Lrp5/ β -Catenin Signaling Controls Lung Macrophage Differentiation and Inhibits Resolution of Fibrosis

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Abstract

Previous studies established that attenuating Wnt/ β -catenin signaling limits lung fibrosis in the bleomycin mouse model of this disease, but the contribution of this pathway to distinct lung cell phenotypes relevant to tissue repair and fibrosis remains incompletely understood. Using microarray analysis, we found that bleomycin-injured lungs from mice that lack the Wnt coreceptor low density lipoprotein receptor-related protein 5 (Lrp5) and exhibit reduced fibrosis showed enrichment for pathways related to extracellular matrix processing, immunity, and lymphocyte proliferation, suggesting the contribution of an immune-matrix remodeling axis relevant to fibrosis. Activation of β -catenin signaling was seen in lung macrophages using the β -catenin reporter mouse, *Axin2*^{+/*LacZ*}. Analysis of lung immune cells by flow cytometry after bleomycin administration revealed that *Lrp5*^{-/-} lungs contained significantly fewer Siglec F^{low} alveolar macrophages, a cell type previously implicated as positive effectors of fibrosis. Macrophage-specific deletion of β -catenin in *CD11c*^{cre}; *β -catenin*^{flox} mice did not prevent development of bleomycin-induced fibrosis but facilitated its resolution by 8 weeks. In a nonresolving model of fibrosis, intratracheal administration of asbestos in *Lrp5*^{-/-} mice also did not prevent the development of fibrosis but hindered the progression of fibrosis in asbestos-treated *Lrp5*^{-/-} lungs, phenocopying the findings in

bleomycin-treated *CD11c*^{cre}; *β -catenin*^{flox} mice. Activation of β -catenin signaling using lithium chloride resulted in worsened fibrosis in wild-type mice, further supporting that the effects of loss of Lrp5 are directly mediated by Wnt/ β -catenin signaling. Together, these data suggest that lung myeloid cells are responsive to Lrp5/ β -catenin signaling, leading to differentiation of an alveolar macrophage subtype that antagonizes the resolution of lung fibrosis.

Keywords: β -catenin; Lrp5; alveolar macrophage; bleomycin; lung fibrosis

Clinical Relevance

The findings from this research demonstrate, for the first time, that a developmental pathway alters lung repair after injury by impacting macrophage differentiation. This evidence adds to the growing body of literature supporting the importance of monocytes–macrophages to lung fibrosis and resolution. Potential future interventions for pulmonary fibrosis may be directed toward strategies that modulate the Wnt/ β -catenin pathway in monocyte–macrophage cells.

Macrophages are important contributors not only to innate immunity, but also to the normal development and repair of tissues (1). Current evidence suggests that

immune cells of the monocyte–macrophage lineage are recruited during tissue injury and contribute dynamic functions that promote either the persistence or resolution

of fibrosis. Recruited monocytes can promote the resolution of fibrotic disease by several potential mechanisms, including differentiating into regulatory

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macrophages, inhibiting T lymphocyte and fibroblast proliferation, degrading interstitial collagen via matrix metalloproteinases (MMPs), phagocytosing extracellular matrix and cellular debris, and promoting myofibroblast apoptosis (2). The importance and diversity of macrophage functions in tissue development and homeostasis is highlighted by studies in which mice lacking distinct macrophage subpopulations revealed surprising developmental abnormalities, such as disrupted bone morphogenesis (3), and in which macrophages expressing Wnt ligands promoted activation of nearby hepatocyte progenitors in regenerating livers (4).

In idiopathic pulmonary fibrosis, developmental pathways, such as Wnt/ β -catenin, have been implicated in the cycle of aberrant wound repair, resulting in persistent fibrosis (5). The Wnt/ β -catenin pathway is best known for determining cell fate decisions during tissue development (6–8) and in homeostasis (9–15). Although lung epithelial- and fibroblast-specific roles of Wnt signaling relevant to fibrosis have been described (16, 17), less is understood about how Wnt signaling affects the immune system of the lung. Recent reports suggest that Wnt/ β -catenin signaling may have opposing effects in different immune cell types (18–21). In a mouse model of inflammatory bowel disease, activation of β -catenin signaling in $CD11c^+$ $CD11b^{+/-}$ intestinal dendritic cells suppressed inflammation and promoted tolerance by inducing T regulatory cells while suppressing the inflammatory T helper 1/T helper 17 responses (19). Moreover, in a full-thickness skin wound model, deletion of β -cat in myeloid cells from *Lys-Cre* mice inhibited macrophage migration and adhesion, ultimately preventing wound healing (20). Together, these studies indicate that a careful understanding of the immune cell and context-specific activities of β -catenin signaling will be required to establish the contribution of this pathway in the pathogenesis of human diseases.

Work from our laboratory has demonstrated that global loss of the Wnt coreceptor, low density lipoprotein receptor-related protein 5 (*Lrp5*), attenuated bleomycin-induced pulmonary fibrosis (22). To determine whether the major Wnt-responsive cell type contributing to fibrogenesis was from the lung parenchyma or hematopoietic in

origin, we generated bone marrow chimeras using *Lrp5*^{-/-} mice. We found that wild-type mice reconstituted with *Lrp5*^{-/-} bone marrow cells were not protected from the development of fibrosis and, 3 weeks after bleomycin treatment, exhibited comparable levels of total lung collagen as chimeras reconstituted with *Lrp5*^{+/+} bone marrow cells (22). However, we noticed that chimeras with *Lrp5*^{-/-} bone marrow cells showed a significant reduction in new collagen synthesis at 3 weeks (22). This suggests that immune cells lacking *Lrp5* may be sufficient to improve the resolution of fibrosis, perhaps by altering immune cell fates that favor tissue repair. Although our previous work has acknowledged the contributing role of Wnt/ β -catenin signaling in lung parenchymal cell types to the injury and repair response (16, 17), how β -catenin signaling modulates the immune response after lung injury is unknown. Here, we show that pathways related to the immune response and matrix processing are activated after bleomycin injury in *Lrp5*^{-/-} lungs relative to *Lrp5*^{+/+} lungs, and that macrophages manifest activation of β -catenin signaling. We also show that *Lrp5*/ β -catenin signaling alters alveolar macrophage (AM) differentiation and that loss of β -catenin in $CD11c^+$ macrophages promotes the resolution of fibrosis.

Materials and Methods

Animals and Cell Culture

All procedures were approved by the Northwestern University Animal Care and Use Committee. C57BL/6 (catalog no. 000664), β -catenin^{flox} (catalog no. 004152), and *CD11c*^{cre} (catalog no. 007567) mice were obtained from Jackson Laboratory (Bar Harbor, ME); *Lrp5*^{-/-} mice were from Bart O. Williams (Van Andel Research Institute, Grand Rapids, MI), *Axin2*^{+/LacZ} mice from EMMA (Germany) and β -catenin^{flox(exon2-5)} were from Anjen Chenn (University of Illinois, Chicago, IL). Age- and sex-matched 8- to 12-week-old mice were used in all the experiments. For bleomycin-induced fibrosis experiments, mice were treated with a single intratracheal injection of 1.5 U/kg of bleomycin in 50 μ l of 0.9% saline or 50 μ l of 0.9% saline alone as a control. Mice were killed and organs were harvested at Day 6, Day 21, or 8 weeks, as applicable. Mice receiving lithium chloride (LiCl;

Mallinckrodt Chemicals, Phillipsburg, PA) for activation of β -catenin signaling were given daily 200 mg/kg intraperitoneal injections of LiCl in PBS or 200 μ l of PBS injected intraperitoneally as control. Mice were injected from either Days 0–6 or Days 7–21 after bleomycin installation. For asbestos-induced fibrosis experiments, mice were treated with 200 μ g of asbestos crocidolite delivered in two 50- μ l volumes via intratracheal catheter, or titanium dioxide (TiO₂) as a control particle. Mice were killed and organs harvested either on Day 21 or 8 weeks after asbestos treatment. Recombinant Wnt3a, IL-4, IL-13, and granulocyte/macrophage colony-stimulating factor were obtained from Peprotech (Rocky Hill, NJ). Bleomycin was obtained from APP Pharmaceuticals (Schaumburg, IL). Asbestos UICC crocidolite was obtained from the U.S. Environmental Protection Agency (Chapel Hill, NC).

Microarray Analysis of Lung Tissue

Total RNA was extracted from tissue using RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Quality control and the probe level processing of the Illumina (Illumina Inc., San Diego, CA) microarray data were further made with R Bioconductor package "lumi". The data processing included a normalization procedure using the quantile normalization method to reduce the obscuring variation between microarrays, which might be introduced during the processes of sample preparation, manufacture, fluorescence labeling, hybridization, and/or scanning. Hierarchical clustering and principal component analysis were performed on the normalized signal data to assess variability of sampling. Probes absent in all samples were filtered out in the downstream analysis according to Illumina detection. Differential gene expression between the different conditions was assessed by a statistical linear model analysis using the Bioconductor package "limma," in which an empirical Bayes method is used to moderate the standard errors of the estimated log-fold changes of gene expression, and results in more stable inference and improved power, especially for experiments with small numbers of microarrays. Function analysis was performed on the differentially expressed gene lists using R Bioconductor package "topGO". Heat map for hierarchical clustering of the differentially expressed genes

Figure 1. Gene expression of mouse lungs with global loss of the Wnt coreceptor low density lipoprotein receptor-related protein 5 (*Lrp5*). (A) Only 75 genes were differentially expressed between the lungs of *Lrp5*^{+/+} and *Lrp5*^{-/-} mice on Day 14 after intratracheal bleomycin administration. (B) Functional enrichment analysis of genes altered in *Lrp5*^{-/-} lungs relative to wild-type (WT) lungs on Day 14. Only genes up-regulated in bleomycin response relative to saline controls were included in the network. Colored nodes, functional gene term sets (functions indicated next to sets); white nodes, "hub" genes; triangles, genes up-regulated in *Lrp5*^{-/-} lungs relative to WT controls during bleomycin response; "V" symbols, genes up-regulated in *Lrp5*^{-/-} lungs relative to WT controls during bleomycin response. (C) Real-time quantitative PCR (qPCR) validation in a second cohort of mice for gene expression of *matrix metalloproteinase 13* (*MMP13*) and *lysyl oxidase* (*LOX*) (*n* = 5–9 animals per condition; **P* < 0.05). Data presented are mean (± SEM). BLEO, bleomycin; Ccr5, C-C motif chemokine receptor 5; Chod1, chondrolectin; EGF, epidermal growth factor; F13a1, family with sequence similarity 13 member A; Fbln2, fibulin 2; GAPDH, glyceraldehyde phosphate dehydrogenase; H2-Ob, histocompatibility 2, O region β locus; H2-Q8, histocompatibility 2, Q region locus 8; Lum, lumican; Lyz1, lysozyme 1; Mfap5, microfibrillar associated protein 5; Plat, plasminogen activator tissue type; Sirpb1a, signal-regulatory protein β 1A; Slpi, secretory leukocyte peptidase inhibitor; Trem2, triggering receptor expressed on myeloid cells 2; Wfdc12, WAP four-disulfide core domain protein 12.

in Figure 1A was generated using Cluster 3.0 software (Michael Eisen; Stanford University, Stanford, CA). Functional gene set enrichment analysis was used to generate the functional gene network in Figure 1B and identify hub genes by metagrouping of

individual gene term sets (Gene Ontology [GO] biological process and Kyoto Encyclopedia of Genes and Genomes [KEGG] pathways), based on function similarity. The GeneTerm Linker algorithm implemented in the "FGNet" R package

(Bioconductor Open Source) was used for this analysis.

Quantification of mRNA

Total RNA was extracted from tissue using RNeasy Kit (Qiagen, Valencia, CA)

according to manufacturer's instructions. Real-time quantitative PCR primer sequences are as follows: mouse glyceraldehyde phosphate dehydrogenase (GAPDH) forward primer 5'-TTGTGATG GGTGTGAACCACGAGA and reverse primer 5'-GAGCCCTTCCACAATG CCAAAGTT; mouse AXIN2 forward 5'-ACCTCAAGTGCAAACCTCTCACCCA and reverse 5'-AGCTGTTTCCGTGGATCTC AACT; mouse MMP-13 forward 5'-GATGGCACTGCTGACATCAT and reverse 5'-CCCACCATAGTTTGGTCCAG; mouse lysyl oxidase (LOX) forward 5'-TGCTATTTCGATCCCACGCTGCTTA and reverse 5'-TCCCTATGCCGTGAAA CCTCACAA. Real-time PCR (quantitative PCR) was performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) with the MyiQ Single Color Real-Time PCR Detection System and software (Bio-Rad). GAPDH was used as the internal control. The relative change in gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

Flow Cytometry, Cell Sorting, Isolation of Bone Marrow-Derived Cells, and Lung Leukocytes

Lung leukocytes were isolated using GentleMACS dissociator (Miltenyi, Auburn, CA) as previously described (30). Bone marrow-derived macrophages were generated from whole bone marrow cells isolated from the femurs and tibias of mice, followed by *in vitro* differentiation in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 20% L929 supernatant containing mouse granulocyte/macrophage colony-stimulating factor. Flow cytometry and cell sorting were performed using LSR II flow cytometer (BD Biosciences, San Jose, CA) as previously described (30) (see Figure E4 and Tables E1–E3 in the online supplement).

Immunohistochemistry and Imaging

β -galactosidase (β -gal) staining was performed as previously described (16). F4/80 immunohistochemistry was performed on 4- μ m-thick paraffin-embedded sections, which were incubated in 20 mg/ml proteinase K in tris-EDTA buffer (pH 8) for 3 minutes, peroxidase was quenched with BLOXALL solution (Vector SP-6000; Vector Labs, Burlingame, CA) for 10 minutes, blocked in RTU Vectastain 2.5% normal goat serum (Vector) for 20 minutes,

incubated with rat anti-mouse F4/80 antibody (AbD Serotec MCA497; Bio Rad, Hercules, CA) diluted 1:10 in RTU Vectastain 2.5% normal goat serum for 1 hour, incubated with ImmPress anti-rat IgG, mouse adsorbed (Vector MP-7444) for 30 minutes, and then finally incubated with ImmPact DAB substrate (Vector SK-4105). Imaging was performed using a Zeiss Axioskop with Cri Nuance multispectral camera (Carl Zeiss, Jena, Germany), as previously published (17).

Collagen Content

Total lung collagen content was performed using the Sircol Collagen assay (Biocolor, Carrickfergus, UK) according to the manufacturer's instructions. In brief, the entire lung was homogenized in 0.5 M acetic acid (pH 3.0) with protease inhibitors, incubated with picosirius red dye for 30 minutes, spun down and rinsed in acid-wash reagent, realkalinized, and then measured at a wavelength of 540 nm.

Glutathione S-Transferase Inhibitor of β -Catenin and TCF Pulldown and Immunoblotting

Antibodies used in this study were as follows: total β -catenin (monoclonal; BD Biosciences/Transduction Labs, San Jose, CA); total β -catenin (polyclonal) and GAPDH (Santa Cruz, Dallas, TX); active β -catenin (ABC, clone 8E7; Upstate/Millipore, Darmstadt, Germany); E-cadherin (BD Biosciences/Transduction Labs). Glutathione S-transferase inhibitor of β -catenin and TCF immunoprecipitation was performed, in brief, by incubation of cell lysate with 10 μ g glutathione S-transferase inhibitor of β -catenin and TCF precoupled to a 50% suspension of glutathione-coupled sepharose beads (GE Healthcare, Chicago, IL) for 2 hours at 4°C. Precipitated proteins were washed and subjected to SDS-PAGE and immunoblot analysis using standard procedures.

Statistical Analysis

Statistical analysis was performed by Student's *t* test or one-way ANOVA followed by Bonferroni's multiple comparison test. A *P* value of less than 0.05 was considered significant. Statistical calculations were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).

Results

Lrp5^{-/-} Lungs Protected from Fibrosis Display Enrichment for Pathways Related to Immunity and Extracellular Matrix Processing

We previously demonstrated that mice lacking *Lrp5* globally were protected from bleomycin-induced pulmonary fibrosis (22). Microarray analysis of whole lung at Day 14 after administration of bleomycin revealed only 75 genes differentially expressed in *Lrp5*^{-/-} compared with *Lrp5*^{+/+} mice (Figure 1A). Functional gene enrichment analysis revealed that, during bleomycin injury, *Lrp5*^{-/-} mice protected from fibrosis showed enrichment for genes not only related to extracellular matrix remodeling and connective tissue degradation (e.g., *Lox* and *Mmp13*), but also the innate inflammatory response, including activation of IL-13 pathway (Figure 1B and Figure E1). As *Lox* and *Mmp13* have established roles in matrix processing (23–26), we sought to corroborate the robustness of the microarray findings (Figure 1A) and confirmed that *Lox* and *Mmp13* expression were also highly increased in the replication cohort (3.9 ± 1.0 -fold versus 1.0 ± 0.3 -fold and 5.8 ± 2.0 -fold versus 1.1 ± 0.2 -fold, respectively; Figure 1C).

β -Catenin Signaling Is Activated in Macrophages of Uninjured Lungs

To determine whether immune cells of the lung receive Wnt/ β -catenin signals under baseline conditions, we used a reporter mouse in which the β -gal gene is knocked into the endogenous *Axin2* gene (27), an established downstream target of β -catenin and T cell factor transcription factor activity (27–29). Analysis by flow cytometry using fluorescein di- β -D-galactopyranoside, which is hydrolyzed by β -gal to fluorescein, demonstrated that β -catenin/T cell factor signaling activity can be detected in many myeloid cell subgroups of the lung, particularly AMs, interstitial macrophages (IMs), and monocytes (Figure 2A). Supporting these findings, immunostaining for β -gal in *Axin2* reporter mouse lungs demonstrated β -gal⁺ cells that are also positive for the mouse macrophage marker, F4/80⁺ (Figure 2D, 2D', and 2D''). Not surprisingly, there are subpopulations of cells that are β -gal⁻ and F4/80⁺, as well as β -gal⁺ and F4/80⁻. In the former group, these cells may represent myeloid cells,

Figure 2. Lung myeloid cells demonstrate active Wnt/ β -catenin signaling. (A) Flow cytometry analysis of lung cells from the *Axin2*- β -galactosidase (β -gal) reporter mouse. Green, *Axin2*- β -gal with fluorescein di- β -D-galactopyranoside (FDG); black, *Axin2*- β -gal without FDG. Cells from control lungs not expressing β -gal showed no fluorescence in the presence of FDG (data not shown). Immunohistochemistry performed on *Axin2*- β -gal lungs using X-gal staining demonstrated (B and B') β -gal-positive (light blue) cells and (C and C') F4/80 positive (brown, arrow) cells. (D, D', and D'') Cells that are both β -gal and F4/80 positive. Dashed insets indicate areas that are magnified in B', C', D', and D''. DC, dendritic cell; Macro, macrophages; MFI, mean fluorescent intensity.

such as eosinophils (30), where β -catenin signaling is not activated (Figures 2C and 2C'); alternatively, this lack of β -gal expression may represent heterogeneity in the basal activation of β -catenin in cells of monocyte lineage (i.e., AMs, IMs, and monocytes). The subpopulation of β -gal⁺ and F4/80[−] cells may represent other leukocytes, such as neutrophils, B lymphocytes, and T lymphocytes, that do not express the macrophage marker, F4/80, but have active β -catenin signaling (Figures 2B and 2B'). Although the majority of β -gal⁺ cells appear morphologically to be of immune cell origin, there are also some nonimmune cell types in the distal lungs,

such as epithelial cells or fibroblasts, which were previously shown to exhibit activation of β -catenin signaling (16, 22).

Differentiation of AMs Is Reduced in *lrp5*^{−/−} Mice after Bleomycin Injury and Contributes to Improved Resolution of Fibrosis

As the Wnt/ β -catenin pathway has been shown to determine cell fate, we speculated that β -catenin signaling might alter lung macrophage differentiation, such that loss of *Lrp5* in lung macrophages may contribute to a milieu that promotes resolution of fibrosis. In the uninjured lung, tissue-resident AMs are believed to be

long lived, populating the lung during early embryogenesis and self-renewing with minimal replenishment from bone marrow-derived cells (31, 32). Intratracheally administered bleomycin induced apoptosis of tissue-resident (Siglec F^{high}) AMs (TR-AM), which were then reconstituted from recruited bone marrow-derived cells (33–36). During the acute phase (Day 5), the number of AMs significantly decreased, whereas the number of IMs increased; during the fibrotic phase (Day 21), AM numbers increased, whereas IM numbers returned to basal levels (30). The increased AM population coincided with the emergence of a new population of Siglec

F^{low} AMs, confirming that recruited monocytes differentiated into IMs, which then gave rise to monocyte-derived (Siglec F^{low}) AMs (Mo-AMs).

Lrp5^{-/-} lungs were injured with intratracheal bleomycin and analyzed on Day 21, a time point when these mice were previously reported to manifest reduced

fibrosis (22) and when AM populations peak in the bleomycin model of injury (30). We found that, even though total CD45⁺ cell numbers did not differ significantly between *Lrp5*^{+/+} and *Lrp5*^{-/-} mice (14.2 ± 1.5 versus $11.6 \pm 1.3 \times 10^6$ cells/lung; Figure 3), the recruited population of Mo-AMs was significantly lower in the *Lrp5*^{-/-} mice

(0.61 ± 0.12 versus $1.02 \pm 0.11 \times 10^6$ cells/lung; Figure 3). This population of recruited Mo-AMs was previously shown to emerge during the fibrotic phase of bleomycin injury (30), suggesting their contributing role in the aberrant wound response. In contrast, neither the numbers of IMs nor TR-AMs differed significantly

Figure 3. Monocyte-derived Siglec F^{low} recruited alveolar macrophage (AM) numbers are lower at Day 21 in injured *Lrp5*^{-/-} lungs. Cell numbers of interstitial macrophages, tissue-resident (Siglec F^{high}) AMs (TR-AMs), and recruited monocyte-derived (Siglec F^{low}) AMs (Mo AMs), as well as T cells and B cells from lungs of *Lrp5*^{+/+} and *Lrp5*^{-/-} mice. Flow cytometry analysis performed on Day 21 after bleomycin ($n = 5$ animals per condition). Data presented are mean ($\pm$ SEM). NK, natural killer cell.

between the two bleomycin-treated genotypes at this fibrotic time point (1.32 ± 0.28 versus $0.85 \pm 0.21 \times 10^6$ cells/lung, $P = 0.21$; 0.27 ± 0.032 versus $0.24 \pm 0.052 \times 10^6$ cells/lung, $P = 0.59$; Figure 3). By Day 56, there were no differences in the populations of IMs, TR-AMs, or recruited Mo-AMs between $Lrp5^{+/+}$ and $Lrp5^{-/-}$ mice (Figure E5). Together, these findings reveal that, during the fibrotic phase of bleomycin lung injury, β -catenin signaling promotes differentiation of recruited Mo-AM populations that contribute to the fibrotic phenotype.

As the $Lrp5^{-/-}$ mice have globally diminished Wnt/ β -catenin signaling, $CD11c^{cre};\beta$ -catenin^{fllox} mice were used to investigate loss of β -catenin specifically in lung macrophages. We confirmed that myeloid cells are responsive to a canonical Wnt ligand *in vitro* using bone marrow-derived macrophages from wild-type mice (Figure E2). The presence of recombinant Wnt3a increased gene expression of the immediate downstream target, *Axin2* (Figure E2A), and stabilized the cytosolic, signaling pool of β -catenin (Figure E2B). Loss of β -catenin in $CD11c^{cre}$ bone marrow-derived macrophages diminished the cytosolic pool of β -catenin basally and after stimulation with recombinant Wnt3a (Figure E3).

When $CD11c^{cre};\beta$ -catenin^{fllox} mice were treated with intratracheal bleomycin, we found no differences in lung collagen content or bronchoalveolar lavage fluid total protein levels during the acute phase of bleomycin injury at Day 6 or during the fibrotic phase at Day 21 (Figures 4A–4D). However, there was improved resolution of bleomycin-induced pulmonary fibrosis at 8 weeks compared with $CD11c^{-/-};\beta$ -catenin^{fllox} littermates (Figures 4E–4G). Recent detailed analysis of the bleomycin model demonstrated that the fibrotic phase continued up to 8 weeks, after which time there was spontaneous resolution (37). Thus, loss of β -catenin in $CD11c^{+}$ macrophages appeared to diminish the persistent fibrotic response to bleomycin and facilitate resolution of injury.

We confirmed that these findings are not limited to bleomycin by using a second model of fibrosis with asbestos. The intratracheal asbestos model has been reported to generate a progressive, nonresolving form of pulmonary fibrosis in which macrophage cell numbers were increased from Weeks 1 to 4 and fibrosis

persisted at least through Week 24 (38–40). We demonstrated that one dose of intratracheal asbestos in $Lrp5^{-/-}$ mice resulted in increased collagen deposition by 3 weeks that was similar to asbestos-treated $Lrp5^{+/+}$ mice, but, consistent with our findings in the $CD11c^{cre};\beta$ -catenin^{fllox} mice, collagen deposition was markedly decreased by 8 weeks in $Lrp5^{-/-}$ mice (Figures 5A–5C). Similar to our findings in the bleomycin model with the $Lrp5$ mice, we found that, by 8 weeks, there were no differences in the populations of IMs, TR-AMs, or recruited Mo-AMs between $Lrp5^{+/+}$ and $Lrp5^{-/-}$ mice (Figure E6).

To support that these results are due specifically to decreased Wnt/ β -catenin signaling, we used the chemical, LiCl, which inhibits glycogen synthase kinase 3 β phosphorylation and has been previously reported to activate β -catenin in the lung (16). We found that daily intraperitoneal LiCl administration in wild-type mice increased collagen deposition and injury at 3 weeks, regardless of whether it was given immediately after bleomycin injury or in a delayed fashion (Figures 5D and 5E), suggesting that, at these early time points, β -catenin signaling has profibrotic effects on multiple cell types. In contrast to the findings associated with inhibition of β -catenin signaling in $Lrp5^{-/-}$ or $CD11c^{cre};\beta$ -catenin^{fllox} mice, activation of β -catenin signaling by LiCl during Weeks 2 and 3 after bleomycin injury in wild-type mice led to prolonged deposition of collagen, such that there was more collagen after 8 weeks than in the lungs of sodium chloride control bleomycin-treated mice (Figure 5F). Overall, these findings provide evidence that Wnt/ β -catenin signaling alters differentiation of AMs and contributes to persistence of fibrosis after lung injury.

Discussion

We previously found that mice with global loss of the Wnt coreceptor, *Lrp5*, were protected from bleomycin-induced pulmonary fibrosis; in these mice, gene pathways related to matrix processing and innate immunity are altered. The finding that Wnt/ β -catenin signaling affects matrix processing is consistent with reports that link β -catenin signaling with the transforming growth factor- β pathway,

fibroblast proliferation, and collagen synthesis (41–45). Among the top up-regulated genes was lysyl oxidase, a collagen cross-linking enzyme that has been linked to disrupted matrix synthesis in a transforming growth factor- β -dependent manner (23, 46–48), although inhibition of another family member, LOXL2, protects from fibrosis (49) and is currently in phase 2 clinical trials for the treatment of idiopathic pulmonary fibrosis (50). Mmp-13, a macrophage-associated metalloproteinase, also known as collagenase-3, is also highly increased in *Lrp5*-null lungs after bleomycin injury, and has been shown to limit the development of fibrosis (25, 26). Our finding, that loss of a Wnt coreceptor is associated with changes in genes that regulate the innate immune response, is unexpected, given the paucity of data linking these two pathways in the lung. Heise and colleagues (51) previously proposed that stretch-induced epithelial mesenchymal transition is mediated by myeloid differentiation primary response 88 in a Wnt/ β -catenin-dependent manner, and, in an influenza A model, β -catenin has also been reported to inhibit viral replication in alveolar epithelial cells via an IFN- γ -dependent mechanism (52). However, abundant literature exists supporting the importance of Wnt/ β -catenin signaling in determining cell fate during embryogenesis (6, 7), as well as adult homeostasis (9, 10). Thus, Wnt/ β -catenin signaling may play an important role in the differentiation of innate immune cells after lung injury.

As macrophages have been shown to contribute to the development of pulmonary fibrosis (53–56), we investigated whether cells of monocyte-macrophage lineage show Wnt/ β -catenin signaling activation. Indeed, myeloid cells of the lung, including AMs, IMs, monocytes, $CD11b^{+}$ dendritic cells, and neutrophils displayed evidence of active β -catenin signaling using an *Axin2* reporter mouse. Furthermore, bone marrow-derived macrophages were able to respond to the canonical ligand, Wnt3a, by increasing stabilization of the cytosolic, signaling pool of β -catenin and by increasing gene expression of the immediate downstream target *Axin2*. This is consistent with previous reports in the literature where manipulation of β -catenin signaling in intestinal dendritic cells and T regulatory lymphocytes altered the innate immune response (18, 19).

Figure 4. Loss of β -catenin in macrophages does not alter development of bleomycin-induced pulmonary fibrosis but improves resolution of fibrosis. *CD11c^{Cre}; β -catenin^{flox}* mice and *CD11c^{-/-}; β -catenin^{flox}* mice were treated with bleomycin or saline, and lungs were harvested for total collagen content by hydroxyproline assay and total protein content of bronchoalveolar (BAL) fluid after (A and B) 6 days and (C and D) 21 days ($n = 5$ animals per condition; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). After 8 weeks, (E) total lung collagen content was measured by hydroxyproline assay, and (F) gene expression of *collagen 1A1* (*COL1A1*) was determined by real-time qPCR (** $P < 0.01$, *** $P < 0.001$). Data presented are mean ($\pm$ SEM). (G) Histology of bleomycin-treated lungs stained with hematoxylin and eosin (H&E; $n = 4$ –5 mice per condition). Insets indicate areas that are magnified in the images to the right.

In determining whether Wnt/ β -cat signaling plays an important role in the differentiation of myeloid cells after lung injury, flow cytometry analysis at Day 21

after bleomycin administration, when there was previously observed decreased fibrosis in the *Lrp5^{-/-}* lungs (22), revealed that there were fewer numbers of recruited

Mo-AMs. These Mo-AMs, which emerge during the fibrotic phase, represent monocyte-derived IMs that differentiated into AMs to replace the TR-AMs that

Figure 5. Persistence of asbestosis-induced fibrosis is attenuated with loss of *Lrp5*, but activation of β -catenin by lithium chloride (LiCl) augments fibrosis persistence. *Lrp5*^{+/+} and *Lrp5*^{-/-} mice were treated with intratracheal asbestos or titanium oxide (TiO₂). Lungs were harvested for total collagen content by hydroxyproline assay on (A) Day 21 and (B) Day 56 ($n = 4-5$ mice per condition; * $P < 0.05$). (C) Histology of asbestos-treated lungs stained with H&E. WT C57BL/6 mice were treated with intraperitoneal LiCl or sodium chloride (NaCl) at the indicated times after bleomycin administration. *Insets* indicate areas that are magnified in the images to the right. (D) Lungs were harvested on Day 21 for total collagen content by hydroxyproline assay and (E) BAL fluid protein content, and (F) on Day 56 for total lung collagen content ($n = 3-6$ mice per condition; * $P < 0.05$, ** $P < 0.01$). Data presented are mean ($\pm$ SEM).

underwent apoptosis after bleomycin injury (30).

As macrophages may promote the resolution of fibrosis by a number of mechanisms (2), we speculated that loss of Wnt/ β -catenin signaling mediated changes in macrophage differentiation that may contribute to an overall proresolution environment. Our finding that loss of β -catenin in CD11c⁺ macrophages improved resolution of bleomycin-induced pulmonary fibrosis confirms an important

role of Wnt/ β -catenin signaling in monocyte-macrophage differentiation to promote aberrant wound healing. We acknowledge that CD11c is also expressed in lung dendritic cells, and thus, a contribution from the dendritic cell compartment to the phenotype cannot be completely excluded in this model. The potential role of β -catenin in the differentiation of dendritic cells has been investigated recently in a mouse model of inflammatory bowel disease (19).

However, the role of dendritic cells in lung wound healing and fibrosis remains poorly studied, and, specifically, the role of β -catenin in the differentiation of dendritic cells in the lung is unknown and an area of active interest in our laboratory.

Nonetheless, similar findings of improved resolution in a second model of pulmonary fibrosis using asbestos in *Lrp5*^{-/-} mice provide strong supporting evidence for this novel role of β -catenin. The sufficiency of β -catenin signaling in

maintaining the persistence of pulmonary fibrosis was demonstrated by the fact that administration of the Wnt/ β -catenin activator, LiCl, from Days 7–21, when recruited Mo-AMs were reported to be highest, resulted in the deposition of more collagen by the end of the fibrotic phase at Day 56.

Overall, Wnt/ β -catenin signaling has effects in multiple cell types of the lung that contribute to the development and persistence of pulmonary fibrosis. We have shown that macrophages with activated

β -catenin signaling play a role in sustaining pulmonary fibrosis after bleomycin and asbestos injury. Wnt signaling in the developing lung occurs in both autocrine and paracrine manners in the epithelium and mesenchyme (6), whereby alveolar epithelial cells, fibroblasts, and macrophages may all be the producers, as well as recipients, of a Wnt signal. Therefore, the potential effects of Wnt/ β -catenin signaling in macrophages may be both cell autonomous and cell nonautonomous, reflecting likely

complex macrophage–epithelial and macrophage–mesenchymal interactions that require further investigation. ■

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