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IKKβ Activation in the Fetal Lung Mesenchyme Alters Lung Vascular Development but Not Airway Morphogenesis

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In the immature lung, inflammation and injury disrupt the epithelial—mesenchymal interactions required for normal development. Innate immune signaling and NF-κB activation disrupt the normal expression of multiple mesenchymal genes that play a key role in airway branching and alveolar formation. To test the role of the NF-κB pathway specifically in lung mesenchyme, we utilized the mesenchymal Twist2-Cre to drive expression of a constitutively active inhibitor of NF-κB kinase subunit β (IKKβca) mutant in developing mice. Embryonic Twist2-IKKβca mice were generated in expected numbers and appeared grossly normal. Airway branching also appeared normal in Twist2-IKKβca embryos, with airway morphometry, elastin staining, and saccular branching similar to those in control littermates. While Twist2-IKKβca lungs did not contain increased levels of *Il1b*, we did measure an increased expression of the chemokine-encoding gene *Ccl2*. Twist2-IKKβca lungs had increased staining for the vascular marker platelet endothelial cell adhesion molecule 1. In addition, type I alveolar epithelial differentiation appeared to be diminished in Twist2-IKKβca lungs. The normal airway branching and lack of *Il1b* expression may have been due to the inability of the Twist2-IKKβca transgene to induce inflammasome activity. While Twist2-IKKβca lungs had an increased number of macrophages, inflammasome expression remained restricted to macrophages without evidence of spontaneous inflammasome activity. These results emphasize the importance of cellular niche in considering how inflammatory signaling influences fetal lung development. *(Am J Pathol 2017, 187: 2635—2644; https://doi.org/10.1016/j.ajpath.2017.08.013)*

In preterm infants, inflammation and injury inhibit normal lung development and lead to the chronic lung disease bronchopulmonary dysplasia.[1—5] Sources of inflammation include elevated inspired oxygen concentrations, mechanical stretch due to positive-pressure ventilation, and both prenatal and postnatal exposure to microbes. In animal models, inflammation in the developing lung arising from these various sources disrupts the normal epithelial—mesenchymal interactions required for airway and alveolar morphogenesis.[6—9] However, the cellular and molecular mechanisms connecting inflammation to abnormal development are still being characterized.

In response to microbial products or environmental exposures, lung macrophages mount an initial innate immune response.[10,11] At the molecular level, the macrophage response is characterized by the activation and nuclear translocation of the transcription factor NF-κB.[12—14] After NF-κB activation, lung macrophages express and release a number of soluble inflammatory mediators that target adjacent cells within the lung tissue.[15,16] Based on experiments using NF-κB reporter

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mice, the initial wave of NF-κB activity in macrophages is followed by a later phase of NF-κB activation in surrounding mesenchymal cells.[17] Spreading inflammation throughout the developing lung inhibits saccular airway branching morphogenesis in late-stage embryonic mouse lungs and alveolar formation in postnatal mouse lungs.[17,18] While multiple inflammatory mediators can affect lung development, inflammasome activation and IL-1β release are required for inflammatory stimuli (both microbial products and environmental injury/hyperoxia) to disrupt airway branching and alveolar formation.[18−20] Lung inflammation inhibits the expression of several key mesenchymal genes important for lung development, including *Fgf10* and *Itga8*.[21,22] In the case of *Fgf10*, activated NF-κB interacts with specificity proteins 1 and 3 to reduce gene transcription.[23,24]

While macrophages play an important role in the initial inflammatory response, we wanted to test whether NF-κB activation in mesenchymal cells could affect lung morphogenesis in a cell-autonomous manner. To bypass macrophages, we crossed mice expressing the mesenchymal-specific Twist2-Cre transgene with a strain expressing a constitutively active inhibitor of NF-κB kinase subunit β (IKKβca) mutant downstream of a *loxP*-flanked STOP cassette. Twist2-Cre animals display Cre activity throughout the lung mesenchyme during development, but spare the developing lung vasculature.[25,26] The resulting double-transgenic mice (Twist2-IKKβca) have increased IKKβ activity and subsequent NF-κB activation in mesenchymal cell populations. Here we report that Twist2-IKKβca mice were viable with normal fetal airway development. Mesenchymal expression of IKKβca was sufficient for stimulating the expression of the inflammatory chemokine-encoding gene *Ccl2* and for recruiting macrophages to the fetal lung. However, Twist2-IKKβca lungs did not express elevated *Il1b* levels or inflammasome complexes outside of the macrophage population. These results emphasize the unique impact of NF-κB activation in different cell populations on tissue inflammation and lung development.

Materials and Methods

Reagents

The following reagents were used for immunofluorescence: rat anti-CD68 (Acris Antibodies, San Diego, CA), rat anti−platelet endothelial cell adhesion molecule 1 (CD31; BD Pharmingen, San Jose, CA), mouse anti−α smooth muscle actin−Cy3 (Sigma-Aldrich, St. Louis, MO), rat anti−E-cadherin (Thermo Fisher Scientific, Waltham, MA), rabbit anti−phospho histone H3 (EMD Millipore, Billerica, MA), hamster anti-podoplanin (T-1α; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), rabbit anti−surfactant protein C (Abcam, Cambridge, MA), rabbit anti−green fluorescent protein (Invitrogen, Waltham, MA), rabbit anti-cryopyrin [(nucleotide-binding oligomerization domain-containing protein)-like receptor protein (NLRP)-3; Santa Cruz Biotechnology, Dallas, TX], and rabbit anti−caspase 1 p10 (Santa Cruz Biotechnology). Prolong Gold mounting media and fluorescent secondary antibodies Alexa Fluor 488 and 555 were purchased from Invitrogen. The nuclear stain DRAQ5 was purchased from Thermo Fisher. Cells and explants were cultured and treated in the following reagents: gel-purified *Escherichia coli* lipopolysaccharide (LPS) (O55:B5; Sigma-Aldrich), ATP (Sigma-Aldrich), Dulbecco's modified Eagle's medium (Corning Life Sciences, Tewksbury, MA), fetal bovine serum (Thermo Fisher), and penicillin−streptomycin (Thermo Fisher).

Mice

All experiments were approved by the Institutional Animal Care and Use Committee at Vanderbilt University and the University of California−San Diego. Twist2-Cre, Rosa$^{mT/mG}$ and B6(Cg)-*Gt(ROSA)26Sor*$^{tm4(Ikbkb)Rsky}$/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Twist2-IKKβca mutant mice were derived from Twist2-Cre^{+}[27] X B6(Cg)-*Gt(ROSA)26Sor*$^{tm4(Ikbkb)Rsky}$/J matings.[28] Genotyping was performed by standard PCR. For timed matings, embryonic day zero (E0) was identified as the morning of vaginal plug confirmation.

Cell and Explant Culture

Fetal lungs were dissected and enzymatically digested with collagenase (0.7 mg/mL). Cells were passed through a 70-μm strainer and centrifuged. Cells were then plated in complete media. After macrophages were allowed to briefly attach, nonadherent cells (including mesenchymal cells) were collected and replated overnight. After passaging, cultures were 95% positive for α smooth muscle actin−expressing mesenchymal cells. Fetal lung explants were isolated and cultured as previously described.[17] Bright-field images were captured at 24 and 72 hours of culture. Branch count analysis was performed using ImageJ software version 1.51 (NIH, Bethesda, MD; *http://imagej.nih.gov.ij*).

RNA Isolation and Real-Time PCR

Total RNA was isolated from whole-lung macrophages and mesenchymal cells using TRIzol (Invitrogen). First-strand cDNA was synthesized using oligo(dT) primers and Superscript III (Invitrogen). Real-time PCR was performed using either SYBR Green or TaqMan probes. PCR was performed using a CFX96 thermocycler (Bio-Rad Laboratories, Hercules, CA). The $2^{-\Delta\Delta Ct}$ method was used for comparing gene expression in samples. All samples were normalized to glyceraldehyde-3-phosphate dehydrogenase. Experiments were performed at least three independent times. Data between groups were compared by analysis of variance or *t*-test for the identification of significant differences.

Immunoassay

Isolated lung mesenchymal cells were cultured until 90% confluent. Cells were then treated with LPS (250 ng/mL), ATP (5 mmol/L), or both LPS + ATP. After treatment culture media were collected, levels of IL-1β were measured using enzyme-linked immunosorbent assay (BD Biosciences, San Jose, CA). Assays were performed in triplicate and in at least three independent experiments.

Tissue Processing and Immunostaining

Paraffin-embedded fetal mouse lungs were dissected and fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). Paraffin sections were then stained with hematoxylin (Dako North America, Carpinteria, CA), eosin (Ricca Chemical, Arlington, TX), and Hart's modified stain (reagents from Electron Microscopy Sciences). Hematoxylin and eosin–stained sections were measured for lung tissue and airspace volume using ImageJ. Frozen embedded fetal mouse lungs were fixed in 4% paraformaldehyde, washed, and processed through a sucrose gradient before being embedded in OCT media (Tissue-Tek; Sakura Finetek USA, Radnor, PA). Frozen sections were stained with antibodies of interest followed by Alexa-conjugated secondary antibodies, and nuclei were stained with DRAQ5.

Imaging and Image Analysis

Confocal images were acquired using a TCS SPE laser-scanning confocal microscope (Leica Microsystems, Buffalo Grove, IL). Widefield fluorescent and bright-field images of whole fetal mouse lung were obtained using an Olympus IX81 inverted microscope (Olympus, Center Valley, PA) with an Orca ER CCD camera (Hamamatsu Photonics, Bridgewater, NJ). All images were saved and imported to Photoshop software version CS6 (Adobe Systems, San Jose, CA) for processing. Identical processing parameters were used for achieving proper image comparison.

Flow Cytometry

The following antibodies were used for flow cytometry: CD45-V500, stem cells antigen-1–fluorescein isothiocyanate (BD Biosciences); CD140b-R-phycoerythrin, CD21-phycoerythrin-cyanin 7, allophycocyanin-cyanin 7 (eBioscience); and E-cadherin–allophycocyanin (R&D Systems, Minneapolis, MN). Viable cells were selected using Live/Dead fixable dead cell stain (Thermo Fisher). Fetal lungs were enzymatically digested into a single-cell suspension using collagenase type 2 (Worthington Biochemical Corporation, Lakewood, NJ). Red blood cells were lysed using ammonium-chloride–potassium lysing buffer (Invitrogen). Cells were placed on ice for 15 minutes in flow cytometry staining buffer (eBioscience) and incubated with antibodies for 30 to 60 minutes. For intracellular antibodies, cells were incubated on ice for 30 to 60 minutes using the Intracellular Fixation and Permeabilization Kit (eBioscience). Flow Cytometry measurements were

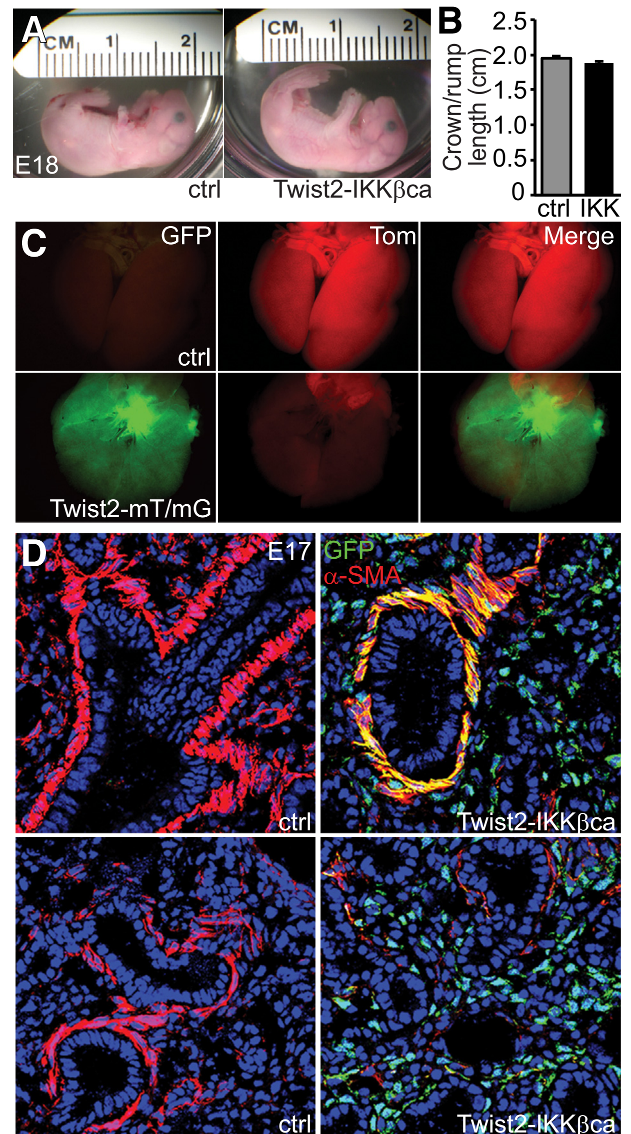

Figure 1 Twist2-driven expression of constitutively active inhibitor of NF-κB kinase subunit β (IKKβca) in fetal lung mesenchyme. Twist2-Cre X B6(Cg)-*Gt(ROSA)26Sor*$^{tm4(Ikbkb)Rsky}$/J matings resulted in the expected genetic distribution of E15 and E18 embryos. E15: 44.7% Twist2-IKKβca and 55.3% Twist2-Cre^{-} (94 embryos from 13 litters). E18: 55.7% Twist2-IKKβca and 44.3% Twist2-Cre^{-} (70 embryos from 8 litters). Genotyping therefore suggested fetal and embryonic viability. **A** and **B:** E18 Twist2-IKKβca fetal pups (IKK) appeared to develop normal with similar crown to rump lengths compared with littermate controls (ctrl). **C:** Twist2-Cre mice were crossed with Rosa26$^{mT/mG}$ reporter mice, demonstrating Tomato fluorescence before Cre recombination and green fluorescent protein (GFP) expression throughout lung mesenchyme in embryos expressing Twist2-Cre. **D:** Twist2-IKKβca mice express internal ribosome entry site–mediated GFP in Twist2-Cre^{+} mesenchymal cells. Laser scanning confocal immunostaining of E17 fetal lung sections showed GFP-positive mesenchymal cells and smooth muscle cells expressing α–smooth muscle actin (SMA) surrounding airways. Nuclei stained with DRAQ5 (blue). Data are expressed as means ± SD. $n = 4$ control mice (**B**). Original magnification: ×4 (**C**); ×40 (**D**). Tom, tomato fluorescence.

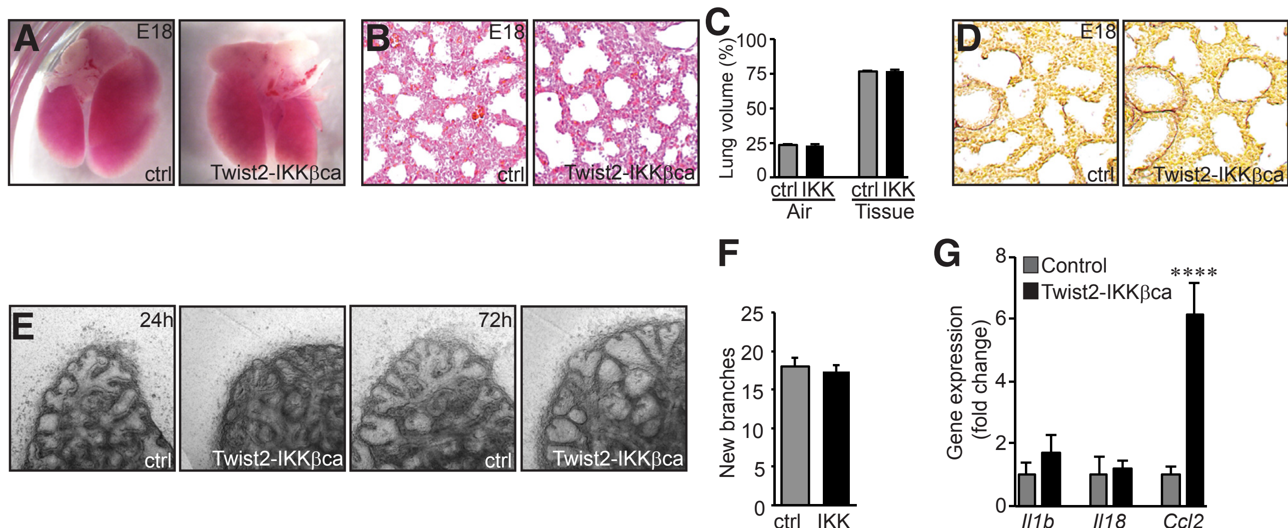

Figure 2 Constitutively active inhibitor of NF-κB kinase subunit β (IKKβca) expression in fetal mesenchyme does not disrupt lung airway morphogenesis. **A:** Similar overall size of E18 Twist2-IKKβca lungs compared with littermate controls. **B:** Hematoxylin and eosin stained sections of E18 lungs show similar airway and interstitial morphology in Twist2-IKKβca and littermate controls. **C:** Morphometry measurements showed no change in Twist2-IKKβca lungs compared with control littermates. **D:** Lung sections were stained with modified Hart's stain to visualize elastic fibers, which appear similar in Twist2-IKKβca and controls. **E** and **F:** E15 fetal mouse lung explants were cultured for 72 hours, with images acquired at 24 and 72 hours. Bright-field images show formation of new saccular airway branches along the periphery. While Twist2-IKKβca airways appeared slightly more dilated, airway branching was similar to controls (**F**). **G:** Expression of pro-inflammatory cytokines and chemokines were measured by real-time PCR using RNA isolated from E17 Twist2-IKKβca and littermate control fetal whole lungs. Twist2-IKKβca lungs had elevated mRNA levels of the chemokine *Ccl2*. Expression levels of *Il1b* and *Il18* were similar to those in control littermates. Data are expressed as means ± SD. $n = 9$ to 15 mice (**C**, **F**, and **G**). ****$P < 0.0001$ versus control. Original magnification: ×20 (**B** and **D**); ×4 (**E**).

conducted on a BD LSR II flow cytometer (BD Biosciences). For gating, doublets were excluded based on forward light scatter area against forward light scatter height followed by side light scatter area against side light scatter height. Data analysis was performed using FlowJo software version 9 (FlowJo, Ashland, OR).

Results

To test whether increased NF-κB activation specifically in developing mesenchymal cells could affect lung development, we crossed Twist2-Cre transgenic mice[27] with B6(Cg)-*Gt(ROSA)26Sor*$^{tm4(Ikbkb)Rsky}$/J mice.[28] The resulting double-transgenic animals were predicted to express both a Ikbkbca mutant allele and enhanced green fluorescent protein in Twist2$^+$ mesenchymal cell populations. Genotyping at embryonal days 15 and 18 detected approximately the predicted numbers of double-transgenic Twist2-Cre:B6(Cg)-*Gt(ROSA)26Sor*$^{tm4(Ikbkb)Rsky}$/J (Twist2-IKKβca) embryos, suggesting developmental viability (E15: 44.7% Twist2-IKKβca and 55.3% Twist2-Cre$^-$, 94 embryos from 13 litters; E18: 55.7% Twist2-IKKβca and 44.3% Twist2-Cre$^-$, 70 embryos from 8 litters). Crown−rump measurements and gross appearance of Twist2-IKKβca embryos were similar to those of control littermates (Figure 1, A and B).

To confirm Cre-mediated transgene expression in the fetal lung mesenchyme during embryogenesis, we crossed Twist2-Cre animals with the *Rosa26*$^{mT/mG}$ reporter strain.[29] In dissected hearts and lungs from control (Twist2-Cre$^-$) embryos, Tomato expression was observed throughout the lung and surrounding structures (Figure 1C). Twist2-Cre$^+$ lungs expressed green fluorescent protein throughout the lung mesenchyme. In E17 Twist2-IKKβca embryos, we detected green fluorescent protein expression from the internal ribosomal entry site sequence downstream of the Ikbkbca allele throughout the lung mesenchyme. E17 Twist2-IKKβca lungs also expressed green fluorescent protein in α smooth muscle actin−positive smooth muscle cells along airways (Figure 1D). We next examined whether mesenchymal expression of the IKKβca affected overall lung and airway morphogenesis. Gross examination of mutant Twist2-IKKβca and control littermate lungs appeared similar at E18 (Figure 2A). Hematoxylin and eosin−stained sections showed a similar overall appearance of both the proximal and distal airways, with comparable airspace and tissue volume as measured by morphometry (Figure 2, B and C). Elastin staining overall was also similar between Twist2-IKKβca and control lungs (Figure 2D).

We previously demonstrated that NF-κB activation in macrophages could disrupt distal airway branching both *in vivo* and in cultured saccular stage explants.[17] To test whether similar effects could be observed with mesenchymal NF-κB activation, we cultured E15 Twist2-IKKβca and littermate control explants, obtaining photomicrographs every 24 hours (Figure 2, E and F). While the airways in Twist2-IKKβca explants appeared slightly more dilated, the number of new distal airway branches formed between 24 and 72 hours of culture was similar to that in controls. These

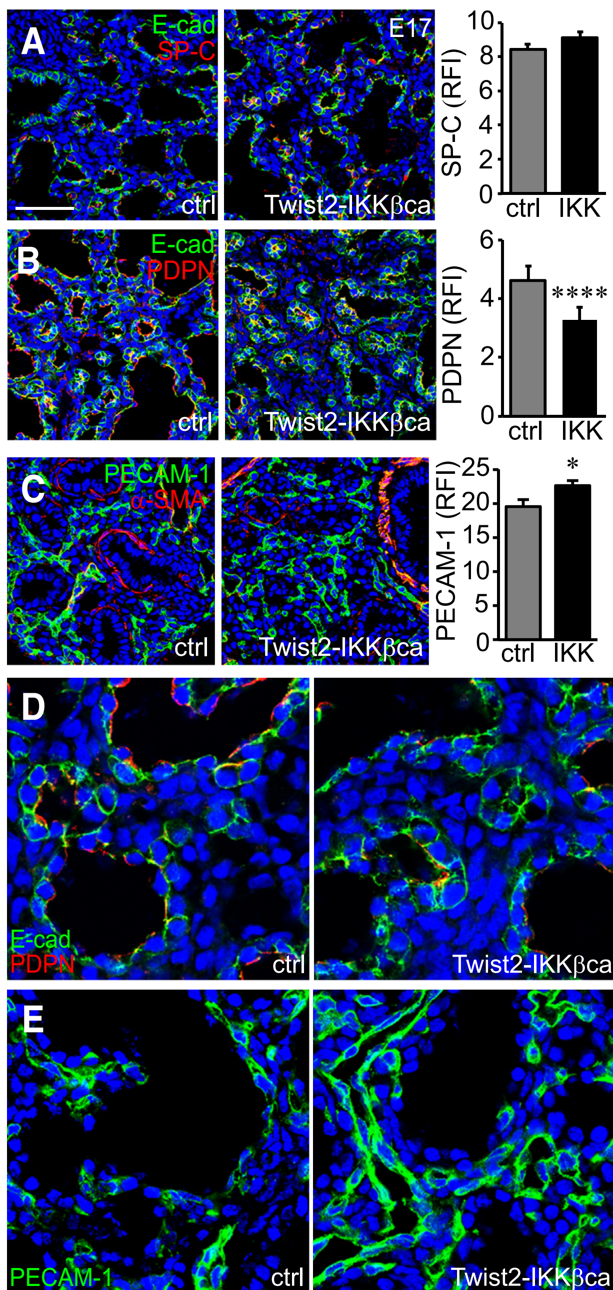

Figure 3 Alterations of Type I epithelial cell and vascular development in Twist2–constitutively active inhibitor of NF-κB kinase subunit β (IKKβca) fetal lungs. E17 lungs from Twist2-IKKβca and littermate control embryos were sectioned, immunostained, and visualized by laser scanning confocal microscopy. Relative fluorescence intensity (RFI) was quantified for each fluorescence channel across each image. **A** and **B:** Twist2-IKKβca embryos had similar expression of the Type II cell marker surfactant protein C (SP-C) (**A**), but reduced staining and RFI for the Type I cell marker podoplanin (PDPN) (**B**). Epithelial cells detected by E-cadherin (E-cad) immunolabeling. **C:** Twist2-IKKβca lungs had increased staining of the endothelial marker platelet endothelial cell adhesion molecule (PECAM)-1. Smooth muscle and mesenchymal cells detected by α–smooth muscle actin (SMA) immunolabeling. **D** and **E:** Higher-magnification images show more detail regarding reduced PDPN staining (**D**) and increased PECAM-1 staining (**E**) in Twist2-IKKβca lungs. Nuclei labeled with DRAQ5 (blue). Data are expressed as means ± SD. $n = 30$ images. *$P < 0.05$, ****$P < 0.0001$ versus control. Scale bar = 50 μm (**A**–**C**). Original magnification, ×63 (**D** and **E**).

results, along with the lung morphometry measurements from lung sections, demonstrated that the mesenchymal expression of a IKKβca mutant was not sufficient for the disruption of fetal airway branching.

We initially hypothesized that Twist2-IKKβca embryos would have abnormal airway branching. To confirm that the IKKβca isoform was sufficient for inducing the expression of NF-κB–dependent inflammatory mediators, we used real-time PCR to measure the expression of several inflammatory genes in E17 whole-lung isolates. Twist2-IKKβca lungs had increased *Ccl2*, but similar *Il1b* and *Il18* expression, compared with control littermates (Figure 2G). Data from previous reports have implicated IL-1β in linking inflammation to altered airway morphogenesis.[18–20] Therefore, the inability of the IKKβca mutant to increase *Il1b* expression could explain the normal airway morphogenesis in Twist2-IKKβca embryos.

While increased IKKβ activity did not seem to affect airway morphogenesis, we did measure changes in cell populations within the developing lung. Immunostaining for surfactant protein C in alveolar type II cells suggested similar type II cell differentiation (Figure 3A). However, staining for podoplanin (T1α), a marker for alveolar type I cells, was diminished in Twist2-IKKβca lungs compared with controls (Figure 3, B and D). Inflammatory chemokines, including *Ccl2*, can stimulate fetal lung angiogenesis.[30] We therefore examined vascular structures in the developing Twist2-IKKβca lungs. Compared with littermate controls, E17 Twist2-IKKβca lungs had more platelet endothelial cell adhesion molecule-1 staining (Figure 3, C and E), consistent with increased numbers of endothelial cells and/or increased angiogenesis.

We next tested whether mesenchymal expression of the IKKβca mutant could cause cell autonomous changes in mesenchymal growth factors. Relative expression levels of *Fgf10* and *Fgf18* were similar in Twist2-IKKβca lungs and controls (Figure 4A), while Twist2-IKKβca lungs had only slightly higher expression of the transcription factor–encoding genes *Sox9* and *Gata4*. Because (sex-determining region Y)-box 9 plays a role in mesoderm development and differentiation,[31] we used flow cytometry to test whether Twist2-IKKβca lungs had changes in the overall mesenchymal progenitor cell populations (Figure 4, B and C). After excluding $CD45^+$ and E-Cadherin/$CD324^+$ cells from E15 and E18 fetal lungs, subpopulations of mesenchymal cells were gated based on the expression of the stem cell markers CD29, stem cells antigen-1, CD104b, and CD44.[32] Twist2-IKKβca mutants and control littermates showed similar percentages of mesenchymal cells expressing each marker. Of note, we measured an increase in stem cells antigen-1–positive mesenchymal cells in E18 lungs both in control and mutants. These results suggest that mesenchymal cell differentiation during the later stages of lung development was not altered by mesenchymal IKKβca expression.

NF-κB activation induces the expression of soluble inflammatory mediators, including chemokines that recruit

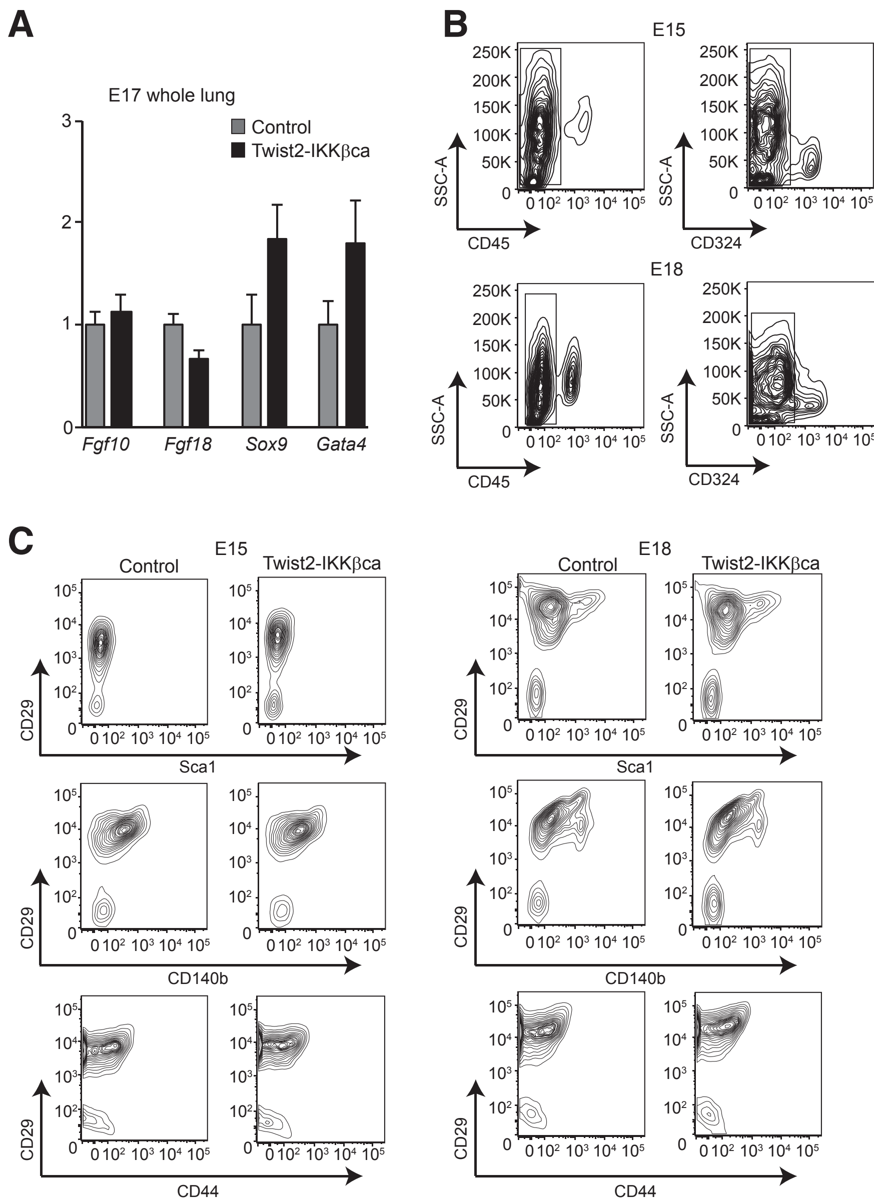

Figure 4 Transgenic inhibitor of NF-κB kinase subunit β (IKK-β) activation in fetal lung mesenchyme did not affect mesenchymal developmental gene expression or cause shifts in mesenchymal stem cell populations. **A:** Measurement of *Fgf10*, *Fgf18*, *Sox9*, and *Gata4* mRNA by real-time PCR in E17 embryonic lungs from Twist2—constitutively active IKKβ (IKKβca) and control littermates. **B:** E15 and E18 whole lungs were digested into single cell suspensions. Flow cytometric gating strategy excluded hematopoietic ($CD45^+$) and epithelial ($CD324^+$) populations. **C:** Mesenchymal stem cell populations from E15 (left) and E18 (right) Twist2-IKKβca and control embryos were compared by flow cytometry using antibodies against CD29, stem cells antigen (Sca)-1, CD140b, and CD44. Data are expressed as means ± SD (**A**). Data shown representative of three independent replicates (**C**). $n = 8$ (**A**). K, 1000.

additional immune cells. Consistent with increased *Ccl2* in Twist2-IKKβca lungs, we detected increased numbers of CD68-expressing macrophages in E17 Twist2-IKKβca lungs compared with controls (Figure 5A). We did not measure differences in the percentages of macrophages staining positive for phospho-histone H3, suggesting that the increase was not due to cell proliferation. Therefore, the increase in macrophage number was most likely due to the recruitment of additional macrophages to the lung. To test whether these additional macrophages were activated in Twist2-IKKβca lungs, we isolated E18 lung macrophages from control and Twist2-IKKβca embryos and measured the expression of several inflammatory genes induced in activated macrophages. Fetal macrophages isolated from E18 lungs showed no differences in *Il1b*, *Cxcl10*, *Ccl3*, or the alveolar macrophage marker *Mrc1* (encoding CD206) between mutants and controls (Figure 5B). Therefore, while NF-κB activation in the fetal lung mesenchyme did recruit macrophages to the lung, it did not appear to be sufficient for causing macrophage activation.

Recent data have implicated inflammasome activation and subsequent IL-1β release as key steps linking inflammation and defective lung development.[18] Inflammasome assembly and activation are required for IL-1β release, and several genes encoding inflammasome components are induced by inflammatory signals. We therefore tested how mesenchymal IKKβ activity might affect inflammasome component expression. Compared with controls, E15 Twist2-IKKβca lungs expressed increased mRNA levels of *Nlrp3* and *Casp11* but similar levels of *Nlrc4* and *Asc* (Figure 6A). However,

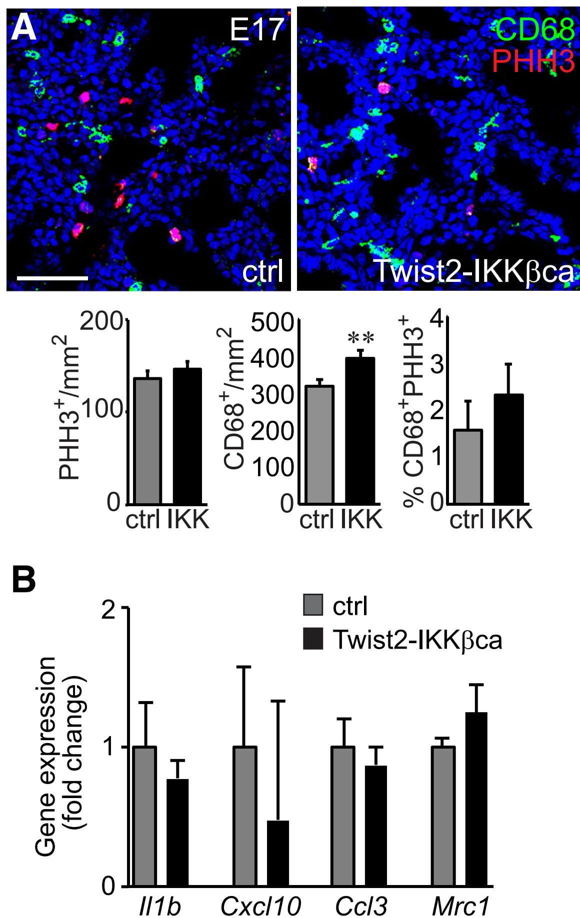

Figure 5 Mesenchyme specific NF-κB activation increases macrophage numbers in fetal lung. **A:** Identification of proliferation and fetal lung macrophages in E17 lung sections from Twist2—constitutively active IKKβ (IKKβca) and littermate control embryos. Sections were immunostained with antibodies against the macrophage marker CD68 and proliferation marker phospho-histone H3 (PHH3). Nuclei were labeled with DRAQ5. Twist2-IKKβca lungs contained increased numbers of $CD68^+$ macrophages, but with no increase in the number of macrophages positive for PHH3. **B:** Gene expression in isolated E18 fetal lung macrophages. Real-time PCR did not measure significantly different mRNA levels of *Il1b*, *Cxcl10*, *Ccl3*, or *Mrc1* in Twist2-IKKβca macrophages compared with littermate controls. Data are expressed as means ± SD. $n = 3$ (**A**); $n = 3$ to 5 (**B**). $^{**}P < 0.01$ versus control. Scale bar = 50 μm.

inflammasome component expression in cultured E15 lung mesenchymal cells was similar in cells from Twist2-IKKβca embryos and controls (Figure 6B). Twist2-IKKβca mesenchymal cells expressed higher *Ccl2*, consistent with our data from whole-lung samples. To test whether mesenchymal IKKβ activity could affect where inflammasome proteins were actually expressed *in vivo*, we used confocal microscopy to identify cells expressing the activated, cleaved form of caspase 1 and NLRP3 (Figure 6C). In E17 lungs, both cleaved caspase 1 and NLRP3 appeared to be restricted to macrophages. Cultured lung mesenchymal cells from Twist2-IKKβca embryos also did not release increased amounts of IL-1β protein upon stimulation with LPS and/or ATP, compared with controls (Figure 6D). Therefore, while IKKβ activity in mesenchymal cells increased the expression of the chemokine−encoding gene *Ccl2* and some inflammasome-encoding genes throughout the lung, we detected significant inflammasome assembly only in lung macrophages.

Discussion

Our data demonstrate that increased IKKβ activity in mesenchymal cells increased *Ccl2* expression but was not sufficient for affecting lung airway morphogenesis. We were surprised to find normal airway development in Twist2-IKKβca mice given data from our group and others using *in vivo* models and lung explants that show reduced saccular airway branching with endotoxin exposure or inflammatory activation in lung macrophages.[7,17,33,34] Inflammation in these other models influenced gene expression in both lung mesenchyme and epithelium. However, bypassing the initial macrophage-mediated response using the mesenchymal Twist2-Cre to drive the expression of IKKβca failed to cause the same degree of defects. Consistent with normal airway branching, embryonic lungs in Twist2-IKKβca mice did not have changes in the expression of *Fgf10*, a gene encoding an important upstream mesenchymal growth factor involved in branching morphogenesis.[22,35,36]

The inflammatory cytokine IL-1β inhibits late-stage lung development.[18,37] While *Il1b* mRNA can be induced by inflammatory stimuli, the release of the bioactive peptide requires cleavage of pro-IL-1β by inflammasome complexes containing activated caspases.[38] Both LPS and the transgenic expression of IKKβca in macrophages lead to IL-1β release in fetal lungs.[17,18] However, the Twist2-IKKβca lungs studied here did not contain increased *Il1b* mRNA. In addition, Twist2-IKKβca mesenchymal cells did not release IL-1β peptide when stimulated with LPS or the inflammasome activator ATP. Adding both agents may have released a small amount of IL-1β peptide, but the levels were not significantly above those in controls. Embryonic lung mesenchymal cells did not appear to express active inflammasome complexes. Several inflammasome gene components were higher in Twist2-IKKβca cells compared to controls. However, immunostaining for the inflammasome components NLRP3 and caspase 1 showed expression only in lung macrophages. So while the increased expression in Twist2-IKKβca lungs may have been consistent with previous data showing LPS induction of inflammasome genes,[18] the expression of the IKKβca transgene was not sufficient for generating the ectopic expression of functional inflammasome complexes in lung mesenchymal cells. We speculate that restricting inflammasome complex expression to professional immune cells such as macrophages both is energetically favorable and helps to limit the spread of inflammation and injury throughout developing tissues.

The chemokine-encoding gene *Ccl2* was elevated in Twist2-IKKβca lungs and mesenchymal cells, suggesting

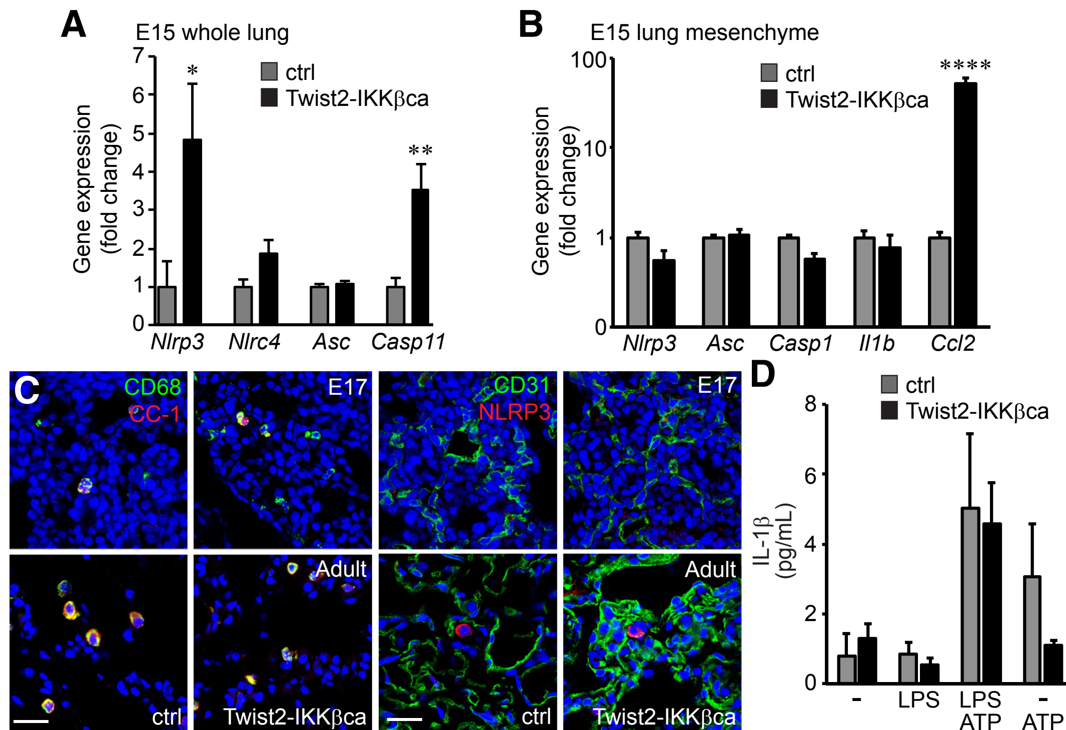

Figure 6 Mesenchyme-specific inhibitor of NF-κB kinase subunit β (IKK)-β activation does not induce inflammasome expression or IL-1β release. **A:** Expression levels of the inflammasome components *Nlrp3*, *Nlrc4*, *Asc*, and *Casp11* in total lung samples were measured by real-time PCR. *Nlrp3* and *Casp11* were expressed at higher levels in Twist2—constitutively active IKKβ (IKKβca) lungs compared with littermate controls. **B:** Expression of inflammasome components in E15 lung mesenchymal cells was similar in Twist2-IKKβca cells compared with controls. Increased expression of the chemokine *Ccl2* expression demonstrated NF-κB activation in Twist2-IKKβca cells. **C:** E17 and adult lung sections were immunostained for colocalization of inflammasome components cleaved caspase (CC)-1, (nucleotide-binding oligomerization domain-containing protein)-like receptor protein (NLRP)-3, macrophages (CD68), and endothelial cells (CD31). Nuclei were labeled with DRAQ5. CC1 and NLRP3 expression is restricted to macrophages in both E17 and adult lungs, both in Twist2-IKKβca and controls. **D:** Measurement of IL-1β in media of E15 lung mesenchymal cells from Twist2-IKKβca and control embryos treated with lipopolysaccharide (LPS) (250 ng/mL) and/or ATP (5 mmol/L) as measured by enzyme-linked immunosorbent assay. Cells from Twist2-IKKβca embryos did not release higher levels of IL-1β compared with control cells. Data are expressed as means ± SD. $n = 4$ (**A**); $n = 9$ (**B**); $n = 3$ to 5 (**D**). $^*P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$ versus control. Scale bars = 25 μm. —, no treatment.

that IKKβ/NF-κB activity in fetal lung mesenchymal cells may activate a different set of inflammatory mediators compared with macrophages or other immune cell populations. We recently published data showing the LPS response in fetal mouse lung mesenchymal cells.[39] While *Il1b*, *Tnf*, *Ccl3*, and *Ccl4* induction has been reported in LPS-treated macrophages,[40] LPS did not increase their expression in fetal mouse lung mesenchymal cells. LPS did, however, strongly increase the expression of other C-C and C-X-C motif chemokine family members, including *Ccl2*, *Ccl7*, *Ccl20*, *Cxcl1*, *Cxcl5*, and *Cxcl10*.[39] These differences suggest that the induction of genes like *IL1B* require transcription factors present in macrophages or other immune cells but perhaps missing in mesenchymal cell populations. Alternatively, mesenchymal cells may have differences in the chromatin landscape that produce a distinct transcriptional response to NF-κB activation.[41–43]

Embryonic lungs in Twist2-IKKβca mice had increased staining for the endothelial marker platelet endothelial cell adhesion molecule-1. This increase, even if subtle, could represent accelerated lung angiogenesis. Similar changes were seen after LPS-induced chemokine expression in lung explants and *in vivo*.[30] CCL2 can have multiple effects on lung cells, stimulating proliferation and migration of lung fibroblasts and airway smooth muscle cells.[44,45] In addition, CCL2—mediated increases in lung endothelial permeability appear to contribute to cancer cell metastases.[46,47] Alterations in lung capillary development and vascular permeability could play a role in bronchopulmonary dysplasia pathogenesis.[30,48–50] While CCL2 and other chemokines might not directly affect airway branching, the changes in lung vasculature and increased numbers of macrophages could predispose the immature lung to injury after a subsequent exposure. In addition, we observed decreased staining for the alveolar type I cell marker T1α. Type I alveolar epithelial cells likely play important roles in facilitating gas exchange and maintaining a dry alveolar space.[51] However, the consequences of the changes we observed and the mechanisms leading to these changes are not yet known.

These data emphasize the importance of considering cell specificity when designing therapeutic approaches for targeting the inflammatory response. Immune cells, including

resident lung macrophages and potentially recruited monocytes and neutrophils, may be the major cell populations that produce inflammatory mediators capable of inhibiting airway branching.[17,52] Likewise, growth factors important for lung development are expressed in specific cellular compartments.[53,54] Therefore, a strategy to affect inflammation-mediated arrest in lung development might need to specifically target macrophages, while increasing growth factor expression could require targeting mesenchymal cells, epithelia, or even vascular endothelia. Mechanistic *in vivo* approaches as presented here will help to characterize cell-specific responses and biological roles in lung injury and disease.

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