Assessment of Brd4 Inhibition in Idiopathic Pulmonary Fibrosis Lung Fibroblasts and in Vivo Models of Lung Fibrosis

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Idiopathic pulmonary fibrosis (IPF) is a chronic lung disease of high unmet medical need. Although bromodomain (Brd) and extra terminal domain isoforms have recently been implicated in mediating inflammatory and oncologic indications, their roles in lung fibrosis have not been comprehensively assessed. We investigated the role of Brd on the profibrotic responses of lung fibroblasts (LFs) in patients with rapidly progressing IPF and a mouse bleomycin model of lung fibrosis. The enhanced migration, proliferation, and IL-6 release observed in LFs from patients with rapidly progressing IPF are attenuated by pharmacologic inhibition of Brd4. These changes are accompanied by enhanced histone H4 lysine5 acetylation and association of Brd4 with genes involved in the profibrotic responses in IPF LFs as demonstrated using chromatin immunoprecipitation and quantitative PCR. Oral administration of 200 mg/kg per day Brd4 inhibitor JQ1 in a therapeutic dosing regimen substantially attenuated lung fibrosis induced by bleomycin in C57BL/6 mice. In conclusion, this study shows that the Brd4 inhibitor JQ1, administered in a therapeutic dosage, is capable of inhibiting the profibrotic effects of IPF LFs and attenuates bleomycin-induced lung fibrosis in mice. These results suggest that Brd4 inhibitors may represent a novel therapy for the treatment of rapidly progressing IPF. (Am J Pathol 2013, 183: 470–479; http://dx.doi.org/10.1016/j.ajpath.2013.04.020)

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suggested that the altered phenotype of the IPF lung myofibroblast may result from epigenetic mechanisms that lead to stable changes in the gene expression profiles,\(^8\) thus inhibiting the epigenetic machinery, which may affect wide-scale reprogramming of fibrotic tissue, which would not be feasible with targeted pathway-based inhibitors. Indeed, expression of several individual antifibrotic and profibrotic signaling components have been identified as regulated epigenetically,\(^9\)-\(^11\) in addition to more wide-scale regulation of the fibrotic transcriptome.\(^12\)

Posttranslational modification of exposed histone tails, which includes acetylation of lysine residues, enables opening of the nucleosomal structure and dynamic regulation of gene transcription through binding of bromodomain (Brd) motif-containing proteins or so-called epigenetic readers that specifically recognize these acetylated lysine residues.\(^13\) These epigenetic readers act as scaffolds and attract components of the transcriptional machinery to these acetylated lysine residues, resulting in modulation of gene transcription.\(^14\),\(^15\) Pharmacologic modulation of epigenetic readers is increasingly being considered a novel therapeutic approach for treatment of a number of inflammatory and oncologic indications, with the Brd and extra terminal domain isoforms gaining most attention.\(^16\) The recent discovery of selective small molecule antagonists of Brd4,\(^17\)\(^-\)\(^19\) which are suitable for in vivo assessment of efficacy, has introduced the possibility of exploring involvement of these epigenetic readers in a wider range of indications.

In a previous study, we demonstrated that several phenotypic responses such as migration, proliferation, and IL-6 release induced after treatment of normal human LFs with platelet-derived growth factor-BB (PDGF-BB) and transforming growth factor-\(\beta\)1 (TGF-\(\beta\)1) could be attenuated by pretreatment of cells using pharmacologic inhibitors of Brd4 or Brd4 siRNA.\(^20\) Genes such as \(z\)SM\(A\) and IL-6 were enriched for acetylated histone H4 lysine 5 (Ac-H4K5) and Brd4 binding after TGF-\(\beta\)1 treatment of normal human LFs, providing some mechanistic insight into how occupation of genes by epigenetic readers such as Brd4 could coordinate expression of the wound healing–related transcriptome.

The present study demonstrated that LFs from patients with IPF exhibit altered proliferation and migration, and increased basal and ligand-induced IL-6 secretion, when compared with control LFs from unaffected donors. Pretreatment of unaffected donor control and IPF LFs with the Brd4 inhibitor JQ1 attenuated many of the phenotypic responses. The level of Ac-H4K5 and Brd4 associated with the Brd4 inhibitor JQ1 attenuated lysine 5 (Ac-H4K5) and Brd4 binding after TGF-\(\beta\)1 treatment of normal human LFs, providing some mechanistic insight into how occupation of genes by epigenetic readers such as Brd4 could coordinate expression of the wound healing–related transcriptome.

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### Materials and Methods

#### Human Lung Tissue

This study was approved by the ethics committees of all institutions involved. IPF lung tissue was obtained, and a diagnosis of usual interstitial pneumonia/IPF was made, as previously outlined.\(^21\) Normal human lung tissue was obtained from macroscopically tumor-free lung samples resected from patients with lung cancer.

#### LF Isolation and Culture

Primary cultures of LFs were isolated from the distal parenchyma of unaffected donor controls (patients with lung cancer) and patients with IPF, as previously described.\(^22\) LFs from unaffected donors (\(n = 3\)) and patients with IPF (\(n = 4\)) were characterized. Because of the abnormal growth characteristics of the IPF LFs, previously described,\(^21\) we were unable to synchronize all of these LF lines with the control LF cultures and to analyze them simultaneously. Cells were cultured in complete medium containing Dulbecco’s modified Eagle’s medium (DMEM) with high levels of glucose and L-glutamine supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotic/antimycotic agents (all from Invitrogen Canada, Inc., Burlington, ON, Canada). All primary LF cultures from patients with IPF and unaffected donor groups were used at passages 8 to 13 under the same cell culture conditions. When indicated, LFs were serum-starved in basal medium, ie, DMEM with high levels of glucose and L-glutamine supplemented with 0.1% (v/v) FBS.

#### Dermal Fibroblast Cell Culture

Dermal fibroblasts from patients with early diffuse systemic sclerosis (SSc) (\(n = 3\)) and healthy individuals (\(n = 3\)) were acquired after informed consent was obtained. All patients fulfilled the criteria of the American College of Rheumatology for the diagnosis of SSc. Fibroblasts were maintained in DMEM supplemented with 10% (v/v) fetal calf serum and used between passages 3 and 7. When indicated, fibroblasts were serum-starved in basal medium, ie, DMEM with high levels of glucose and L-glutamine supplemented with 0.2% (v/v) FBS.

#### Antibodies and Reagents

Human PDGF-BB, TGF-\(\beta\)1 (PeproTech, Inc., Rocky Hill, NJ), JQ1 [(S)-4-(4-chloro-phenyl)-2,3,9-trimethyl-6H-1-thia-5,7,8,9a-tetraaza-cyclopenta[e]azulen-6-yl]-acetic acid tert-butyl ester] was synthesized as previously described.\(^17\) Also used were BioCoat Collagen I 96-well clear black
plates, 8-μm pore size; 24-well BD Falcon cell culture inserts, and Cytofix/Cytoperm fixation/permeabilization solution kit (all from BD Biosciences, Franklin Lakes, NJ); BrdU proliferation assay kit (Calbiochem, San Diego, CA); Odyssey blocking buffer (LI-COR Biosciences, Inc., Lincoln, NE), DAPI; Lipofectamine RNAiMax, polyvinylidene fluoride, 4% to 12% (w/v) SDS-PAGE gels (Invitrogen, Grand Island, NY); MILLIPLEX Map Human Cytokine/Chemokine Magnetic Bead Panel, HCYTOMAG-60K kit (EMD Millipore Corp., Billerica, MA); Gurr fixative solution; methylene blue stain (BDH Chemicals, Bridgeport, NJ); RNasea Plus Mini Kit, quantitative PCR probe/primer sets for human fibronectin 1, collagen 1A1, 18S rRNA, Brd2, Brd3, Brd4, predesigned short interfering RNA for human MYC (alias c-myc), EpiTect ChIP One-Day kit (Qiagen, Inc., Valencia, CA), AffinityScript qPCR cDNA synthesis kit (Stratagene, Santa Clara, CA); radioimmunoassay buffer; and BCA protein assay kit (Thermo Fisher Scientific, Inc., Hudson, NH). Antibodies for chromatin immunoprecipitation (ChIP) were rabbit anti-human AcH4K5 antibody (Epigentek, Farmingdale, NY), rabbit anti-human Brd4 (Active Motif, Carlsbad, CA), and rabbit anti IgG (Abcam, Cambridge, MA); EpiTect ChIP qPCR primers for the human Il-6 promoter within −1 kb and +1 kb of the transcriptional start sites (Qiagen), bleomycin sulfate (MP Biomedicals, Inc., Solon, OH), sodium pentobarbital (Abbott Laboratories, Inc., North Chicago, IL), anti-collagen I antibody (rabbit polyclonal; Genetex, Inc., Irvine, CA), cytochrome oxidase reagent (Zap-Oglobin II; Beckman Coulter, Inc., Fullerton, CA), cytoxin slides (Thermo Shandon Scientific, Ltd., Runcorn, UK), and hemacolor stain set (EMD Chemicals Inc., Gibbstown, NJ).

Assessment of Proliferation Using BrdU Incorporation

LFs were seeded in BioCoat Collagen I 96-well clear black plates at a density of 8 x 10^5 cells per well and were incubated overnight in complete medium, after which the cells were starved with basal medium for 24 hours. The cells were incubated with JQ1 for 2 hours at the concentrations indicated and were then stimulated with 100 ng/mL PDGF-BB for 48 hours. Proliferation was assessed by determining the incorporation of BrdU using a BrdU proliferation assay kit in accordance with the manufacturer’s protocol. The degree of BrdU incorporation was assessed using an EnVision Multilabel Reader (PerkinElmer, Inc., San Jose, CA).

LF Migration

LF migration was performed using the BD Falcon HTS Multitwell Insert System (BD Biosciences) as previously described. In brief, 1.5 x 10^5 cells resuspended in 0.5 mL basal medium vehicle, 10 μmol/L JQ1, was added to the top chamber of the transwell plate, and 1 mL basal medium containing 0.1% (v/v) FBS was added to the bottom chamber. After 2 hours of incubation, vehicle or 100 ng/mL PDGF-BB was added to the bottom chamber of the transwell plate, and the plate was incubated overnight at 37°C.

Determination of IL-6 and CCN2 Secretion from Dermal Fibroblasts

Fibroblasts were plated at a density of 2 x 10^5 cells per well in 6-well plates and were maintained in DMEM supplemented with 10% (v/v) FBS. After 24 hours, the medium was exchanged with serum-starved medium for another 24 hours. When indicated, cells were treated for 1 hour with either vehicle or JQ1 at the dosages indicated, after which they were stimulated with 1 ng/mL TGF-β1. Plates were incubated for an additional 24 hours at 37°C before removal of supernatant for analysis of IL-6, assessed using a standard alphaLISA kit (PerkinElmer) according to the recommended protocol.

Determination of IL-6 and CCN2 Secretion from Dermal Fibroblasts

Fibroblasts were plated at a density of 1 x 10^4 cells per well in 96-well plates in complete medium and incubated overnight at 37°C. The medium was replaced with fresh medium containing 2% (v/v) FBS and conditioned for the following 48 hours before collection. Supernatant was analyzed using a Milliplex Map magnetic bead custom cytokine kit (EMD Millipore), following the manufacturer’s recommended protocol. Data were collected and analyzed using the Flexmap 3D system (Luminex, Austin, TX). Cells were assessed for viability using the Cell Titer-Glo assay kit (Promega Corp., Madison, WI) following the recommended protocol, and luminescence was read using the PHERAstar multimode plate reader (BMG Labtech, Inc., Cary, NC). When indicated, the cells were treated with either vehicle or JQ1 for 1 hour at 37°C, after which they were stimulated with 10 ng/mL TGF-β1. Plates were incubated for an additional 24 hours at 37°C before removal of supernatant for analysis of IL-6, assessed using a standard alphaLISA kit (PerkinElmer) according to the recommended protocol.
incubated for an additional 24 hours, and conditioned medium was analyzed for IL-6 levels (R&D Systems) and CCN2 levels (PeproTech) according to the manufacturer’s instructions.

3D Collagen Gel Contraction

LF contraction was assessed using a commercially available 3D collagen gel contraction kit according to the manufacturer’s instructions. In brief, 2.5 × 10^5 LFs per well were mixed 1:4 with collagen gel lattice mixture using the volumes indicated in the manufacturer’s instructions, and 0.5 mL per well was plated in a 24-well plate and incubated for 1 hour at 37°C to enable gel polymerization. After gel polymerization, 1 mL basal medium containing 0.1% (v/v) FBS was added to each well. The gels were detached, and either vehicle or 10 ng/mL TGF-β1 was added. After 18 hours, images of the contracted gel disks were obtained, and quantitation was performed by assessing the weight of the gel disks.

Real-Time qPCR

Total RNA was extracted using the RNeasy Plus Mini Kit and then transcribed to cDNA using the AffinityScript QPCR cDNA Synthesis Kit (Stratagene). Real-time quantitative PCR (qPCR) reactions were performed using an Mx3000P Real-Time PCR System (Agilent Technologies, Inc., Santa Clara, CA). The resulting amplification and melt curves were analyzed to ensure specific PCR product. Threshold cycle values were used to calculate the fold change in transcript levels.

ChIP/qPCR

ChIP was performed using a ChIP EpiTect ChIP One-Day kit (Qiagen) according to the manufacturer’s instructions. In brief, cells were treated with 1% (v/v) formaldehyde for 10 minutes at 37°C to cross-link proteins to DNA. The cross-linking reaction was terminated by addition of stop buffer. To generate DNA fragments approximately 500 to 1500 bp long, the cross-linked chromatin was sonicated on ice for 7 minutes using an Ultrasonic processor (Sigma, St. Louis, MO) at 15 seconds on and 30 seconds off. ChIP assays were performed using 1 × 10^6 cells per 8 μL antibody using ChIP-grade antibodies. Antibody complexes were recovered via incubation with protein-A agarose with rocking for at least 1 hour at 4°C. After reversal of the cross-links, qPCR was performed using an Mx3000P Real-Time PCR System (Agilent Technologies). The resulting amplification and melt curves were analyzed to ensure specific PCR product. Threshold cycle values were used to calculate the fold enhancement.

Murine Model of Pulmonary Fibrosis

Animals

Male C57BL/6J mice aged 7 to 9 weeks were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed under pathogen-free conditions with food and water ad libitum. All animal care and experimental procedures were approved by the Roche Animal Care and Use Committee, which is a facility accredited by the American Association for the Accreditation of Laboratory Animal Care.

Administration of Bleomycin

On day 0, animals received 2 U/kg bleomycin or saline solution (baseline controls) via intratracheal administration, as described previously. On day 5, the normal chow diet was replaced with Purina Rodent Chow No. 5001 mixed with JQ1 and a coloring dye to identify the correct formulation for each group. The two formulations contained either 999.283 g of the Purina chow, 0.417 g JQ1, and 0.3 g FD&C Blue Dye No. 1 to achieve a daily dose of 50 mg/kg or 998.033 g of the Purina chow, 1.66667 g JQ1, 0.15 g FD&C Blue Dye No. 1, and 0.15 g FD&C Red Dye No. 40 to achieve a daily dose of 200 mg/kg. These dosages were calculated on the assumption that the mice would consume 2.5 g/day, and food consumption was monitored. Control groups received the Purina chow. Animals received this diet every day until the end of the study (day 21 after bleomycin administration).

Assessment of Inflammation and Fibrosis

Animals were euthanized via overdose of sodium pentobarbital, 250 mg/kg. Subsequently, bronchoalveolar lavage fluid was performed, and total and differential cell counts were obtained as previously described. Whole lungs were inflated under 25 cm H2O pressure with 10% (v/v) neutral buffered formalin through the tracheal cannula and were immersed in formalin for at least 24 hours. After being processed into paraffin blocks, the lungs were cut into 5-μm sections and were either stained with H&E or immunolabeled with an anti-collagen I antibody to assess fibrotic changes in the lungs, as previously described. For analysis of 4-hydroxyproline, bronchoalveolar lavage fluid (BALF) samples were extracted using acetonitrile at a concentration of 1:6. After centrifugation, 0.1% (v/v) acetic acid in water was added to the supernatants at a concentration of 1.5:1. Samples were mixed and centrifuged before injection for liquid chromatography—tandem mass spectrometry analysis, performed using gradient high-performance liquid chromatography with selective reaction monitoring. The calibration range was 40 to 2000 ng/mL. Because the control BALF matrix contained a basal level of hydroxyproline (approximately 200 ng/mL), the calibration curve was corrected with the basal concentration determined by the standard addition. The liquid chromatography—tandem mass spectrometry system used for the analysis consisted of an AB SCIEX API4000 instrument (AB SCIEX, Framingham, MA) with an electrospray source connected to an Agilent 1200 pump, a Waters 2777 autosampler (Waters Corp., Milford, MA), and an Agilent 1100 series column oven. Chromatographic analysis was performed via hydrophilic interaction liquid chromatography high-performance
liquid chromatography using an Ascentis Express HILIC column (length \( \times \) i.d., \( 30 \times 2.1 \) mm; particle size, \( 2.7 \) \( \mu m \)) (Sigma). The selective reaction monitoring transition was m/z 132 to m/z 41. Pulmonary fibrosis was graded as previously described,\textsuperscript{25} with minor differences. In brief, to determine the fibrosis histopathology score for the lung of each mouse, the entire left and right longitudinal lung sections were scored separately (score range, 0 to 9) at \( \times 100 \) magnification, and the scores were combined (total score range, 0 to 18). Grading criteria were as follows: grade 0 = no apparent fibrosis; grade 1 = minimal fibrosis with rare foci of mostly interstitial alveolar septal fibrosis affecting less than 5% of the entire lung section; grade 3 = mild fibrosis characterized by multiple foci with thickening of alveolar septa by fibrosis and progressing to regions with fibrous deposition within the alveolar spaces, with some damage to the alveoli, affecting 5% to 25% of the entire lung section; grade 5 = moderate fibrosis with multiple or single

![Figure 1](image1)

**Figure 1** A: PDGF-BB–mediated proliferation and migration are increased in LFs from donors with IPF (grey bars) compared with unaffected donors (black bars). Data represent the fold proliferation induced by PDGF-BB compared to vehicle control and are given as the means \( \pm \) SEM of all donors combined for three independent experiments. Statistical analysis was performed using two-way analysis of variance (ANOVA) followed by the Bonferroni post hoc multiple comparison test. \(* P < 0.01\) versus unaffected donor control. B: Migration of LFs was assessed using the transwell filter system. Images are representative of three independent experiments. C: Quantification of migration. Data represent the fold migration mediated by PDGF-BB compared with the corresponding vehicle control and are given as the means \( \pm \) SEM of all donors combined for three independent experiments. Statistical analysis was performed using an unpaired \( U \)-test. \( \text{**} P < 0.001\) versus unaffected donor control.

![Figure 2](image2)

**Figure 2** Basal cytokine and growth factor secretion and TGF-\( \beta \)-mediated contraction are enhanced in LFs from donors with IPF (grey bars) compared with unaffected donors (black bars). Conditioned medium was collected from LFs from unaffected donors and IPF donors for assessment of levels of cytokine and growth factors. A: Data represent the means \( \pm \) SD of secreted proteins of all donors combined for three independent experiments. Statistical analysis was performed using an unpaired \( U \)-test. \( \text{**} P < 0.0001\) versus unaffected donors. B: Contraction of LFs from an unaffected donor and an IPF donor treated with vehicle or stimulated with 10 ng/mL TGF-\( \beta \). Data are representative of three independent experiments. C: Wet weight of each gel disk was measured, and the contraction ratio is expressed as the wet weight of the TGF-\( \beta \)- or vehicle-treated sample. Data represent the means \( \pm \) SEM of all unaffected donors or IPF donors for at least eight independent experiments performed in duplicate. Statistical analysis was performed using an unpaired \( U \)-test. \( \text{**} P < 0.0001\) versus unaffected donors.
coalescing large areas of fibrosis effacing the alveoli, with definitive damage to pulmonary architecture, affecting 25% to 50% of the entire lung section; grade 7 = marked fibrosis with severe distortion of the pulmonary parenchyma by large contiguous fibrous areas, affecting 50% to 75% of the entire lung section; and grade 9 = massive pulmonary fibrosis similar to grade 7, affecting 75% to 100% (not observed in this study) of the entire lung section. Grades 2, 4, 6, and 8 were intermediate grades assigned on the basis of the predominant histopathologic changes if features described for two distinct grades were present in the section, to account for the progressive nature of the fibrotic lesion.

Statistical Analysis

Statistical comparisons were performed as outlined in the figure legends, using commercially available software (Prism version 5; GraphPad Software, Inc., San Diego, CA). Probability values were considered significant at $P < 0.05$.

Results

Phenotypic Characterization of Unaffected Donor and IPF Donor LFs

Phenotypic assays were performed to assess the responses of unaffected donor and IPF donor LFs. Doubling time of IPF LFs was prolonged compared with LFs isolated from unaffected donor controls, and we also noted a similar phenomenon during cell culture using these donor IPF LF samples (unpublished observations). We observed that, compared with unaffected donor LFs, the IPF LFs demonstrated an enhanced proliferative response to 300 ng/mL PDGF-BB when normalized to basal proliferative levels: 1.78-fold ± 0.15-fold for unaffected donor LFs, compared with 2.34-fold ± 0.17-fold for IPF donor LFs ($P < 0.05$) (Figure 1A). Similarly, we also observed a significant increase in the migration of the IPF donor LFs compared with unaffected donor LFs when treated with 100 ng/mL PDGF-BB (Figure 1, B and C). Previously published work has reported that interstitial fibroblasts are capable of participating in an inflammatory response by promoting direct communication from fibroblasts to immune cells through synthesis and secretion of inflammatory mediators. In the present study, IPF donor LFs demonstrated...
a statistically significant increase in baseline secretion of a number of inflammatory cytokines and growth factors including IL-6, IL-8, MCP-3, and VEGF-A compared with that observed in supernatants from unaffected donor controls (Figure 2A). Compared with unaffected donor LFs, the IPF donor LFs also demonstrated an enhanced contractile response in 3D collagen gels after treatment with 10 ng/mL TGF-β1 (Figure 2B and C), as has been demonstrated for LFs from different IPF patient cohorts and fibroblasts from other fibrotic tissues.

JQ1 Inhibits Phenotypic Responses of Unaffected Donor and IPF Donor LFs

JQ1, a previously reported small molecule Brd4 inhibitor, was used to assess the role of Brd4 in mediating the phenotypic responses of LFs isolated from unaffected and IPF donors. The migration and proliferation of unaffected donor and IPF donor LFs after stimulation with 100 ng/mL PDGF-BB was significantly attenuated by pretreatment of the cells with 10 μmol/L JQ1 (Figure 3A–C), which suggests that Brd4 is involved in driving these responses in LFs. Studies have implicated the Brd family of epigenetic readers in regulating the expression of proinflammatory cytokines through direct binding to genes at areas where the chromatin has been modified by acetylation such as Ac-H4K5. The enhanced baseline increase in inflammatory cytokines including IL-6 (Figure 2A) prompted us to assess the effect of JQ1 on basal and TGF-β1-mediated IL-6 secretion. Pretreatment of unaffected donor and IPF donor LFs with increasing concentrations of JQ1 attenuated basal and TGF-β1–mediated IL-6 secretion (Figure 3D). The observed potency of JQ1 to inhibit the responses by 50% was 67.9 nmol/L in unaffected donor LFs and 36.8 nmol/L in donor LFs. We have also shown that dermal fibroblasts isolated from patients with early diffuse SSC also exhibit increased IL-6 secretion at baseline and enhanced TGF-β1–stimulated levels that can be attenuated with JQ1 pretreatment (Supplemental Figure S1). Thus, our data suggest that aberrant Brd activity may be a common mechanism underlying signaling defects in multiple tissues in various fibrotic indications.

Enrichment of Ac-H4K5 and Brd4 on the IL6 gene in IPF Donor LFs

We were intrigued by the elevated baseline levels of secreted IL-6 in the IPF donor LFs compared with LFs from unaffected donors (Figure 2A) and that JQ1 could attenuate basal and TGF-β1–mediated IL-6 secretion in IPF donor LFs (Figure 3D). We hypothesized that the elevated baseline levels of IL-6 observed in the supernatants from LFs of IPF donors, compared with LFs of unaffected donors, could be due to aberrantly increased levels of Ac-H4K5 and Brd4 association with the IL-6 gene at baseline. Indeed, ChIP qPCR experiments with antibodies recognizing Ac-H4K5 and Brd4 demonstrated enrichment of Ac-H4K5 and Brd4 on areas of the IL-6 gene within 1 kb of the transcriptional

Figure 5 Increased inflammatory cell infiltration in the lung after intratracheal bleomycin challenge can be inhibited by JQ1 in a dose-dependent manner. Infiltrates in BALF include total inflammatory cells (A), lymphocytes (B), macrophages (C), and neutrophils (D). Statistical analysis was performed using one-way ANOVA followed by the Bonferroni post hoc multiple comparison test. *P < 0.05, **P < 0.01, ***P < 0.001 versus bleomycin vehicle or saline vehicle.

Figure 6 Bleomycin-induced lung fibrosis after intratracheal administration can be inhibited by JQ1. Lung histologic sections (×0.5 magnification) stained with anti-collagen I antibody demonstrate that collagen I induction in the lung after intratracheal bleomycin challenge at day 21 can be inhibited by 200 mg/kg JQ1. A: Animals challenged with intratracheal saline solution and fed vehicle-infused chow. B: Animals challenged with intratracheal bleomycin and fed vehicle-infused chow. C: Animals challenged with intratracheal bleomycin and fed JQ1-infused chow calculated to provide a daily dose of 200 mg/kg from day 5 after bleomycin challenge.
start site using nuclear extracts from IPF donor LFs compared with unaffected donor LFs (Figure 4, A and B). These data suggest that IPF donor LFs may be sensitized to exhibit profibrotic phenotypic responses, such as elevated cytokine secretion levels, through aberrant histone modification and consequent Brd4 association and transcription of affected genes.

Effects of JQ1 in a Murine Model of Bleomycin-Induced Pulmonary Fibrosis

We used a bleomycin-induced murine model of pulmonary fibrosis28 to assess the effect of Brd4 inhibition on subsequent development of the disease. As expected, compared with vehicle-treated animals, bleomycin-challenged animals exhibited a significant increase in infiltration of inflammatory cells in BALF (Figure 5A), in particular lymphocytes (Figure 5B), macrophages (Figure 5C), and neutrophils (Figure 5D). Total cell counts, lymphocytes, and macrophage infiltration in BALF after bleomycin challenge were reduced in a dose-dependent manner at day 5 in animals fed JQ1-infused chow compared with animals fed a normal chow diet.

More important, animals fed a normal chow diet exhibited evidence of lung fibrosis, as shown at histologic analysis of lung sections assessed for collagen I staining (Figure 6, A and B), which could be reversed in animals fed a JQ1-infused chow diet calculated to provide a daily dose of 200 mg/kg, at day 5 after bleomycin challenge (Figure 6C). Quantitation of the assessment of lung fibrosis using the Ashcroft scoring method (Figure 7A), 4-hydroxyproline measurements in BALF (Figure 7B), and the percentage of lung tissue collagen I staining (Figure 7C) all demonstrated a consistent increase in lung fibrosis in animals at 21 days after bleomycin challenge compared with vehicle-challenged animals. In contrast, animals fed JQ1-infused chow at day 5 after bleomycin challenge, calculated to provide a daily dose of 200 mg/kg, demonstrated significantly decreased lung fibrosis at 21 days after bleomycin challenge compared with vehicle-challenged animals. In contrast, animals fed JQ1-infused chow at day 5 after bleomycin challenge, calculated to provide a daily dose of 200 mg/kg, demonstrated significantly decreased lung fibrosis at 21 days after bleomycin challenge compared with vehicle-challenged animals. In contrast, animals fed JQ1-infused chow at day 5 after bleomycin challenge, calculated to provide a daily dose of 200 mg/kg, demonstrated significantly decreased lung fibrosis at 21 days after bleomycin challenge compared with vehicle-challenged animals.

Discussion

Several recent studies have implicated Brd4 in the development of experimental models of sepsis,18 multiple myeloma,29 and chronic kidney disease.30 Herein we have demonstrated for the first time that pharmacologic inhibition of Brd4 after systemic administration in mice of Brd4 small molecule inhibitors can attenuate experimental lung fibrosis induced by bleomycin challenge when given at therapeutic dosage. The clinical implications of the findings of the present study are also important because they suggest that LFs from patients with IPF may be sensitized to exhibit profibrotic features such as enhanced cytokine and growth factor secretion through aberrant alterations in chromatin markers such as Ac-H4K5 that attract epigenetic readers such as Brd4 to these genes to facilitate transcription. Our findings also suggest that phenotypic responses such as migration, proliferation, and cytokine secretion of IPF LFs can be antagonized by Brd4 inhibition.

Characterization of LFs isolated from the lungs of patients with IPF has been reported by several groups.10,31
and has shown alterations in phenotypic responses that are preserved over several population doublings, although other groups have suggested that these effects disappear through continued passage. Maintenance of an abnormal, relatively stable phenotype of LFs isolated from lungs with IPF is transmissible across population doublings strongly implicates genetic or epigenetic alterations as responsible for these effects. Indeed, LFs isolated from lungs with IPF are relatively unresponsive to antifibrotic effects of exogenously applied prostaglandin E2 in culture through the silencing of prostaglandin E2 receptor expression mediated by methylation of CpG islands. The ability to transfer a progressive lung fibrotic phenotype to immune-incompetent mice after tail vein injection of \textit{ex vivo} cultured LFs isolated from human lungs with IPF also suggests that these cells harbor stable alterations, mediated either genetically or epigenetically, that permit perpetuation of patients with a clinical diagnosis of rapidly progressive disease, within 12 months, to compare with LFs isolated from the lungs of unaffected donors. Our data suggest that LFs isolated from lungs of patients with rapidly progressing IPF exhibit altered responses in a range of cell-based phenotypic assays, compared with LFs isolated from the lungs of unaffected donor controls. The phenotype of the IPF LFs may result from the altered transcriptome of these cells, which is driven in part by histone acetylation and binding of Brd4 to these promoters to modulate transcription. The data are intriguing, in particular in combination with \textit{in vivo} data that demonstrated for the first time that therapeutic dosing of a small molecule Brd inhibitor can attenuate experimental lung fibrosis in mice. That said, it is worth recognizing the limitations in the present study. First, the cells we used were isolated from surgical lung biopsy specimens and were cultured for several passages before measurements were made, and it is possible that some of the observed differences could be an artifact of cell culture. Second, because of the abnormal growth rates of IPF LFs and the requirement for substantial amounts of biological material to perform experiments such as ChIP qPCR, it was only possible in some cases to compare LFs from single donors.

Inasmuch as we assessed only IPF LFs from patients with clinically defined rapidly progressive disease, it will be of great interest to determine whether alterations in the level of Ac-H4K5 and Brd4 binding with genes in IPF LFs is also associated with the rate of disease progression and prognosis, as has been suggested for other targets and processes. In the study by Boon et al., in which complex networks of genes seemed to be aberrantly regulated, it is tempting to speculate that such wide-scale alterations in the cellular transcriptome may be orchestrated by epigenetic readers such as Brd4 as a result of aberrant histone modifications mediated through repeated environmental insults. In this regard, a recent study has identified that mitogen- and stress-activated kinase 1 links cigarette smoke to alterations in the acetylation status of histone H4. Cigarette smoke has been recognized as one of the strongest risk factors that predisposes to development of IPF.

Understanding the molecular mechanisms that drive aberrant histone modifications will also be key to identifying further novel targets other than Brd4. Some evidence suggests that histone acetyltransferases such as P300 may be responsible for mediating changes in the acetylation status of histone residues including Ac-H4K5 to drive profibrotic gene expression including alterations in extracellular matrix components. Indeed, elevation in the levels of P300 has been demonstrated in fibroblasts derived from the human dermis of patients with SSc, which suggests that targeting P300 and/or Brd4 may have wider therapeutic usefulness in fibrotic indications.

In conclusion, LFs isolated from patients with rapidly progressive IPF exhibit evidence of dysfunctional Brd4 activity, with functional consequences likely as a result of aberrant histone H4 acetylation. The phenotypic responses of LFs from unaffected donors and IPF donors, including migration, proliferation, and cytokine induction, can be attenuated with pharmacologic antagonists of Brd4. We also provide the first experimental demonstration that pharmacologic inhibition of Brd4, administered in therapeutic dosages, can reverse experimental lung fibrosis in mice. Considered together, our data suggest that Brd4 inhibitors represent a novel therapeutic approach for the treatment of rapidly progressive IPF, for which there are currently no effective treatment options.

**Supplemental Data**

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2013.04.020.

**References**


21. Brd4 in Rapid IPF


29. Brd4 in Rapid IPF


35. Brd4 in Rapid IPF