposed lungs. IL-1Ra abolished TGF- β 1 activation and α v β 6 upregulation, which occurred as a result of exposure to hyperoxia, whereas 1D11 had no discernible effect on the expression of either α v β 6 or IL-1 β even following O₂-exposure, suggesting that IL-1 β was initially induced. Additionally, double staining revealed the presence of epithelium-derived elastin-producing cells, which was confirmed via *in vitro* IL-1 β stress-induced epithelial-mesenchymal transformation (EMT) morphological and molecular marker changes, which may explain the altered lung elastin deposition and defective septation observed in BPD. **Conclusions:** These data support the hypothesis that IL-1 β was initially induced by hyperoxia; α v β 6 subsequently interacted with and activated TGF- β 1, acting as an epithelial/mesenchymal signaling molecule that contributed to excessive alveolar elastogenesis, the primary pathological feature of BPD.

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Introduction

Bronchopulmonary dysplasia (BPD) is a chronic lung disease that most often occurs in premature neonates suffering from incomplete lung development and hyperoxia-induced toxicity [1, 2], as well as trauma-induced tissue damage [3, 4]. BPD is characterized by disruptions in alveologenesis, disordered elastin expression and the absence of alveolar septations during the terminal stage of lung development [5]. Pre- and postnatal inflammatory conditions such as maternal chorioamnionitis, oxygen exposure and mechanical ventilation, as well as subsequent abnormal epithelial development, contribute to the development of BPD [6-10]; however, the mechanisms by which inflammation disrupts alveologenesis are poorly understood.

Among the mediators of pulmonary inflammation, interleukin-1 β (IL-1 β) is a critical cytokine associated with both the initiation and the persistence of inflammation [11-13]. The conditional expression of IL-1 β causes severe pulmonary inflammation, alveolar hypoplasia, and progressive lung fibrosis, whereas the *in vivo* blockage or depletion of IL-1 β alleviates inflammation and fibrosis and ultimately prevents murine bronchopulmonary dysplasia [14, 15]. Regarding the direct effects of IL-1 β on the regulation of extracellular matrix (ECM) expression and composition, conflicting data exist regarding the contributions of IL-1 β to the development of myofibroblasts, which are critical for alveolar formation [16-18]. Additionally, IL-1 β induces myofibroblast formation and elastogenesis via both an epithelial-mesenchymal transition (EMT) [19] and an endothelial-mesenchymal transition (EndoMT) [20, 21].

The α v β 6 integrin is an epithelially restricted integrin and is also recognized as an activator of transforming growth factor (TGF)- β 1, which has been implicated in abnormal lung development and fibrosis in newborn mice [22, 23]. Once liberated, active TGF- β binds to both type II and type I TGF- β receptors to activate the intracellular Smad2/3 and the TAK1-p38 MAPK pathways [23]. In normal lung epithelial cells, α v β 6 integrin expression is low but increases significantly when alveolar epithelial cells are injured [24]. The neutralization of the α v β 6 integrin reportedly attenuates the lung injury induced by IL-1 β [25]. Additionally, TGF- β activity is increased in the lungs of premature infants with BPD [26, 27]. During the later phases of pulmonary development, excessive IL-1 β / α v β 6/TGF- β signaling impacts both alveologenesis and alveolar elastogenesis, which eventually results in lung disease. However, the exact mechanisms by which IL-1 β causes the perturbation of alveolar elastogenesis are unknown.

We have determined that IL-1 β induces TGF- β activation via α v β 6 integrin in alveolar type II (ATII) epithelial cells, which subsequently develop mesenchymal features and produce elastin. These epithelial-derived mesenchymal cells may contribute to the development of BPD. Ultimately, determining the exact mechanisms underlying the roles played by these dysregulated pathways in the pathogenesis of BPD will provide clinicians with novel targets and markers for both disease monitoring and therapy.

Materials and Methods

BPD model

All animal studies were performed in accordance with the criteria approved by the Chongqing Medical University Animal Use Committee. Within 24 hours of delivery, full term pups (weighing 1.32 $\pm$ 0.10 g) were randomly divided into paired chambers (containing either air or 85% O₂) for scheduled exposure periods (1, 3, 7, 14, 21, or 28 d). The nursing dams were rotated daily between the groups to avoid the influence of maternal mouse oxygen toxicity on the pups' nursing [2, 28]. In the first experimental setting, the pups were pooled and randomly divided into groups receiving daily subcutaneous injections of either IL-1Ra (10 mg/kg, R&D Systems, Minneapolis, MN) or volume-matched vehicle. In the second experimental setting, pregnant C57BL/6J dams (embryonic days 17 and 19) were intraperitoneally injected with either 1D11 (10 mg/kg, R&D Systems) or MOPC21 (Sigma, St. Louis, MO), an isotype-matched IgG. Within 24 hours

of delivery, the pups were allocated to either air or 60% O₂. The subsequent processing of the lung tissue samples for histology, quantitative real-time PCR (qPCR), and Western blotting, as well as immunohistology, has been described previously [2, 29].

Cell lines and culture techniques

The mouse lung epithelial cell line, MLE-12, and the rat fetal lung fibroblast cell line, RFL-6, were each obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The MLE-12 and RFL-6 cells were maintained at 37 °C in Dulbecco's modified Eagle's medium with 2 mM glutamine supplemented with 10% fetal bovine serum, 100 units/ml of penicillin G and 100 units/ml of streptomycin sulfate. Mouse alveolar type II epithelial cells obtained from 3-week-old C57BL/6 mice were isolated as described previously [14], with minor modifications. The alveolar type II (ATII) epithelial cells represented the nonadherent population and were recovered for culture and mRNA extraction. The cultures were selectively stressed with recombinant mouse IL-1 β (10 ng/ml) (401-ML-025, R&D Systems) and α v β 6 siRNA for the indicated time periods as reported previously [30].

RNA interference

The α v β 6 knockout was achieved in the MLE-12 cells using siRNA (25 nM) for α v β 6, which was provided by Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The optimized sequences of the α v β 6 siRNA have been described previously (synthesized by GenePharma) [31]. The same siRNA reagents were added to the medium at 24 hours post-transfection for 24 hours. The gene-silencing effects were evaluated via Western blotting following 48 h of transfection. The appropriate controls were included during the entire siRNA knockout process, confirming the specificity of the siRNA.

Quantitative Real-time PCR

Freshly dissected lungs and cultured cells were subjected to RNA extraction at scheduled time points using TRIzol® (Invitrogen, Carlsbad, CA). Quantitative real-time PCR, using proprietary primers and probes (Taqman Gene Expression Assays; Applied Biosystems, Foster, CA), was utilized to measure mRNA expression with primers as demonstrated in Table 1 (synthesized by GenePharma, Inc. Shanghai, China); 18S ribosomal RNA was used as an internal control. The relative quantification method [2, 28, 29] was used to determine the Ct values for the PCR products of the target genes and β -actin. The relative quantification formula was as follows: $2^{-\Delta Ct} \times 100\%$, where $\Delta Ct = Ct_{(\text{target gene})} - Ct_{(\beta\text{-actin})}$.

Transmission electron microscopy

The protocol was performed using previously published data [2]. Briefly, the lungs were fixed in situ with 2.5% glutaraldehyde and sequentially stained with 1% osmium tetroxide and 1% tannic acid prior to dehydration and Epon embedding. Thin sections (60 nm) were counterstained with 7% methanolic uranyl acetate, followed by lead citrate. The images were visualized and captured using a Hitachi 7500 transmission electron microscope (Hitachi, Ltd., Japan).

Table 1. The primer sequences used for the qRT-PCR analysis

Genes	Forward	Reverse
β 6 integrin	TAG CTT CCA GCC AAG GTG GG	TCT GAG GGA CTG GTA TGT GTG TCC
Lox	GCACTGCACACACAGGGA	TTAGTGTAGTCTGATTACAGG
IL-1 β	TGGTGTGTGACGTTCCCAT	CAGCAGGAGGCTTTTTTGTG
TGF- β 1	GATCCTGTCCAACTAAGGCTC	ACCTCTTTAGCATAGTAGTCCGC
E-cadherin	ATG TTC ACT GTC AAT AGG GAC ACT	TCA GTC ACC TTG AGT GTG GCA
vimentin	CCAACCTTTTCTCCCTGAA	TTGAGTGGGTGTCAACCAGA
α SMA	ATTGTGCTGGACTCTGGAGATGGT	TGATGTCACGGACAATCTCACGCT
β -actin	CATCCTCTTCTCCCTGGAGAAGA	ACAGGATTCCATACCCAAGAAGGAAGG

Table 2. The paired antibodies for dual confocal immunofluorescence

paired	Primary	Host	Catalog	Manufacturer	Secondary antibodies (Invitrogen)
1	p-Smad2/3	Goat	sc-11769	Santa Cruz	Donkey anti-Goat AlexaFluor 488
	proSP-C	Rabbit	ab40879	Abcam	Donkey anti-rabbit AlexaFluor 594
2	α v β 6	Mouse	mab2074z	Millipore	Donkey anti-mouse AlexaFluor 488
	elastin	Rabbit	ab21610	Abcam	Donkey anti-rabbit AlexaFluor 594
3	proSP-C	Rabbit	ab40879	Abcam	Chicken anti-rabbit AlexaFluor 488
	elastin	Mouse	ab9519	Abcam	Goat anti-mouse AlexaFluor 594

Dual confocal immunofluorescence

Paraffin-embedded sections were fixed, permeabilized and incubated with dual primary antibodies overnight at 4°C, followed by several washes with phosphate-buffered saline (PBS), followed by incubation for 1 h with secondary antibodies coupled to either Alexa Fluor 488 or Alexa Fluor 594 probes (Invitrogen) counterstained with DAPI (Sigma) and mounted with glycerol. The primary antibodies used here are listed in Table 2. The staining was visualized using a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). Each of the digital images was processed and merged using Photoshop software, version 6.0 (Adobe Systems Inc., San Jose, CA).

An analysis of whole-lung and cultured cells via flow cytometry

Lung single cell suspensions were prepared at predetermined time points following oxygen exposure as previously described [2]. Following their initial isolation, the cells were incubated for 30 min on ice with the following primary antibodies: proSP-C (ab40879, Abcam, Cambridge, MA) and elastin primary antibody (ab9519, Abcam). The resuspended cells were incubated for 20 min on ice with the appropriate FITC- and phycoerythrin-conjugated secondary antibodies (Biovol, Shanghai, China). In separate experiments, the cultured MLE-12 cells were washed and suspended to stain with α v β 6 integrin (MAB2074Z, Merck Millipore) and the appropriate FITC- conjugated secondary antibodies (Biovol, Shanghai, China). Cell sorting was performed using a FACScan flow cytometer (R&D Systems) using Cellquest 3.2.1f1 software. Dead cells and erythrocytes were excluded from the analysis by excluding either the cell fragments (low forward scatter) or the events characterized by high PI fluorescence. The cells within this live cell gate were displayed on a Hoechst-Red- Hoechst-Blue histogram, and 200 side population (SP) cells per recipient were sorted for transplantation studies.

The analysis of the raw data was completed using FlowJo software (Treestar, Ashland, OR).

Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunofluorescence analyses were performed as described previously [2, 28]. Briefly, the slides were incubated for 2 h at room temperature with the following primary antibodies: IL-1 β (sc-7884, Santa Cruz Biotechnology, Santa Cruz, CA), α v β 6 integrin (MAB2074Z, Merck Millipore, Billerica, MA), TGF- β 1 (sc-146, Santa Cruz), and α SMA (clone 1A4, Sigma). In separate experiments, the cultured MLE-12 cells were washed and suspended to stain with unconjugated pSmad2/3 (sc-11769, Santa Cruz). For immunohistochemical detection, the slides were viewed using a DAB Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA). The sections were lightly counterstained with hematoxylin before mounting. For the immunofluorescence analysis, the staining was followed by a second incubation for 1 h with the appropriate secondary antibodies, which were labeled with AlexaFluor 594 (Invitrogen), counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) for 20 sec and mounted with glycerol. The slides were analyzed using a Nikon 551 microscope with a DS-Fi1c camera and NIS-Elements F software. All digital images were processed and merged using Photoshop 6.0 (Adobe Systems Inc., San Jose, CA).

ELISA for IL-1 β and TGF- β 1

At 14 and 28 days following oxygen exposure, the lung homogenates of the mouse pups were prepared as described previously [2, 31]. Specimens corresponding to 60 μ g of protein were used for the IL-1 β measurements, using an IL-1 β Quantikine ELISA kit (R&D Systems) according to the manufacturer's protocol. In a separate analysis, the MLE-12 cells were incubated with IL-1 β for 1, 4 and 7 days. The serum-free culture media were collected and neutralized for the TGF- β 1 concentration measurements using a commercially available enzyme-linked ELISA kit (R&D Systems) according to the manufacturer's protocol [33].

Western Blotting

The snap-frozen tissues and cultured cells were treated with lysis buffer containing a protease inhibitor (Roche). Equivalent amounts of protein from each sample (30 mg per lane) were separated on SDS-PAGE gels (Invitrogen) and blotted electrophoretically onto a polyvinylidene fluoride membrane [28]. The membranes were incubated overnight at 4°C with specific diluted primary antibodies, including pSmad3 (9520, Cell Signaling Technology, Boston, MA), Smad2/3 (#3102, Cell Signaling), E-cadherin (sc-8426, Santa Cruz), vimentin (sc-32322, Santa Cruz), Tropoelastin (ab21600, Abcam), α SMA (clone 1A4, Sigma), β -Actin (sc-47778, Santa Cruz) and lamin B (sc-6216) (Santa Cruz). Following washing, the membranes were incubated with the corresponding secondary antibodies conjugated with horseradish peroxidase (Biovol) for 1 h at room temperature. The relative intensities of the bands were evaluated using Kodak 1D software, version 3.5.4 (Kodak Scientific Imaging System, Rockville, MD).

Statistical analysis

The experimental values are presented as the means $\pm$ SEMs. A one-way analysis of variance multiple comparison tests was used to identify differences in both mRNA expression and protein levels, as well as quantitative histological measurements, whereas Student's unpaired t-test was used to determine whether significant differences existed between the two groups. The statistical analyses were performed using the Prism software package, version 4 (GraphPad Software Inc., San Diego, CA). $P < 0.05$ was considered statistically significant.

Results

The gene expression and localization profiles of IL-1 β /TGF- β 1 signaling in the hyperoxia-treated mouse pup lungs

We determined the mRNA expression of the potential TGF- β 1 signaling related genes using quantitative real-time PCR in the hyperoxia-treated mouse pup lungs. We observed that the expression patterns of IL-1 β , $\alpha\beta$ 6 integrin, and TGF- β 1 were strongly impacted by exposure to 85% O₂. Compared with the age-matched pups, IL-1 β mRNA expression

Fig. 1. Ontogenies expression of IL-1 β , TGF- β 1 and $\alpha\beta$ 6 integrin, during lung development and hyperoxia in mouse pup lungs. Comparison of the mRNA expression of IL-1 β (A), $\alpha\beta$ 6 integrin (B), TGF- β 1(C), all expressed relative to the housekeeping gene β -actin mRNA, in mouse pup lungs obtained from air- and 85% O₂-exposed pups at indicated time causes. Columns: average values of a minimum of three independent experiments; bars: $\pm$ SEM. * $p < 0.01$ compared with the corresponding

air-exposed control (one-way ANOVA). D. Comparison of IL-1 β concentrations in the lungs of air- or O₂-exposed at P14. Total lung homogenate active IL-1 β levels were measured by ELISA. Columns: average values of a minimum of three independent experiments; bars: $\pm$ SEM. * $p < 0.01$ compared with the corresponding air-exposed control (one-way ANOVA).

Fig. 2. Assessment position or amount of IL-1 β , TGF- β 1 and α v β 6 integrin in the oxygen-injured mouse pup lungs. Immunostaining for IL-1 β (A), α v β 6 integrin (B) and TGF- β 1 (C) in paraformaldehyde-fixed lung sections (Bar = 50 μ m) revealed increased distal air space staining (Arrows), which were indicative of greater deposition, in the septa of 14-day-old mouse pup lungs exposed to 85% O₂, compared with control pups. n=6 per group. One representative slide per group is depicted. D. Dual immunofluorescence was performed for p-Smad2/3 (red) and proSP-C (green) staining, with cell nuclei indicated (blue), and the merged images under fluorescence microscopy. Typical fluorescence microscopic images indicated that co-localization of the cell markers was demonstrated on the merged image, illustrating prominently augmented linking of elastin with α v β 6 integrin in the 14-day-old mouse pup lungs exposed to 85% O₂ (White arrows), which may account for the disordered elastin accumulation. n=6 per group. One representative slide per group is depicted. Bar = 50 μ m.

progressively increased from postnatal day 7 (P7) until P28 in the 85% O₂-exposed pups (Fig. 1A, Fig. 1B). The mRNA levels of TGF- β 1 (Fig. 1A, B) and α v β 6 (Fig. 1C) were comparable in the lungs of mouse pups between the pups exposed to air and 85% O₂ before P14 but subsequently increased and exhibited 1- to 2-fold elevations among the pups exposed to 85% O₂ (one-way ANOVA). Over time, this change gradually decreased following P28 (Fig. 1A, 1B and 1C). Compared with the air-exposed littermates, persistently elevated IL-1 β protein levels were evident in 85% O₂-exposed pups, in which a maximal effect was noted at P14 following 85% O₂ exposure (Fig. 1D).

To understand the functional relationship between IL-1 β and TGF- β 1, we investigated the cellular localization of IL-1 β , integrin α v β 6, and TGF- β 1 using immunostaining. IL-1 β staining was localized to the developing septae and the blood vessel walls of the lungs, as well as the inflammatory infiltrate regions (Fig. 2A). Compared with the air-exposed mouse pups, there appeared to be more intense staining for IL-1 β in the 85% O₂-exposed pup lungs (Fig. 2A). Abundant α v β 6 protein was observed within the lining of the alveolar ducts and the alveoli, as well as the airway epithelium in the lungs exposed to hyperoxia, whereas the large airways and intraalveolar macrophages exhibited only limited amounts of α v β 6 (Fig. 2B), and undetectable immunostaining levels of α v β 6 protein were observed in the control mouse pups on P14 (Fig. 2B). Likewise, the sections of the lungs exposed to 85% O₂

Fig. 3. Improved alveolar morphogenesis and elastin deposition in the injured mouse pup lungs treated with IL-1Ra. A. H&E staining for morphometric analyses of the 14-day-old mouse pup lungs treated as indicated; n=3 per group. One representative slide per group is shown. Bar = 50 μ m. B. Scans of whole lungs were analyzed for the secondary crest/field (left panel), the radial alveolar counts (RAC) from the 14-day-old mouse pup lungs treated as indicated; n = 3 per group. Columns: average values of a minimum of three independent experiments; bars: $\pm$ SEM; *P < 0.05, #P < 0.05, one-way ANOVA. C. morphological comparison of elastin deposition using Gomori staining of the 14-day-old mouse pup lungs treated as indicated. Elastin foci are indicated at the distal respiratory unit walls (Asterisks) and the tips of the septa (Arrows). n = 3 per group. One representative slide per group is shown. Bar = 50 μ m. D. Quantitative image analysis revealed that elastin density, expressed as a percentage of the lung parenchyma, was significantly decreased in IL-1Ra treated pups compared with the corresponding control. Columns: average values of a minimum of three independent experiments; bars: $\pm$ SEM. n = 3, *P < 0.05, #P < 0.05, one-way ANOVA.

exhibited intense TGF- β 1 staining distributed throughout the walls of the distal air spaces, the parenchyma and the ATII and ATI pneumocytes (Fig. 2C), which may have influenced alveolar development.

We subsequently determined whether the activity of TGF- β was restricted to the epithelial cells using dual immunostaining for both p-Smad2/3 and proSP-C, a marker of ATII. The merged images exhibited a certain degree of co-localization of both p-Smad2/3 and proSP-C in the neonatal lungs; O₂ exposure promoted this co-localization, although most of the cells were stained with a single color (Fig. 2D). These findings confirm that the spatial distribution of the ATII cells was consistent with the regions of TGF- β activity in the O₂-exposed lungs.

IL-1Ra attenuated hyperoxia-induced injury via the downregulation of α v β 6 and TGF- β 1 activity

To determine if IL-1 β plays a role in hyperoxia-induced lung injury as indicated previously, we facilitated IL-1 β blockage using IL-1Ra (an IL-1 receptor antagonist that blocks both IL-1 α and IL-1 β) [31] and evaluated the histological appearances of the

Fig. 4. Improved α SMA amount and elastin ultrastructure position in the hyperoxia injured mouse pup lungs treated with IL-1Ra. A: Immunofluorescent staining of α SMA in the 14-day-old mouse pup lungs treated as indicated. In contrast with air-exposed lungs, 85% O_2 exposure increased α SMA staining. Treatment with IL-1Ra was associated with an improvement to nearly normal of the staining pattern of α SMA in the O_2 -injured developing lungs. Bar=50 μ m. n = 3 per group. One representative slide per group is shown. B: Ultrastructure of alveolar tips on transmission electron microscopy in 14-day-old mouse pup lungs treated as indicated. Note that elastin was enmeshed within microfibril bundles, whereas in the O_2 -exposed lungs, small elastin globules (indicated by white arrows) formed large aggregates that were localized next to the microfibrils (Asterisks) and IL-1Ra restored the elastin structure in the O_2 -exposed lungs to nearly normal. Arrows point to elastin and * represent microfibrils. Bar = 1 μ m.

lungs treated with O_2 . As expected, compared with the vehicle control samples, improved alveolar numbers and secondary crest numbers (Fig. 3A, 3B), as well as increased elastin localization (Fig. 3C, 3D), were evident in the lungs of the mouse pups treated with IL-1Ra. We subsequently determined whether the alveolar myofibroblasts, which produce elastic fibers, were influenced by IL-1Ra by staining for the marker of "myo" cells, alpha-smooth muscle actin (α SMA). It was apparent that the myofibroblasts that stained positive for α SMA appeared to be less intense with respect to IL-1Ra (Fig. 4A). Using electron microscopy, we observed that O_2 induced both fragmentation and disorganization into the ultrastructure of the elastic matrices, whereas the lungs treated with IL-1Ra exhibited organized and thick fibrous structures, even following O_2 exposure (Fig. 4B).

To determine whether IL-1Ra induces $\alpha\beta 6$ and TGF- $\beta 1$ activity, which have been implicated in elastogenesis, the lung homogenate transcript levels of TGF- $\beta 1$ and $\alpha\beta 6$ integrin were measured via RT-PCR in the IL-1Ra treated mice. Compared with the control group, the transcript levels of $\alpha\beta 6$ were unchanged in the P14 mice with respect to IL-1Ra (Fig. 5A); the increased mRNA expression observed in the lungs of the O_2 -treated pups on P28 was inhibited with IL-1Ra. The transcriptional pattern of TGF- $\beta 1$ was coincident with the transcriptional regulation of $\alpha\beta 6$ by IL-1Ra (Fig. 5A). Consistent with the mRNA expression data, both the abundance and the localization of TGF- $\beta 1$ induced by O_2 exposure were prevented following 28 days of IL-1Ra treatment (Fig. 5B). Using a sandwich ELISA

Fig. 5. IL-1Ra inhibited TGF- β 1 activation *in vivo*. A. Comparison of the mRNA expression of TGF- β 1, α v β 6 integrin, all expressed relative to the housekeeping gene β -actin mRNA, in 14, or 28 day-old pup lungs treated as indicated. Columns: average values of a minimum of three independent experiments; bars: $\pm$ SEM. $n=3$ per group. * $P < 0.05$, # $P < 0.05$, one-way ANOVA. B. Immunohistochemistry staining of TGF- β 1 in 14 day-old pup lungs treated as indicated. TGF- β 1 staining was scant in the air exposure lung but considerably more prominent (Arrows) in the cytoplasm and extracellular of alveolar septa along the walls of the distal air spaces in O₂ exposed mouse pup lungs. Further, IL-1Ra treatment decreased the TGF- β 1 reactivity in O₂-exposed lungs almost close to pattern of normal control. Bar = 50 μ m. $n = 3$ per group. One representative slide per group is shown. C. Comparison of active TGF- β 1 concentrations in 14 day-old pup lungs treated as indicated. Total lung homogenate active TGF- β 1 levels were measured by ELISA. Columns: average values of a minimum of three independent experiments; bars: $\pm$ SEM. * $p < 0.01$ compared with the corresponding air-exposed control (Student's unpaired t-test).

assay, the global levels of TGF- β activation in the lung homogenates subjected to O₂ exposure decreased to normal levels secondary to IL-1Ra treatment (Fig. 5C).

The evidence of the epithelial origins of elastin-producing cells in the hyperoxia-treated mouse pup lungs

Because the α v β 6 integrin is epithelially expressed, we subsequently studied the contribution of α v β 6-positive epithelial cells to elastin production. We observed that the cells positive for both α v β 6 and elastin were located in regions of matrix remodeling (Fig. 6A). Furthermore, the α v β 6 activity induced by O₂ was inhibited by IL-1Ra. Double immunostaining for proSP-C (green) and elastin (red) revealed that proSP-C-positive ATII expressed elastin (appears yellow in Fig. 6B). A subset of proSP-C-positive cells from the hyperoxia-treated lungs demonstrated a distinct elongated mesenchymal phenotype (arrows). These results confirmed that the ATII-derived cells directly contributed to the deposition of elastin in BPD. To quantitatively estimate the number of elastin-producing cells in the lungs, the phenotypes of these cells were evaluated via a fluorescence-activated cell sorter analysis (Fig. 6C). More

Fig. 6. Determination the phenotype of elastin producing cells. **A.** Immunofluorescent colocalization of $\alpha v \beta 6$ (red) and elastin (green), with cell nuclei indicated (blue) in the 14-day-old mouse pup lungs treated as indicated. The colocalization for both $\alpha v \beta 6$ and Elastin immunostaining in the alveolar epithelium (yellow, double positive cells, arrowheads) on the merged image were indicated. IL-Ra-treated O_2 -exposed lungs had fewer double positive staining than those exposed to O_2 . Bar = 50 μm . $n = 3$ per group. One representative slide per group is shown. **B.** Immunofluorescent colocalization of proSP-C (green) and elastin (red), with cell nuclei indicated (blue) in the 14-day-old mouse pup lungs treated as indicated. Few dual staining positive cells (yellow, double positive cells, arrowheads) were seen in the lungs of air or O_2 -exposed mice. Bar = 50 μm . $n = 3$ per group. One representative slide per group is shown. **C.** Single lung cell suspensions from 14-day-old mouse pup lungs treated as indicated were analyzed by flow cytometry for elastin and proSP-C. Up panel, representative FACS gating histogram used to exclude cell fragments and red blood cells. Low panel, representative of three independent experiments.

Table 3. The phenotypes of the elastin-producing cells of epithelial origin as percentages of the proSP-C+ Elastin-positive cells. The data represent the means $\pm$ SEs ($n=6$). * $P < 0.05$ vs the Air group, # $P < 0.05$ vs the O_2 group (one-way ANOVA)

Days	Air	O_2	O_2 +IL-1Ra
1	3.4 $\pm$ 1.1	2.5 $\pm$ 0.7	2.6 $\pm$ 0.9
7	3.1 $\pm$ 1.6	2.9 $\pm$ 0.6	2.8 $\pm$ 0.9
14	3.9 $\pm$ 0.9	8.3 $\pm$ 3.4*	5.2 $\pm$ 2.1#
21	4.8 $\pm$ 2.2	7.2 $\pm$ 2.1*	6.1 $\pm$ 2.2
28	6.1 $\pm$ 1.9	7.9 $\pm$ 2.3	4.9 $\pm$ 1.6

than 70% of the cells did not express proSP-C; however, among the proSP-C-positive cells, which represented 22.8% of the cell population analyzed, approximately 3-6% of the proSP-C-positive cells were elastin producing in the control pups, indicating that the cells were of epithelial origin (Fig. 6B). By contrast, 3-8 % of the cells were dual proSP-C and elastin positive in the hyperoxia-treated pups (Table 3). IL-1Ra slightly decreased the proportion of epithelium-derived elastin-producing cells, which supports the idea that some ATII cells undergo an EMT to promote elastin production in hyperoxia-treated lungs.

Fig. 7. 1D11 suppressed the induction of lox and α SMA by hyperoxia but no influence with IL-1 β and α v β 6. **A.** Comparison of the mRNA expression of TGF- β 1, α v β 6 integrin and lox, all expressed relative to the housekeeping gene β -actin mRNA, in 14 or 28 day-old pup lungs treated as indicated. Columns: average values of a minimum of six independent experiments; bars: $\pm$ SEM. * p < 0.01 compared with the corresponding 85% O₂-exposed control (one-way ANOVA). **B.** Immunohistochemistry was used to detect α v β 6 in 14-old pup lungs treated as indicated. Positive staining of alveolar epithelium and walls of distal air spaces were indicated (Arrows). Considerably more prominent staining in 85% O₂ exposed lungs exhibited compared with control. Treatment of 1D11 could not prevent the increased α v β 6 signaling caused by O₂ exposure. Bar = 50 μ m. n = 3 per group. One representative slide per group is shown. **C.** Immunofluorescent staining of α SMA in the 14-day-old mouse pup lungs treated as indicated. In contrast with control, 85% O₂ had increased α SMA staining. Treatment with 1D11 was associated with an improvement in the staining pattern of α SMA in the O₂-injured developing lung. Bar = 100 μ m. n = 3 per group. One representative slide per group is shown. **D.** Immunofluorescent colocalization of proSP-C (green) and elastin (red), with cell nuclei indicated (blue) in the 14-day-old mouse pup lungs treated as indicated. The colocalization in the alveolar epithelium (yellow, double positive cells, arrowheads) on the merged image were indicated. Bar = 50 μ m. n = 3 per group. One representative slide per group is shown.

TGF- β neutralization alleviated excessive elastin deposition and prevented the induction of TGF- β 1 activity but not IL-1 β activity

We subsequently measured both α v β 6 and IL-1 β expression during the neutralization of TGF- β signaling via 1D11, a TGF- β 1,2,3-neutralizing IgG1 antibody. As depicted in Fig. 7A, no impact of TGF- β neutralization on the mRNA levels of either α v β 6 or IL-1 β was noted, whereas treatment with 1D11 inhibited the 85% oxygen-induced pulmonary lox mRNA

Fig. 8. IL-1 β increases TGF- β 1 activation in rat AII cell monolayers via α v β 6 integrin dependent mechanisms in epithelial cells but not fibroblast. A. Western blot analyses with pSmad3 in total protein extracts from MLE-12 cells and RFL-6 cells treated with IL-1 β (10 ng/ml) for the indicated time periods. A representative example from minimum of three independent experiments is shown. B. MLE-12 cells, stimulated with IL-1 β (10 ng/ml) for 48 hours, were fractionated into cytoplasmic and nuclear fractions and subjected to Western blot with antibody against pSmad3 and Smad2/3. β -actin and Lamin B was used as

loading control for the cytoplasmic and nuclear fractions, respectively. C. Fluorescent immunocytochemistry of pSmad2/3 (Green, which also recognizes pSmad2/3) in MLE-12 cells stimulated with IL-1 β (10 ng/ml) for 48 hours. Nuclei were labeled with the DNA dye DAPI. Arrowheads denote nuclear translocation of pSmad2/3. Bar=50 μ m. n = 3 per group. One representative slide per group is shown. D. IL-1 β mediated induction of β 6 mRNA expression on MLE-12 cells and RFL-6 cells is determined using RT-PCR. The graph showed the expression of β 6 mRNA induced by IL-1 β treatment relative to the housekeeping gene β -actin mRNA. Columns: average values of a minimum of three independent experiments; bars: $\pm$ SEM. *p < 0.01 compared with the corresponding air-exposed control (Student's unpaired t-test). E. Evidence for α v β 6 integrin depletion. RT-PCR analysis of β 6 mRNA expression 96 h after siRNA transfection. Columns: average values of a minimum of three independent experiments; bars: $\pm$ SEM, *p < 0.01 versus scrambled siRNA (Up panel). Western blot analyses with α v β 6 in cell lysates after siRNA transfection for 96 hours. Data were replicated three times and a representative example is shown (Low panel). F. Western blot analyses with pSmad3 in total protein extracts from MLE-12 cells, expressing scrambled or α v β 6-specific shRNA, treated with IL-1 β or its vehicle for 48 hours. A representative example from minimum of three independent experiments is shown. β -actin was used as loading control. G. Western blot analyses with α SMA and tropoelastin in total protein extracts of MLE-12 cells, expressing scrambled or α v β 6-specific shRNA, treated with IL-1 β or its vehicle for the indicated time periods. Data were replicated three times and a representative example is shown.

Fig. 9. IL-1 β induced morphologic and marker proteins changes towards EMT in alveolar epithelial cells but not fibroblast. A. Morphologic changes of MLE-12 cells after 1, 4, and 7 days of stimulation by IL-1 β (10 ng/mL). Cellular protrusions and spreading are indicated (Arrows). The images are representatives of many pictures taken in triplicate experiments. B. Comparison with the mRNA expression of E-cadherin, vimentin and α SMA in α v β 6 integrin depleted MLE-12 cells treated with IL-1 β (10 ng/mL) for indicated time causes. Expression levels were calculated as mean level of induction in comparison to housekeeping gene β -actin. Columns: average values of a minimum of three independent experiments; bars: $\pm$ SEM. *p < 0.01 compared with the corresponding air-exposed control (one-way ANOVA). C. Western blot analyses with E-cadherin or vimentin in cell lysates from α v β 6 integrin depleted MLE-12 cells treated with IL-1 β (10 ng/mL) for 48 hours. Data were replicated three times and a representative example is shown. D. Assessment of supernatant TGF- β 1 in MLE-12 cells stimulated with IL-1 β (10 ng/mL) for the indicated time using ELISA. Columns: average values of a minimum of three independent experiments; bars: $\pm$ SEM. *p < 0.01 compared with the corresponding air-exposed control (one-way ANOVA).

expression, a key mediator of elastin synthesis, which suggests that elastin synthesis and assembly are mediated by the TGF- β pathway. Moreover, minimal α v β 6 staining was detected in the control mice, whereas markedly increased epithelial staining intensity was observed throughout the alveolar epithelium following 14 days of O₂ exposure. There appeared to be no changes in 1D11 compared with the control (nonimmune, MOPC21) pups exposed to 85% O₂ (Fig. 7B). These results suggest that TGF- β interrupts O₂-triggered signaling at the level of either IL-1 β or α v β 6.

The myofibroblast measurements confirmed that there was significant thickening of the septal tips (Fig. 7C) and increased α SMA staining in the mice exposed to O₂; these effects were attenuated by 1D11 (Fig. 7C). These data support previous observations suggesting that the neutralization of TGF- β signaling in a hyperoxia model of BPD partially restores

the normal structures of developing lungs [26, 32]. We subsequently performed double immunostaining for proSP-C (green) and elastin (red) and determined that the expression of both was downregulated by 1D11 (Fig. 7D).

IL-1 β 's effects on the TGF- β 1-Smad2/3 pathway were suppressed in the epithelial cell line but not in the fibroblasts

To determine the molecular basis of the regulation of myofibroblast differentiation and excessive elastogenesis, we performed *in vitro* IL-1 β stress testing using MLE-12 cells and RFL-6 cells and measured TGF- β mediated Smad2/3 signaling. As shown in Fig. 8A, IL-1 β (10 ng/ml) significantly increased Smad3 phosphorylation, a marker of TGF- β activation in epithelial cells (Fig. 8A). Furthermore, a nucleus fractionation assay confirmed that greater levels of pSmad3 translocation to the nucleus occurred in epithelial cells exposed to IL-1 β stress for 4 days (Fig. 8B), whereas pSmad3 nuclear translocation was scarce in the primary fibroblasts; this finding is demonstrative of a clear difference in sensitivity to IL-1 β between epithelial cells and fibroblasts and consistent with the data obtained via a confocal microscopy analysis, indicating that IL-1 β enhanced pSmad2/3 nuclear translocation (Fig. 8C) in epithelial cells.

To determine whether increased TGF- β activity was caused by higher levels of the α v β 6 integrin on the cell surface, we subsequently investigated the effects of IL-1 β on α v β 6 expression in both MLE-12 cells and RFL-6 cells. IL-1 β induced a higher level of β 6 mRNA expression in the MLE-12 cells, as determined via RT-PCR (Fig. 8D), whereas the RFL-6 cells did not express β 6 integrin either basally or in response to IL-1 β , illustrating that heterogeneity exists with respect to β 6 expression. We also observed that α v β 6 siRNA selectively reduces β 6 mRNA expression levels but not those of control siRNA (Fig. 8E); the Smad3 phosphorylation induced by IL-1 β was prevented by blocking the α v β 6 integrin in the MLE-12 cells (Fig. 8F), suggesting that the α v β 6 integrin may account for the different responses to IL-1 β observed in epithelial cells and fibroblasts. Furthermore, α v β 6 depletion with siRNA reduced both α SMA and elastin expression in the MLE-12 cells (Fig. 8G), demonstrating that IL-1 β activates TGF- β via an α v β 6-dependent mechanism in the epithelium.

Continuous stimulation by IL-1 β resulted in cell remodeling and an EMT in the epithelial cells

Given the above findings, we performed a detailed analysis of the ability of IL-1 β to induce an EMT using both the MLE-12 cells and the primary ATII cells isolated from the lung tissue specimens. Stimulation of the MLE-12 cell line with IL-1 β induced the development of spindle fibroblast-like morphological changes with reduced cell-cell contact consistent with an EMT (Fig. 9A), whereas the control cells retained their typical epithelial cell characteristics.

IL-1 β treatment of the MLE-12 cells was associated with reductions in both E-cadherin transcript levels and cell surface protein levels at 4 d (Fig. 9B), findings indicative of the loss of cell-to-cell contact. Simultaneously, IL-1 β stimulated significant increases in the expression of vimentin and α SMA expression, findings indicative of the acquisition of a mesenchymal phenotype by the cells (Fig. 9B). As expected, α v β 6 integrin depletion alleviated the induction of cell remodeling and the development of EMT-related characteristics facilitated by IL-1 β (Fig. 9B). Furthermore, IL-1 β plus TGF- β 1 stimulated significant increases in the expression of vimentin and reductions in the expression of E-cadherin (Fig. 9C), the key manifestations of EMT. The primary ATII cells also underwent an EMT in response to IL-1 β (data not shown).

We also determined the amount of TGF- β 1 secreted by the primary rat ATII cell monolayer in response to IL-1 β using ELISA. We observed that 10 ng/mL of IL-1 β promoted the significant secretion of TGF- β 1 in the supernatant for 24 hours (Fig. 9D). Taken together, these results indicate that TGF- β 1 was released by the ATII cells via an IL-1 β -dependent mechanism, which may either induce EMT in alveolar epithelial cells or convert fibroblasts into myofibroblasts via the paracellular pathway.

Discussion

IL-1 β is one of the most biologically active cytokines in the tracheal aspirates of infants with BPD and the amniotic fluid samples of patients who subsequently develop BPD [33-36]. In this study, we demonstrated that O₂ exposure in newborn mice caused increased IL-1 β signaling and disorganized elastin deposition and that IL-1Ra rescued this phenotype with improved pulmonary alveolar development in newborn mice. Furthermore, this study provided evidence regarding a possible causal relationship between excessive IL-1 β and TGF- β activation in ATII cells. We also confirmed that lung epithelial cells transition into a mesenchymal phenotype to produce elastin, a process promoted by IL-1 β .

The temporal and spatial expressions of IL-1 β during lung development have been described previously [6]. We defined functional IL-1 β performance in the context of damaged alveolar formation in the setting of BPD. Elevated IL-1 β levels were elicited by O₂, a finding suggestive of a relationship between IL-1 β and the pathogenesis of BPD. Consistent with this finding, the inhibition of IL-1 β attenuated the abnormal airspace enlargements caused by O₂ [11], suggesting that IL-1 β function is critical for alveogenesis during the neonatal period. In BPD, abnormal alveolar formation is caused by alveolar elastogenesis perturbations that result from disordered elastic fiber accumulation [5, 37, 38]. We detected excessive α SMA-expressing septal cells (myofibroblasts), which are responsible for normal alveolarization; therefore, the disorganized elastin deposition observed in the alveolar septae of the O₂ exposed lungs may be alleviated via IL-1 β blockage mediated by IL-1Ra. Additionally, IL-1Ra treatment restored the thickened alveolar septae in the O₂-exposed mice to a normal elastin structure. Regarding the performance of IL-1 β in promoting elastin expression, conflicting data regarding the inductive effects of IL-1 β exist depending on different cell type and specificity. One study demonstrated the absence of an effect by IL-1 β on elastin expression in lung fibroblasts [16, 39], whereas the findings of another study were suggestive of the elastogenic effects of IL-1 β in lung fibroblasts [40]. In the alveolar epithelial cell monolayers, IL-1 β activated TGF- β 1 via RhoA/ α v β 6 integrin-dependent mechanisms [33, 41]. We observed *in vitro* inductive responses to IL-1 β indicative of the pro-fibrotic features of TGF β 1 in the epithelial cells, whereas no changes were observed in the fibroblasts. In response to IL-1 β , epithelial cells undergo shape changes consistent with those of mesenchymal cells and are characterized by decreased expression levels of epithelial cell-associated proteins (proSP-C, E-cadherin) and increased expression levels of mesenchymal cell-associated proteins (vimentin, N-cadherin, and α SMA). Given that as many as 30% of the fibroblasts were derived from EMT in a bleomycin lung fibrosis model [42], this finding was suggestive of the essential role played by epithelial cells in alveolar elastogenesis.

In the present BPD model, the expression of β 6 integrin at both the mRNA and protein levels was commonly up-regulated in and restricted to injured epithelial cells. Furthermore, recent studies have demonstrated that the blockade of α v β 6 attenuates pulmonary fibrosis [8] and renal fibrosis [43] *in vivo*. The *in vitro* depletion of the α v β 6 integrin by siRNA significantly decreased the IL-1 β -induced increases in the activation of both the TGF- β protein and the mRNA expression characteristic of mesenchymal cells and TE production; these findings are suggestive of a role associated with an EMT secondary to TGF- β activation. Among the three isoforms of TGF- β that promoted EMT in conjunction with elastogenesis, TGF- β 1 was the most prominent type [27, 44]. TGF- β 1 promotes the differentiation of fibroblasts into activated myofibroblasts, enhances collagen synthesis, and reduces collagen degradation via the downregulation of proteases and the upregulation of protease inhibitors [45]. The epithelial-derived TGF- β 2 also drives collagen synthesis in subepithelial fibroblasts [46]. TGF- β 3 has a limited ability to mediate progressive pulmonary fibrosis [47]. TGF- β neutralization attenuated excessive elastogenesis, resulting in improved alveolar development in O₂-treated neonatal lungs, a finding that supports this point and may also explain why it was observed that O₂ increased the levels of both IL-1 β and α v β 6, irrespective of the presence of TGF- β .

ATII cells are associated with the generation of microenvironments with many cytokines and chemokines; these environments are conducive to the promotion of myofibroblasts [48, 49]. We observed increased TGF- β 1 levels in the supernatant of ATII cells, which was induced by IL-1 β . Although the origin of TGF- β 1 in this cell culture was not formally characterized, it is possible that the paracrine TGF- β 1 that we observed in our culture model is physiologically relevant. ATII cells produced TGF- β 1 that may move freely through basement membranes and contribute to the differentiation of myofibroblasts via the recruitment and the proliferation of either nonresident or resident fibroblastic cells. Recent data indicate that resident fibroblasts within the lungs are essential for the maintenance of the myofibroblast population following injury [50]. The dysregulation of TGF- β 1 in the ATII cells *in vivo* noted in our data suggests that the ATII cells may play an important role in the regulation of elastogenesis, either via the epithelial-mesenchymal transformation of alveolar epithelial cells or the provision of the necessary paracrine signals for such a process to occur [48]. The results of the *in vitro* IL-1 β stress studies indicated that the induction of elastin expression by IL-1 β is not a primary event in fibroblast cells, *per se*, but may result from paracrine TGF- β 1 signaling in epithelial cells. We have not undertaken definitive lineage tracing studies to investigate the potential contributions of resident fibroblasts to the excessive elastogenesis observed in the setting of BPD; however, this should be explored in future studies.

The limitations of this study were as follows: first, when identifying the phenotypes of elastin and the TGF- β -generating cells using dual immunofluorescence, we could not exclude nonspecifically labeled cells or autofluorescent cells. We may have exaggerated the numbers of epithelium-derived cells and the numbers of EMT events contributing to elastin production. Lineage-specific tracking would be more suitable for tracking both the origins and the phenotypes of these cells [51]. Second, *in vitro* IL-1 β stress testing induced EMT and elastin expression in the epithelial cells. However, the cell culture system may have unavoidably removed other elements that would have been present under real physiological conditions. It is not clear whether the performance of the cells in the setting of *in vitro* stress resembled elastin expression *in vivo*. It would be premature to confirm the relative contribution of EMT to the increasing alveolar elastin expression levels observed in mice with BPD.

In summary, these findings contribute to an overall picture of excessive elastogenesis in which epithelial-derived IL-1 β /TGF- β signaling is a key factor in driving lung mesenchymal cell differentiation. We demonstrated that IL-1 β dysregulation contributes to excessive lung elastic fiber production. We also provided evidence that IL-1 β increased TGF- β 1 activation in lung endothelial cells via α v β 6 integrin-dependent mechanisms. These findings warrant further exploration to ameliorate the prognoses of infants with BPD.

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Disclosure Statement

The authors declare that they have no competing interests.

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