Interleukin-1β Promotes Epithelial-Derived Alveolar Elastogenesis via ανβ6 Integrin-Dependent TGF-β Activation

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Key Words
Bronchopulmonary dysplasia • IL-1β • Elastin • EMT ανβ6

Abstract

Background/Aims: IL-1β 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-1β were investigated in vitro in lung epithelial and mesenchymal cells. Results: Elevated levels of IL-1β, ανβ6 and TGF-β1 were each associated with aberrant elastin production in O2-exposed lungs. IL-1Ra abolished TGF-β1 activation and ανβ6 upregulation, which occurred as a result of exposure to hyperoxia, whereas 1D11 had no discernible effect on the expression of either ανβ6 or IL-1β even following O2-exposure, suggesting that IL-1β was initially induced. Additionally, double staining revealed the presence of epithelium-derived elastin-producing cells, which was confirmed via in vitro IL-1β 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-1β was initially induced by hyperoxia; ανβ6 subsequently interacted with and activated TGF-β1, acting as an epithelial/mesenchymal signaling molecule that contributed to excessive alveolar elastogenesis, the primary pathological feature of BPD.
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-1β (IL-1β) is a critical cytokine associated with both the initiation and the persistence of inflammation [11-13]. The conditional expression of IL-1β causes severe pulmonary inflammation, alveolar hypoplasia, and progressive lung fibrosis, whereas the *in vivo* blockage or depletion of IL-1β alleviates inflammation and fibrosis and ultimately prevents murine bronchopulmonary dysplasia [14, 15]. Regarding the direct effects of IL-1β on the regulation of extracellular matrix (ECM) expression and composition, conflicting data exist regarding the contributions of IL-1β to the development of myofibroblasts, which are critical for alveolar formation [16-18]. Additionally, IL-1β induces myofibroblast formation and elastogenesis via both an epithelial-mesenchymal transition (EMT) [19] and an endothelial-mesenchymal transition (EndoMT) [20, 21].

The αvβ6 integrin is an epithelially restricted integrin and is also recognized as an activator of transforming growth factor (TGF)-β1, 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, αvβ6 integrin expression is low but increases significantly when alveolar epithelial cells are injured [24]. The neutralization of the αvβ6 integrin reportedly attenuates the lung injury induced by IL-1β [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-1β/αvβ6/TGF-β signaling impacts both alveologenesis and alveolar elastogenesis, which eventually results in lung disease. However, the exact mechanisms by which IL-1β causes the perturbation of alveolar elastogenesis are unknown.

We have determined that IL-1β induces TGF-β activation via αvβ6 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±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% $O_2$. 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-1β (10 ng/ml) (401-ML-025, R&D Systems) and αβ6 siRNA for the indicated time periods as reported previously [30].

**RNA interference**

The αβ6 knockout was achieved in the MLE-12 cells using siRNA (25 nM) for αβ6, which was provided by Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. The optimized sequences of the αβ6 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: $2^{-ΔΔCt} \times 100\%$, where $ΔΔCt = Ct_{\text{target} \text{gene}} - Ct_{\text{β-actin}}$.

**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                     |
|-------------|------------------|-----------------------------|
| β6 integrin | TAG CTT CCA GCC AAG GTG GG | TCT GAG GGA CTG GTA TGT GTG TCC |
| Lox         | GCACTGACACACACACAGGA | TTAGTGTAGCTGATCGAGG         |
| IL-1β       | TGGTGTTGAGTGGCTCAGTT | CAGCAGAAGGCTTTTGGTG          |
| TGF-β1      | GATCTGGTCCAACAATAGGGCTC | ACCTTCCAGTACCTGAGCCGC       |
| E-cadherin  | ATGTTCACTGTAAAGGAGACT | TCA GTC ACC TTG AGT GTG GCA |
| vimentin    | CCAACCTTTCTGTCCCTGAA | TTAGTGATTGTTCTCACCAGA       |
| αSMA        | ATTTGCTGACTCCTGGAGATGGT | TGATGTCAGCAGAACATCAGCGT    |
| β-actin     | CATCCTCTTCTCCTGGAGAAGA | ACAGGATTCCATACCAGAGAAGGAGG  |
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 ME-12 cells were washed and suspended to stain with αvβ6 integrin (MAB2074, 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 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-1β (sc-7884, Santa Cruz Biotechnology, Santa Cruz, CA), αvβ6 integrin (MAB2074Z, Merck Millipore, Billerica, MA), TGF-β1 (sc-146, Santa Cruz), and αSMA (clone 1A 4, 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 55i 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-1β and TGF-β1

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 μg of protein were used for the IL-1β measurements, using an IL-1β Quantikine ELISA kit (R&D Systems) according to the manufacturer’s protocol. In a separate analysis, the MLE-12 cells were incubated with IL-1β for 1, 4 and 7 days. The serum-free culture media were collected and neutralized for the TGF-β1 concentration measurements using a commercially available enzyme-linked ELISA kit (R&D Systems) according to the manufacturer’s protocol [33].

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 |
| 2      | αvβ6     | Mouse | mab2874Z | Millipore | Donkey anti-mouse AlexaFluor 488 |
| 3      | proSP-C  | Rabbit | ab08079 | Abcam | Donkey anti-rabbit AlexaFluor 594 |
|        | elastin  | Rabbit | ab21610 | Abcam | Chicken anti-rabbit AlexaFluor 488 |

αSMA

MAB2074

ELISA
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), αSMA (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 (Biovid) 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 ± 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-1β/TGF-β1 signaling in the hypoxia-treated mouse pup lungs

We determined the mRNA expression of the potential TGF-β1 signaling related genes using quantitative real-time PCR in the hypoxia-treated mouse pup lungs. We observed that the expression patterns of IL-1β, αvβ6 integrin, and TGF-β1 were strongly impacted by exposure to 85% O₂. Compared with the age-matched pups, IL-1β mRNA expression

![Fig. 1. Ontogenies expression of IL-1β, TGF-β1 and αvβ6 integrin, during lung development and hypoxia in mouse pup lungs.](image)

Comparison of the mRNA expression of IL-1β(A), αvβ6 integrin (B), TGF-β1(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: ± SEM. *p < 0.01 compared with the corresponding air-exposed control (one-way ANOVA). D. Comparison of IL-1β concentrations in the lungs of air- or O₂-exposed at P14. Total lung homogenate active IL-1β levels were measured by ELISA. Columns: average values of a minimum of three independent experiments; bars: ± SEM. *p < 0.01 compared with the corresponding air-exposed control (one-way ANOVA).
progressively increased from postnatal day 7 (P7) until P28 in the 85% O₂-exposed pups (Fig. 1A, Fig. 1B). The mRNA levels of TGF-β1 (Fig. 1A, B) and αvβ6 (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-1β 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-1β and TGF-β1, we investigated the cellular localization of IL-1β, integrin αvβ6, and TGF-β1 using immunostaining. IL-1β 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-1β in the 85% O₂-exposed pup lungs (Fig. 2A). Abundant αvβ6 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 αvβ6 (Fig. 2B), and undetectable immunostaining levels of αvβ6 protein were observed in the control mouse pups on P14 (Fig. 2B). Likewise, the sections of the lungs exposed to 85% O₂
exhibited intense TGF-β1 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.


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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 $\alpha$-1Ra. We subsequently determined whether the alveolar myofibroblasts, which produce elastic fibers, were influenced by $\alpha$-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 $\alpha$-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 $\alpha$-1Ra exhibited organized and thick fibrous structures, even following $O_2$ exposure (Fig. 4B).

To determine whether $\alpha$-1Ra induces αvβ6 and TGF-β1 activity, which have been implicated in elastogenesis, the lung homogenate transcript levels of TGF-β1 and αvβ6 integrin were measured via RT-PCR in the $\alpha$-1Ra treated mice. Compared with the control group, the transcript levels of αvβ6 were unchanged in the P14 mice with respect to $\alpha$-1Ra (Fig. 5A); the increased mRNA expression observed in the lungs of the $O_2$-treated pups on P28 was inhibited with $\alpha$-1Ra. The transcriptional pattern of TGF-β1 was coincident with the transcriptional regulation of αvβ6 by $\alpha$-1Ra (Fig. 5A). Consistent with the mRNA expression data, both the abundance and the localization of TGF-β1 induced by $O_2$ exposure were prevented following 28 days of $\alpha$-1Ra treatment (Fig. 5B). Using a sandwich ELISA

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**Fig. 4.** Improved α-SMA amount and elastin ultrastructure position in the hyperoxia injured mouse pup lungs treated with $\alpha$-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 $\alpha$-1Ra was associated with an improvement to nearly normal of the staining pattern of α-SMA in the $O_2$-injured developing lungs. Bar=50μm. 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 $\alpha$-1Ra restored the elastin structure in the $O_2$-exposed lungs to nearly normal. Arrows point to elastin and * represent microfibrils. Bar = 1 μm.
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 αvβ6 integrin is epithelially expressed, we subsequently studied the contribution of αvβ6-positive epithelial cells to elastin production. We observed that the cells positive for both αvβ6 and elastin were located in regions of matrix remodeling (Fig. 6A). Furthermore, the αvβ6 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
than 70% of the cells did not express proP-C; however, among the proP-C-positive cells, which represented 22.8% of the cell population analyzed, approximately 3-6% of the proP-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 pro P-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.

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 ± SEs (n=6). *P<0.05 vs the Air group, #P<0.05 vs the O2 group (one-way ANOVA)
We subsequently measured both αvβ6 and IL-1β expression during the neutralization of TGF-β signaling via 1D11, a TGF-β1,2,3-neutralizing IgG1 antibody. As depicted in Fig. 7A, no impact of TGF-β neutralization on the mRNA levels of either αvβ6 or IL-1β was noted, whereas treatment with 1D11 inhibited the 85% oxygen-induced pulmonary lox mRNA expression.

**Fig. 7.** 1D11 suppressed the induction of lox and αSMA by hyperoxia but no influence with IL-1β and αvβ6. A. Comparison of the mRNA expression of TGF-β1, αvβ6 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: ± SEM. *p < 0.01 compared with the corresponding 85% O₂-exposed control (one-way ANOVA). B. Immunohistochemistry was used to detect αvβ6 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 αvβ6 signaling caused by O₂ exposure. Bar = 50 μm. n = 3 per group. One representative slide per group is shown. C. Immunofluorescent staining of αSMA in the 14-day-old mouse pup lungs treated as indicated. In contrast with control, 85% O₂ had increased αSMA staining. Treatment with 1D11 was associated with an improvement in the staining pattern of αSMA in the O₂-injured developing lung. Bar=100μm. 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 μm. n = 3 per group. One representative slide per group is shown.

TGF-β neutralization alleviated excessive elastin deposition and prevented the induction of TGF-β1 activity but not IL-1β activity.

We subsequently measured both αvβ6 and IL-1β expression during the neutralization of TGF-β signaling via 1D11, a TGF-β1,2,3-neutralizing IgG1 antibody. As depicted in Fig. 7A, no impact of TGF-β neutralization on the mRNA levels of either αvβ6 or IL-1β was noted, whereas treatment with 1D11 inhibited the 85% oxygen-induced pulmonary lox mRNA expression.
Fig. 8. IL-1β increases TGF-β1 activation in rat ATII cell monolayers via αvβ6 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-1β (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-1β (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. A representative example from minimum of three independent experiments is shown. 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-1β (10 ng/ml) for 48 hours. Nuclei were labeled with the DNA dye DAPI. Arrowheads denote nuclear translocation of pSmad2/3. Bar=50 μm. n = 3 per group. One representative slide per group is shown. D. IL-1β mediated induction of β6 mRNA expression on MLE-12 cells and RFL-6 cells is determined using RT-PCR. The graph showed the expression of β6 mRNA induced by IL-1β treatment relative to the housekeeping gene β-actin mRNA. Columns: average values of a minimum of three independent experiments; bars: ± SEM. *p < 0.01 compared with the corresponding air-exposed control (Student’s unpaired t-test). E. Evidence for αvβ6 integrin depletion. RT-PCR analysis of β6 mRNA expression 96 h after siRNA transfection. Columns: average values of a minimum of three independent experiments; bars: ± SEM, *p <0.01 versus scrambled siRNA (Up panel). Western blot analyses with αvβ6 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 αvβ6-specific shRNA, treated with IL-1β 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 αsMA and tropoelastin in total protein extracts of MLE-12 cells, expressing scrambled or αvβ6-specific shRNA, treated with IL-1β or its vehicle for the indicated time periods. Data were replicated three times and a representative example is shown.
expression, a key mediator of elastin synthesis, which suggests that elastin synthesis and assembly are mediated by the TGF-β pathway. Moreover, minimal αβ6 staining was detected in the control mice, whereas markedly increased epithelial staining intensity was observed throughout the alveolar epithelium following 14 days of O₃ exposure. There appeared to be no changes in 1D11 compared with the control (nonimmune, MOPC21) pups exposed to 85% O₃ (Fig. 7B). These results suggest that TGF-β interrupts O₃-triggered signaling at the level of either IL-1β or αβ6.

The myofibroblast measurements confirmed that there was significant thickening of the septal tips (Fig. 7C) and increased αSMA staining in the mice exposed to O₃; 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-1β's effects on the TGF-β1-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-1β stress testing using MLE-12 cells and RFL-6 cells and measured TGF-β mediated Smad2/3 signaling. As shown in Fig. 8A, IL-1β (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-1β 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-1β between epithelial cells and fibroblasts and consistent with the data obtained via a confocal microscopy analysis, indicating that IL-1β enhanced pSmad2/3 nuclear translocation (Fig. 8C) in epithelial cells.

To determine whether increased TGF-β activity was caused by higher levels of the α6 integrin on the cell surface, we subsequently investigated the effects of IL-1β on α6β6 expression in both MLE-12 cells and RFL-6 cells. IL-1β induced a higher level of β6 mRNA expression in the MLE-12 cells, as determined via RT-PCR (Fig. 8D), whereas the RFL-6 cells did not express β6 integrin either basally or in response to IL-1β, illustrating that heterogeneity exists with respect to β6 expression. We also observed that α6β6 siRNA selectively reduces β6 mRNA expression levels but not those of control siRNA (Fig. 8E); the Smad3 phosphorylation induced by IL-1β was prevented by blocking the α6β6 integrin in the MLE-12 cells (Fig. 8F), suggesting that the α6β6 integrin may account for the different responses to IL-1β observed in epithelial cells and fibroblasts. Furthermore, α6β6 depletion with siRNA reduced both αSMA and elastin expression in the MLE-12 cells (Fig. 8G), demonstrating that IL-1β activates TGF-β via an α6β6-dependent mechanism in the epithelium.

**Continuous stimulation by IL-1β 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-1β 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-1β 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-1β 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-1β stimulated significant increases in the expression of vimentin and αSMA expression, findings indicative of the acquisition of a mesenchymal phenotype by the cells (Fig. 9B). As expected, α6β6 integrin depletion alleviated the induction of cell remodeling and the development of EMT-related characteristics facilitated by IL-1β (Fig. 9B). Furthermore, IL-1β plus TGF-β1 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-1β (data not shown).

We also determined the amount of TGF-β1 secreted by the primary rat ATII cell monolayer in response to IL-1β using ELISA. We observed that 10 ng/ml of IL-1β promoted the significant secretion of TGF-β1 in the supernatant for 24 hours (Fig. 9D). Taken together, these results indicate that TGF-β1 was released by the ATII cells via an IL-1β-dependent mechanism, which may either induce EMT in alveolar epithelial cells or convert fibroblasts into myofibroblasts via the paracellular pathway.
Discussion

IL-1β 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-1β 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-1β 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-1β.

The temporal and spatial expressions of IL-1β during lung development have been described previously [6]. We defined functional IL-1β performance in the context of damaged alveolar formation in the setting of BPD. Elevated IL-1β levels were elicited by O₂, a finding suggestive of a relationship between IL-1β and the pathogenesis of BPD. Consistent with this finding, the inhibition of IL-1β attenuated the abnormal airspace enlargements caused by O₂ [11], suggesting that IL-1β 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 αSMA-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-1Ra 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-1β in promoting elastin expression, conflicting data regarding the inductive effects of IL-1β exist depending on different cell type and speciﬁcity. One study demonstrated the absence of an effect by IL-1β on elastin expression in lung ﬁbroblasts [16, 39], whereas the ﬁndings of another study were suggestive of the elastogenic effects of IL-1β in lung ﬁbroblasts [40]. In the alveolar epithelial cell monolayers, IL-1β activated TGF-β1 via RhoA/αvβ6 integrin-dependent mechanisms [33, 41]. We observed in vitro inductive responses to IL-1β indicative of the pro-fibrotic features of TGFβ1 in the epithelial cells, whereas no changes were observed in the ﬁbroblasts. In response to IL-1β, 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 αSMA). 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 β6 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 αvβ6 attenuates pulmonary ﬁbrosis [8] and renal ﬁbrosis [43] in vivo. The in vitro depletion of the αvβ6 integrin by siRNA signiﬁcantly decreased the IL-1β-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-β1 was the most prominent type [27, 44]. TGF-β1 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-β2 also drives collagen synthesis in subepithelial ﬁbroblasts [46]. TGF-β3 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-1β and αvβ6, 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-β1 levels in the supernatant of ATII cells, which was induced by IL-1β. Although the origin of TGF-β1 in this cell culture was not formally characterized, it is possible that the paracrine TGF-β1 that we observed in our culture model is physiologically relevant. ATII cells produced TGF-β1 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-β1 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-1β stress studies indicated that the induction of elastin expression by IL-1β is not a primary event in fibroblast cells, per se, but may result from paracrine TGF-β1 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-1β 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-1β/TGF-β signaling is a key factor in driving lung mesenchymal cell differentiation. We demonstrated that IL-1β dysregulation contributes to excessive lung elastic fiber production. We also provided evidence that IL-1β increased TGF-β1 activation in lung endothelial cells via αvβ6 integrin-dependent mechanisms. These findings warrant further exploration to ameliorate the prognoses of infants with BPD.

Acknowledgements

We thank Prof. Xianqing Jin for providing technical assistance and insightful discussions during the preparation of the manuscript. We thank Dr. Xiaoyong Zhang of the Wistar Institute (USA), who provided medical writing services.

This research was supported by the National Natural Science Foundation of China (No. 81270058, 30770950) and by the Chongqing Natural Science Foundation (CSTC, 2009BB6072).

Disclosure Statement

The authors declare that they have no competing interests.
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