

Hypoxic Epithelial Necrosis Triggers Neutrophilic Inflammation via IL-1 Receptor Signaling in Cystic Fibrosis Lung Disease

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Abstract

Rationale: In many organs, hypoxic cell death triggers sterile neutrophilic inflammation via IL-1R signaling. Although hypoxia is common in airways from patients with cystic fibrosis (CF), its role in neutrophilic inflammation remains unknown. We recently demonstrated that hypoxic epithelial necrosis caused by airway mucus obstruction precedes neutrophilic inflammation in *Scnn1b*-transgenic (*Scnn1b*-Tg) mice with CF-like lung disease.

Objectives: To determine the role of epithelial necrosis and IL-1R signaling in the development of neutrophilic airway inflammation, mucus obstruction, and structural lung damage in CF lung disease.

Methods: We used genetic deletion and pharmacologic inhibition of IL-1R in *Scnn1b*-Tg mice and determined effects on airway epithelial necrosis; levels of IL-1 α , keratinocyte chemoattractant, and neutrophils in bronchoalveolar lavage; and mortality, mucus obstruction, and structural lung damage. Furthermore, we analyzed lung tissues from

21 patients with CF and chronic obstructive pulmonary disease and 19 control subjects for the presence of epithelial necrosis.

Measurements and Main Results: Lack of IL-1R had no effect on epithelial necrosis and elevated IL-1 α , but abrogated airway neutrophilia and reduced mortality, mucus obstruction, and emphysema in *Scnn1b*-Tg mice. Treatment of adult *Scnn1b*-Tg mice with the IL-1R antagonist anakinra had protective effects on neutrophilic inflammation and emphysema. Numbers of necrotic airway epithelial cells were elevated and correlated with mucus obstruction in patients with CF and chronic obstructive pulmonary disease.

Conclusions: Our results support an important role of hypoxic epithelial necrosis in the pathogenesis of neutrophilic inflammation independent of bacterial infection and suggest IL-1R as a novel target for antiinflammatory therapy in CF and potentially other mucoobstructive airway diseases.

Keywords: airway inflammation; airway epithelium; mucus obstruction; cystic fibrosis; chronic obstructive pulmonary disease

Recent studies in infants with cystic fibrosis (CF) diagnosed by newborn screening identified neutrophilic inflammation and air

trapping caused by small airways mucus obstruction as early abnormalities that are present in most infants often in the absence

of detectable bacterial infection and respiratory symptoms (1, 2). Neutrophilic inflammation has been implicated in

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At a Glance Commentary

Scientific Knowledge on the

Subject: Recent studies in infants with cystic fibrosis (CF) detected neutrophilic inflammation and small airways mucus obstruction as early abnormalities that are often present in the apparent absence of bacterial infection. IL-1 α derived from necrotic cells has been identified as a potent trigger of sterile neutrophilic inflammation. Although mucus-obstructed CF airways exhibit areas of hypoxia (i.e., a common cause of necrotic cell death), the role of hypoxic epithelial necrosis, IL-1 α , and IL-1R signaling in CF airway inflammation remains unknown.

What This Study Adds to the

Field: This study identifies hypoxic epithelial necrosis with release of IL-1 α and activation of IL-1R–myeloid differentiation primary response gene 88 signaling as a potent trigger of neutrophilic airway inflammation in mice with CF-like lung disease. Furthermore, we demonstrate an increased abundance of necrotic epithelial cells in mucus-obstructed airways from patients with CF and chronic obstructive pulmonary disease. These results support an important role of hypoxic epithelial necrosis in the *in vivo* pathogenesis of neutrophilic inflammation independent of bacterial infection and suggest IL-1R as a novel target for antiinflammatory therapy in patients with CF and potentially other mucoobstructive lung diseases.

several disease processes in CF lung disease including dysregulated epithelial ion transport and structural lung damage, and neutrophil elastase (NE), a major neutrophil product, was identified as a key risk factor for bronchiectasis and lung function decline in children with CF (3–9). Furthermore, evidence suggests that mediators released in neutrophilic airway inflammation contribute to goblet cell metaplasia and mucus hypersecretion under certain, but not all pathophysiologic conditions (9–11). However, the *in vivo* pathogenesis of neutrophilic inflammation

in the absence of apparent bacterial infection in early CF lung disease remains unknown.

Besides bacterial infection, cell injury and necrosis constitutes another principal stimulus of inflammation. Evidence from common diseases caused by tissue hypoxia, such as myocardial infarction and ischemic stroke, demonstrated that hypoxic cell death provides a potent trigger of sterile neutrophilic inflammation (12, 13). Whereas inflammation in infection is stimulated by binding of microbial components to diverse Toll-like receptors (TLR) that signal through the common adaptor protein myeloid differentiation primary response gene 88 (MyD88) (14), recent studies in mouse models of sterile peritonitis and liver necrosis identified activation of IL-1R as a key pathway linking necrosis with inflammation in the absence of infection (12, 15). IL-1R is a cytokine receptor that also uses MyD88 signaling and is activated by IL-1 α induced by dying cells. Although reduced oxygen tension caused by airway mucus plugging and increased epithelial oxygen consumption caused by increased absorption of airways surface liquid is a characteristic abnormality of CF airways (16, 17), the role of hypoxic necrosis and IL-1R–MyD88 signaling in CF airways inflammation has not been studied.

In previous studies using *Scnn1b*-Tg mice as a model of CF lung disease (18, 19), we demonstrated that airway surface dehydration, a key disease mechanism in CF, causes airway mucus obstruction in the first days of life, that early mucus plugging is associated with systemic and cellular hypoxia of airway epithelia, and that a subset of Clara cell undergoes hydropic degeneration and necrosis (20). Furthermore, we observed that the appearance of necrotic cells preceded the onset of airway neutrophilia in neonatal *Scnn1b*-Tg mice (20). The abundance of necrotic epithelial cells correlated with the severity of airway mucus obstruction (21) and was decreased when mucus plugging was reduced by preventive amiloride treatment in neonatal *Scnn1b*-Tg mice (22).

The aim of this study was, therefore, to determine the role of airway epithelial necrosis and IL-1R–MyD88 pathway activation in the *in vivo* pathogenesis of neutrophilic inflammation associated with mucus obstruction. To achieve this goal, we crossed *Scnn1b*-Tg mice with

IL-1R–deficient mice (*Il1r1*^{-/-}) and determined effects of genetic deletion of *Il1r1* on airway epithelial necrosis, IL-1 α levels, airway neutrophilia, mucus obstruction, structural lung damage, and mortality. Furthermore, we used the IL-1R antagonist (IL-1Ra) anakinra (15) to determine effects of pharmacologic inhibition of IL-1R signaling in adult *Scnn1b*-Tg mice with established lung disease. In addition, we compared expression profiles of genes implicated in airway inflammation in lungs from neonatal *Scnn1b*-Tg mice with mild versus severe mucus plugging using whole-genome microarray analysis. Finally, we used *in situ* hybridization and immunohistochemistry to localize IL-1 α expression in mouse lungs, and evaluated airways sections from patients with CF and chronic obstructive pulmonary disease (COPD) for the presence of necrotic epithelial cells. Some of the results of these studies have been previously reported in the form of an abstract (23).

Methods

Human Lung Tissues

Lung tissues from 13 patients with CF (29.2 \pm 1.9 yr) who underwent lung transplantation and eight patients with COPD (66.6 \pm 3.3 yr) diagnosed according to Global Initiative for Chronic Obstructive Lung Disease guidelines (24) were included in this study. Lung tissues from 16 never smokers (68.6 \pm 2.4 yr) with normal lung function who underwent surgical resection for suspected lung cancer and from three donors with no lung disease (15.3 \pm 5.5 yr) were included as control subjects. This study was approved by the ethics committees of the University of Heidelberg and the Hannover Medical School, and the institutional review board of the University of North Carolina at Chapel Hill. Informed written consent was obtained from all subjects. Additional information is provided in the online supplement.

Experimental Animals

All animal studies were approved by the Regierungspräsidium Karlsruhe, Germany. *Scnn1b*-Tg mice (18) were backcrossed onto the C57BL/6 background (21) and intercrossed with *Il1r1*^{-/-} mice on the C57BL/6 background (Jackson Laboratory, Bar Harbor, ME) (25) to

generate *Scnn1b-Tg/Il1r1^{-/-}*, *Scnn1b-Tg, Il1r1^{-/-}*, and wild-type (WT) mice in the second filial generation. Offspring were genotyped as described (18, 25) and mice were studied at neonatal (5 d) or adult (6 wk) ages. Microarray analyses were performed in neonatal (3 d old) and anakinra treatment studies in adult (4–6 wk old) *Scnn1b-Tg* mice and WT littermate control animals on the original mixed genetic background (C57BL/6N × C3H/HeN) (18, 20). Mice were housed in a specific pathogen-free animal facility and had free access to chow and water.

Anakinra Treatment

Adult (4–6 wk old) *Scnn1b-Tg* mice and WT littermates were treated with the IL-1Ra anakinra (Kineret; Swedish Orphan Biovitrum AB, Stockholm, Sweden) (15) by subcutaneous injection of 200 mg/kg body weight, or equal volumes of vehicle (NaCl 0.9%) alone, twice per day for a period of 7 days. Twelve hours after the last treatment, bronchoalveolar lavage (BAL) was performed and lungs were removed for further analysis as described in the online supplement.

BAL Cell Counts and Cytokine Measurements

Mice were anesthetized via intraperitoneal injection of 120 mg/kg ketamine and 16 mg/kg xylazine. BAL, differential cell counts, and ELISA of cytokines were performed as previously described (20). Additional details are provided in the online supplement.

Histology and Morphometry

Degenerative airway epithelial cells were identified by morphologic criteria (i.e., cell swelling with cytoplasmic vacuolization) and quantified by counting the number of degenerative cells per millimeter of the basement membrane. Histologic and morphometric analyses of airway mucus content and assessment of distal airspace enlargement and alveolar wall destruction were performed as previously described (20, 26, 27) and detailed in the online supplement.

Immunohistochemistry

Immunohistochemical staining for IL-1 α was performed using a goat polyclonal antimouse-IL-1 α antibody (R&D Systems, Minneapolis, MN) as previously described (28) and detailed in the online supplement.

RNA In Situ Hybridization

Nonradioactive *in situ* hybridization of IL-1 α transcripts was performed on 10- μ m sections of paraffin-embedded lung tissues using custom designed 5' and 3' DIG-labeled IL-1 α antisense and sense locked nucleic acid–modified oligonucleotide probes (Exiqon, Woburn, MA) as previously described (29) and detailed in the online supplement.

Microarray Analysis

Microarray analysis was performed using Mouse Genome 430 2.0 Microarrays (Affymetrix, Santa Clara, CA) and quantitative real-time reverse transcriptase polymerase chain reaction was performed for confirmation of candidate genes as described in the online supplement.

Statistics

Data were analyzed with SigmaStat version 3.1 (Systat Software, Erkrath, Germany) and are reported as mean \pm SEM. Statistical analyses were performed using Student *t* test, Mann-Whitney rank sum test, one-way analysis of variance, chi-square test, and Pearson product-moment correlation as appropriate and *P* less than 0.05 was accepted to indicate statistical significance.

Results

Lack of IL-1R Reduces Neutrophilic Airway Inflammation Associated with Epithelial Necrosis in *Scnn1b-Tg* Mice

To determine if IL-1R is implicated in the onset of neutrophilic airway inflammation associated with airway epithelial necrosis in early lung disease in *Scnn1b-Tg* mice (20), we compared numeric densities of degenerative airway epithelial cells, BAL inflammatory cell counts, macrophage size, and levels of the keratinocyte chemoattractant (KC), IL-1 α , IL-1 β , and IL-1Ra in 5-day-old neonatal *Scnn1b-Tg/Il1r1^{-/-}* mice with *Scnn1b-Tg, Il1r1^{-/-}* and WT littermates (Figure 1). Degenerative airway epithelial cells were rarely observed in WT and *Il1r1^{-/-}* mice, but were significantly increased in *Scnn1b-Tg* mice (Figures 1A and 1B). Epithelial necrosis in neonatal *Scnn1b-Tg* mice was associated with an increase in macrophages and neutrophils, increased macrophage size (Figure 1C), and elevated levels of KC, IL-1 α , IL-1 β , and IL-1Ra in BAL (Figure 1D). Lack of IL-1R had no effect on increased levels of degenerative epithelial

cells, BAL macrophages, and IL-1 α , but airway neutrophilia was abrogated and KC, IL-1 β , and IL-1Ra were significantly reduced in *Scnn1b-Tg/Il1r1^{-/-}* mice compared with *Scnn1b-Tg* littermates.

To localize IL-1 α mRNA and protein expression in lungs of neonatal WT and *Scnn1b-Tg* mice, we performed *in situ* hybridization and immunohistochemistry (Figure 2). With both techniques, we obtained distinct IL-1 α signals in the epithelium lining the conducting airways and to a lesser extent in lung parenchyma of WT and *Scnn1b-Tg* mice (Figure 2). Consistent with the translocation of IL-1 α protein to the nucleus (13), immunoreactive signals were clearly detected in the cytoplasm and nucleus of epithelial cells, whereas only faint staining was observed in macrophages sporadically detected in lung sections from WT and *Scnn1b-Tg* mice (Figure 2B; see Figure E1 in the online supplement). Taken together, these results show constitutive expression of IL-1 α by the airway epithelium and lung parenchyma, and suggest that epithelial cells undergoing necrosis constitute a major source of increased IL-1 α levels in neonatal *Scnn1b-Tg* mice.

In 6-week-old adult *Scnn1b-Tg* mice, absolute numbers of degenerative airway epithelial cells were lower than at neonatal ages, but remained elevated compared with WT control animals (Figure 3A). Similar to the results obtained in the neonatal studies, lack of IL-1R had no effects on the level of degenerative airway cells (Figure 3A), but neutrophils and KC were significantly reduced in BAL from *Scnn1b-Tg/Il1r1^{-/-}* mice compared with *Scnn1b-Tg* mice (Figures 3B and 3C). Collectively, these results demonstrate that IL-1R signaling plays an important role in the initiation and perpetuation of airway neutrophilia and suggest that necrotic cell death and release of IL-1 α in mucus-obstructed hypoxic airways contribute to IL-1R–dependent neutrophilic airway inflammation in *Scnn1b-Tg* mice.

Lack of IL-1R Reduces Airway Mucus Obstruction and Mortality in *Scnn1b-Tg* Mice

Because chronic neutrophilic inflammation is associated with airway mucus plugging, we determined effects of genetic deletion of IL-1R on mucus obstruction and related mortality in *Scnn1b-Tg* mice (20). Consistent with spontaneous postnatal

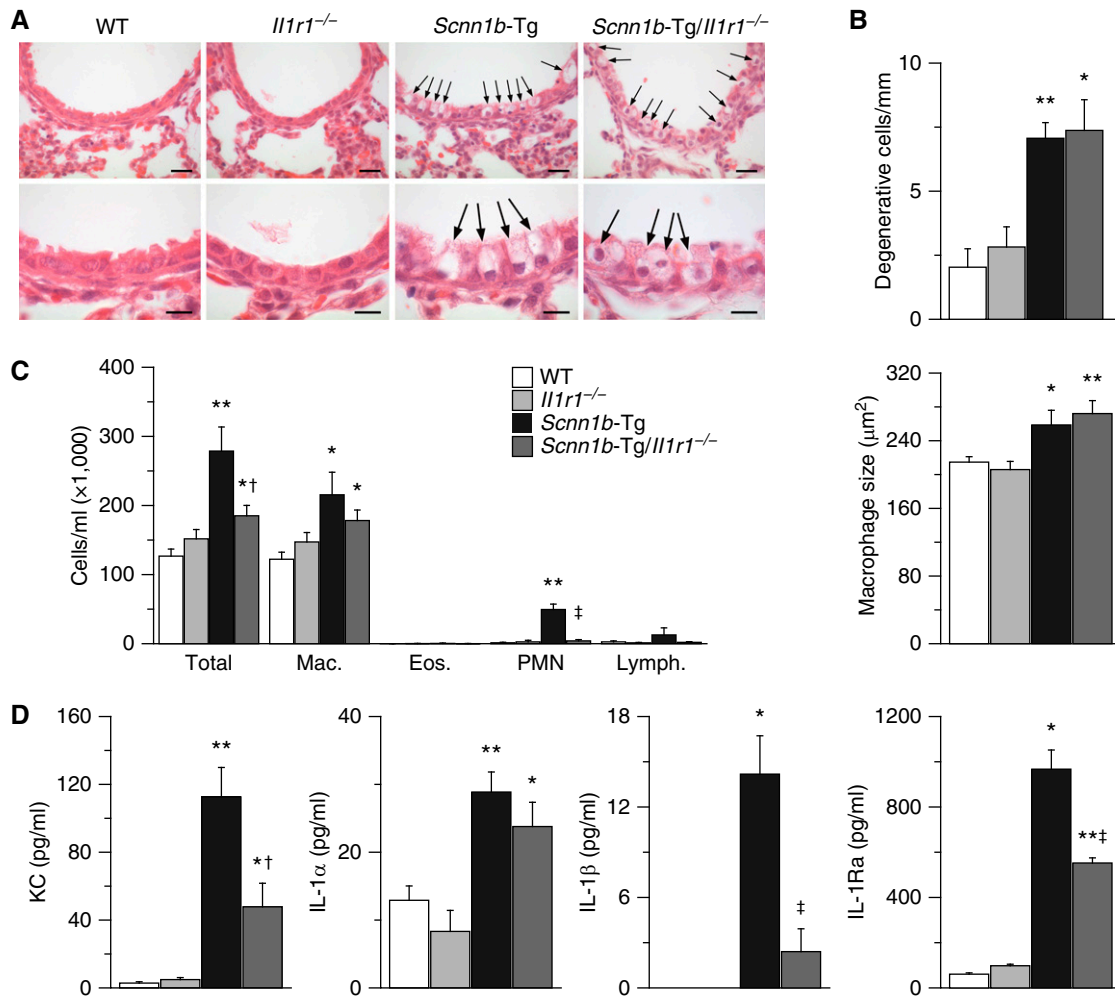


Figure 1. Lack of IL-1R reduces neutrophilic airway inflammation associated with epithelial necrosis in neonatal *Scnn1b*-Tg mice. Comparison of airway epithelial necrosis (A and B), bronchoalveolar lavage (BAL) inflammatory cell counts and macrophage size (C) and keratinocyte chemoattractant (KC), IL-1 α , IL-1 β , and IL-1Ra concentrations (D) in 5-day-old wild-type (WT), *Il1r1*^{-/-}, *Scnn1b*-Tg, and *Scnn1b*-Tg/*Il1r1*^{-/-} mice. (A) Representative airway sections were stained with hematoxylin and eosin to determine the number of degenerative epithelial cells (arrows). Scale bars = 20 μ m (top) and 10 μ m (bottom). (B) Summary of degenerative epithelial cells per millimeter of the basement membrane. n = 5–10 mice per group from 16 litters. * P < 0.01 and ** P \leq 0.001 compared with WT mice. (C) BAL cell counts and macrophage size. n = 8–18 mice per group from 22 litters. * P < 0.01 and ** P \leq 0.001 compared with WT mice. † P < 0.05 and ‡ P \leq 0.001 compared with *Scnn1b*-Tg mice. (D) KC, IL-1 α , IL-1 β , and IL-1Ra concentration in BAL. n = 4–10 mice per group from 15–20 litters. * P < 0.05 and ** P \leq 0.001 compared with WT mice. † P < 0.05 and ‡ P < 0.01 compared with *Scnn1b*-Tg mice. Eos. = eosinophils; Lymph. = lymphocytes; Mac. = macrophages; PMN = neutrophils.

mortality observed in previous studies (18, 20), the percentage of surviving *Scnn1b*-Tg mice was significantly lower than the expected mendelian ratio for the intercross of *Scnn1b*-Tg with *Il1r1*^{-/-} mice, whereas *Scnn1b*-Tg/*Il1r1*^{-/-} mice were protected from postnatal mortality (Figure 4A). Morphometric analysis of airway sections from 6-week-old adult mice demonstrated that airway mucus obstruction in *Scnn1b*-Tg mice was significantly reduced in *Scnn1b*-Tg/*Il1r1*^{-/-} mice (Figures 4B and 4C). The notion that IL-1R signaling is implicated in airway mucus obstruction

was supported by independent studies using whole-genome cDNA microarray analysis of lungs from neonatal *Scnn1b*-Tg mice on a mixed genetic background (C57BL/6N \times C3H/HeN) that develop more heterogeneous disease (18, 20) and were selected for exhibiting either mild (\leq 20% of luminal area) or severe (\geq 80% of luminal area) tracheal mucus obstruction (Figures 5A and 5B).

Seeking for genes that were differentially expressed in *Scnn1b*-Tg mice with mild versus severe mucus plugging, with a focus on genes implicated in inflammation using

custom gene set and gene set enrichment analysis, we found that *Il1ra* was differentially up-regulated in *Scnn1b*-Tg mice with mild mucus obstruction (Figure 5D). Significant up-regulation of *Il1ra* (approximately threefold) in lungs from *Scnn1b*-Tg mice with mild versus severe airway obstruction was confirmed by real-time reverse transcriptase polymerase chain reaction (Figure 5E). Of note, the number of necrotic airway cells did not differ in *Scnn1b*-Tg mice with mild versus severe tracheal obstruction indicating that differences in *Il1ra* expression, rather than

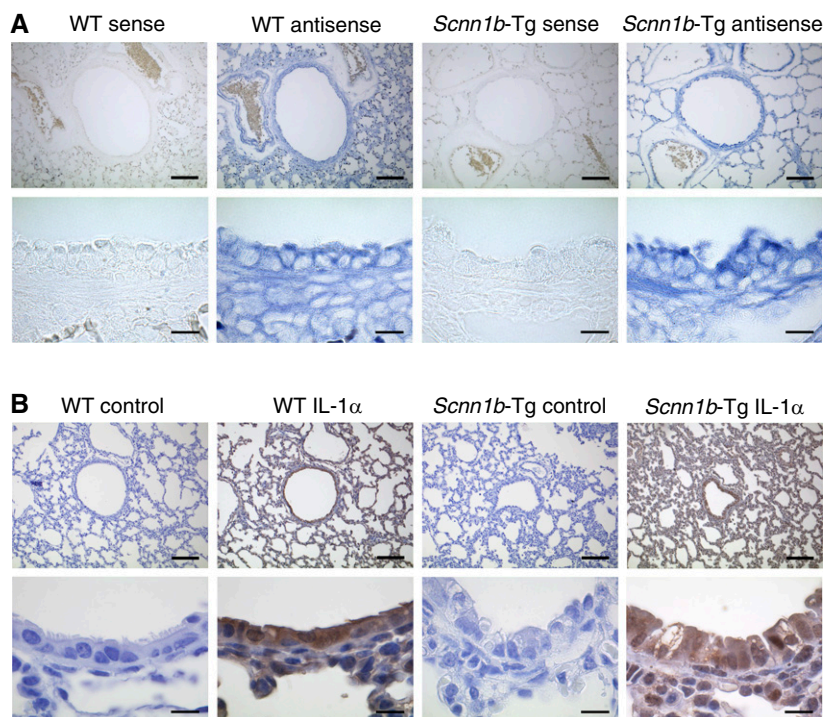


Figure 2. Localization of IL-1 α in lungs from neonatal wild-type (WT) and *Scnn1b*-Tg mice. (A) *In situ* hybridization of lung sections from neonatal (5 d old) WT and *Scnn1b*-Tg mice with locked nucleic acid-modified sense and antisense IL-1 α mRNA detection probes. Representative localization of IL-1 α transcripts (dark blue) in airway and lung parenchymal cells in WT and *Scnn1b*-Tg lungs hybridized with antisense probe. Staining was not detected in lung sections hybridized with sense probes. Scale bars = 100 μ m (top) and 10 μ m (bottom). (B) Immunohistochemical detection of IL-1 α in lung sections from neonatal WT and *Scnn1b*-Tg mice. Representative localization of IL-1 α (brown) in cytoplasm and nuclei of airway epithelial cells and some parenchymal cells in WT and *Scnn1b*-Tg lungs stained with primary anti-IL-1 α antibody (IL-1 α). Staining was not detected in lung sections incubated with secondary antibody only (control). All sections were counterstained with hematoxylin. Scale bars = 100 μ m (top) and 10 μ m (bottom). Representative of n = 3–4 mice per group from six litters.

necrotic cell death, modulated early mucus obstruction in neonatal *Scnn1b*-Tg mice (Figure 5C). Collectively, these results demonstrate that IL-1R signaling is

implicated in airway mucus obstruction and associated mortality in *Scnn1b*-Tg mice, and suggest IL-1Ra as a potential modifier of mucus plugging.

Lack of IL-1R Reduces Emphysema in *Scnn1b*-Tg Mice

Chronic neutrophilic inflammation is associated with emphysema in *Scnn1b*-Tg mice (20, 30). To determine if IL-1R signaling is implicated in the *in vivo* pathogenesis of emphysema, we compared mean linear intercepts as a measure of distal airspace enlargement and destructive index as a measure of alveolar wall destruction in 6-week-old *Scnn1b*-Tg/*Il1r1*^{-/-} mice with *Scnn1b*-Tg, *Il1r1*^{-/-} and WT littermates. As expected from previous studies, mean linear intercepts and destructive index were significantly increased in *Scnn1b*-Tg mice compared with WT (Figure 6). In *Scnn1b*-Tg/*Il1r1*^{-/-} mice, emphysema parameters were significantly reduced compared with *Scnn1b*-Tg mice (Figure 6B). These results suggest an important role of IL-1R signaling in structural lung damage characteristic of chronic lung disease in *Scnn1b*-Tg mice.

Pharmacologic Inhibition of IL-1R Signaling Reduces Neutrophilic Airway Inflammation and Emphysema in Adult *Scnn1b*-Tg Mice

To determine effects of pharmacologic inhibition of IL-1R signaling, we treated adult (4–6 wk old) *Scnn1b*-Tg mice and WT littermates by subcutaneous injections of the IL-1Ra anakinra for a period of 7 days. Similar to genetic deletion of IL-1R, treatment with anakinra had no effects on epithelial necrosis, but abrogated airway neutrophilia almost completely and reduced KC levels in BAL significantly in *Scnn1b*-Tg mice (Figures 7A–7C).

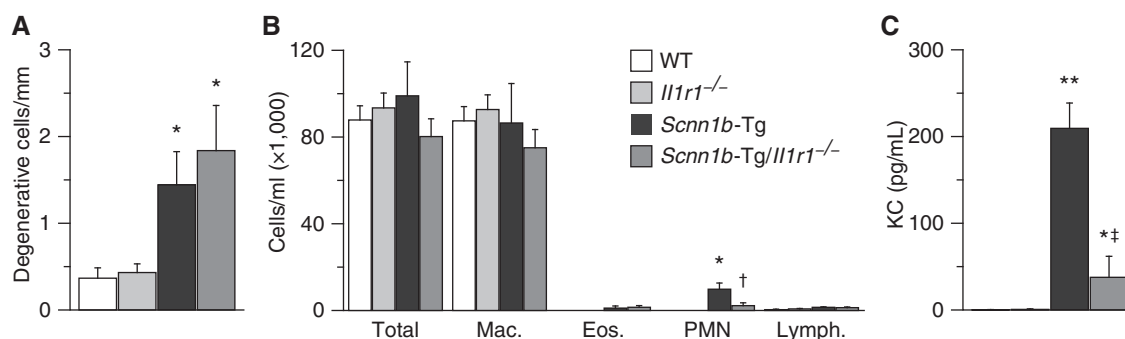


Figure 3. Lack of IL-1R reduces neutrophilic airway inflammation in adult *Scnn1b*-Tg mice. Comparison of airway epithelial necrosis (A), bronchoalveolar lavage (BAL) inflammatory cell counts (B), and keratinocyte chemoattractant (KC) levels (C) in 6-week-old wild-type (WT), *Il1r1*^{-/-}, *Scnn1b*-Tg, and *Scnn1b*-Tg/*Il1r1*^{-/-} mice. (A) Summary of degenerative epithelial cells per millimeter of basement membrane. n = 5–6 mice per group from 14 litters. **P* < 0.05 compared with WT mice. (B and C) Inflammatory cell counts (B) and concentration of KC (C) in BAL. n = 4–13 mice per group from 11–15 litters. **P* < 0.01 and ***P* ≤ 0.001 compared with WT mice. †*P* < 0.01 and ‡*P* ≤ 0.001 compared with *Scnn1b*-Tg mice. Eos. = eosinophils; Lymph. = lymphocytes; Mac. = macrophages; PMN = neutrophils.

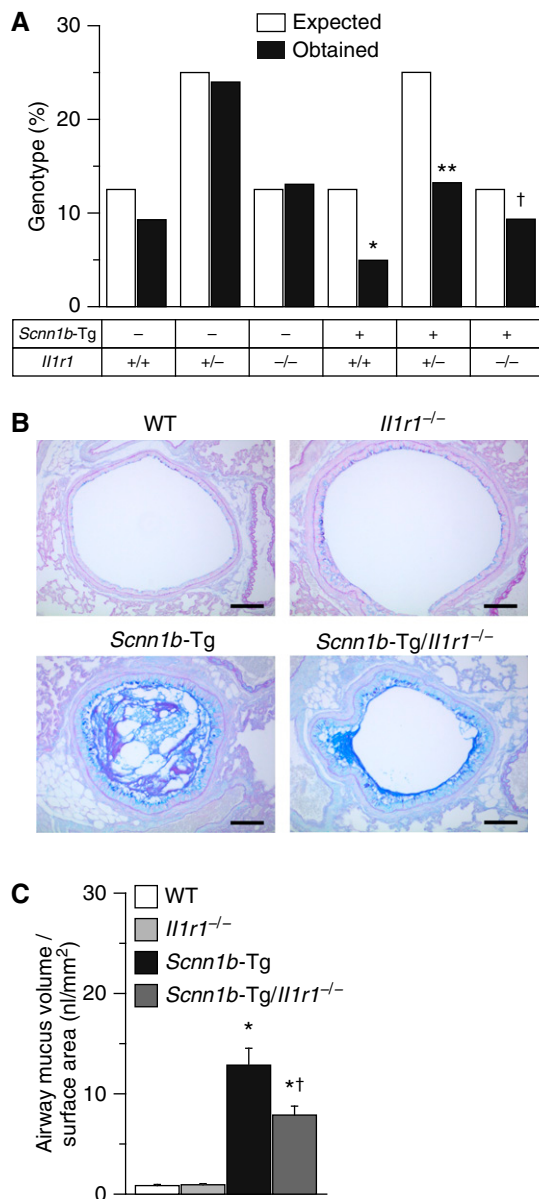


Figure 4. Lack of IL-1R reduces mortality and airway mucus obstruction in *Scnn1b*-Tg mice. Comparison of survival (A) and airway mucus obstruction (B and C) in 6-week-old wild-type (WT), *Il1r1*^{-/-}, *Scnn1b*-Tg, and *Scnn1b*-Tg/*Il1r1*^{-/-} mice. (A) Frequencies of expected versus obtained genotypes of mice derived from the intercross of *Scnn1b*-Tg with *Il1r1*^{-/-} mice that survived until the age of 6 weeks. $n = 32\text{--}154$ mice per group from 66 litters. * $P < 0.05$ for expected versus obtained genotype frequencies in *Scnn1b*-Tg compared with WT mice. ** $P \leq 0.001$ for expected versus obtained genotype frequencies in *Scnn1b*-Tg/*Il1r1*^{-/-} compared with *Il1r1*^{+/-} mice. † $P < 0.05$ for expected versus obtained genotype frequencies in *Scnn1b*-Tg/*Il1r1*^{-/-} compared with *Scnn1b*-Tg mice. (B) Representative sections of proximal main axial airways stained with alcian blue periodic acid-Schiff. Scale bar = 100 μm . (C) Airway mucus content determined from the presence of alcian blue periodic acid-Schiff-positive material in airways from 6-week-old mice. $n = 20\text{--}28$ mice per group from 23 litters. * $P \leq 0.001$ compared with WT mice. † $P < 0.01$ compared with *Scnn1b*-Tg mice.

Furthermore, anakinra treatment reduced emphysema, as determined from measurements of mean linear intercepts and the destructive index (Figures 7D and

7E; see Figure E2). In addition, we observed a trend toward reduction of airway mucus obstruction ($\sim 40\%$; $P = 0.058$) in anakinra-treated versus vehicle-treated *Scnn1b*-Tg

mice (Figure 7F). These results demonstrate that pharmacologic inhibition of IL-1R signaling reduces neutrophilic inflammation and associated structural lung damage in adult *Scnn1b*-Tg mice with chronic lung disease.

Epithelial Degeneration Is Present in Mucus-obstructed Airways of Patients with CF and COPD

Our studies in mice with CF-like lung disease indicate that epithelial necrosis in mucus-obstructed airways may play an important role in the *in vivo* pathogenesis of neutrophilic inflammation. However, the occurrence of necrotic epithelial cells in airways from patients with CF and other chronic obstructive lung diseases remains unknown. We therefore evaluated sections of small airways (< 2 mm diameter) from patients with CF or COPD and nonsmoking control subjects for the presence of degenerative epithelial cells (Figures 8A and 8C) and airway mucus (Figures 8A and 8B). Hydropic, highly vacuolated degenerative epithelial cells similar to the cells observed in *Scnn1b*-Tg mice (Figure 1A) were rarely observed in control subjects, but were significantly increased in mucus-obstructed airways from patients with CF (Figures 8A and 8C). Similarly, numeric densities of degenerative cells were increased in COPD compared with control subjects (Figures 8A and 8C). Of note, the number of necrotic airway epithelial cells was significantly correlated ($r = 0.53$; $P < 0.01$) with the severity of airway mucus obstruction (Figure 8D). These results provide initial evidence that epithelial necrosis may be implicated in the pathogenesis of CF and potentially other chronic obstructive lung diseases in humans.

Discussion

Neutrophilic airway inflammation plays a key role in the onset and progression of lung disease in CF and other chronic obstructive lung diseases, even in the apparent absence of bacterial infection (1, 4, 5, 9, 31–33). However, stimuli other than infection that can trigger airway neutrophilia remain poorly understood and therapeutic benefits of current antiinflammatory and antiinfective therapies remain limited. The importance of neutrophilic inflammation in early CF

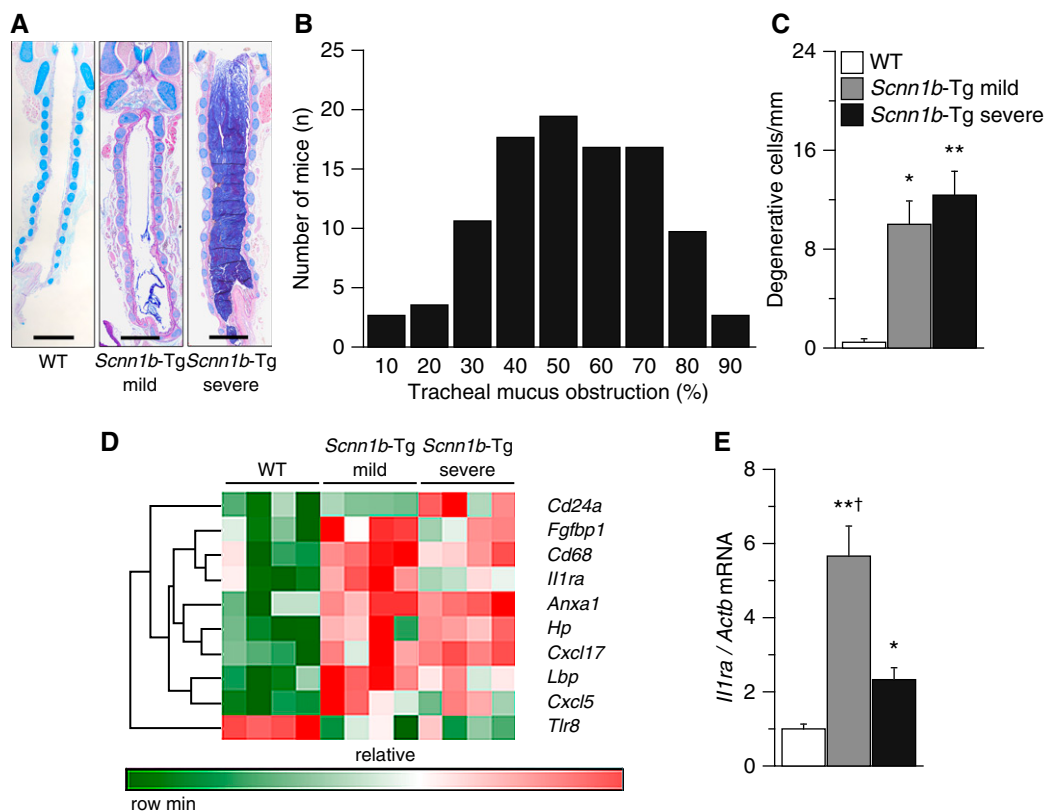


Figure 5. Severity of lung disease is associated with IL-1Ra transcript levels in neonatal *Scnn1b*-Tg mice. (A–C) Quantification of tracheal mucus obstruction and microarray gene expression analysis of 3-day-old *Scnn1b*-Tg and wild-type (WT) mice maintained on a mixed genetic background (C57BL/6N × C3H/HeN). (A) Representative sections of trachea from a 3-day-old WT mouse, a *Scnn1b*-Tg mouse with mild mucus obstruction, and a *Scnn1b*-Tg mouse with severe mucus obstruction stained with alcian blue periodic acid–Schiff. Scale bar = 500 μ m. (B) Distribution of severity of tracheal mucus obstruction as determined from the percentage of the cross-sectional area of the tracheal lumen obstructed with alcian blue periodic acid–Schiff–positive material in 3-day-old *Scnn1b*-Tg mice. $n = 102$ mice from 31 litters. (C) Summary of degenerative epithelial cells per millimeter of the basement membrane in airways from WT mice and *Scnn1b*-Tg mice with mild ($\leq 20\%$ of luminal area) or severe ($\geq 80\%$ of luminal area) mucus obstruction. $n = 6$ –15 mice per group from 15 litters. * $P < 0.01$ and ** $P \leq 0.001$ compared with WT mice. (D) Heat map of genes related to inflammation that showed significant changes in expression in WT mice compared with *Scnn1b*-Tg mice with mild ($\leq 20\%$ of luminal area) or severe ($\geq 80\%$ of luminal area) mucus obstruction. $n = 4$ mice per group. (E) Quantitative real-time reverse transcriptase polymerase chain reaction for *Il1ra* expression in lungs from WT mice, *Scnn1b*-Tg mice with mild mucus obstruction, and *Scnn1b*-Tg mice with severe mucus obstruction. $n = 6$ –7 mice per group from 10 litters. * $P < 0.01$ and ** $P \leq 0.001$ compared with WT mice. † $P < 0.01$ compared with severely obstructed *Scnn1b*-Tg mice.

pathogenesis was buttressed by recent studies demonstrating that airway neutrophilia and mucus obstruction are already present in infants with CF, mostly in the absence of detectable bacterial infection (1, 2). However, these observational studies in infants with CF did not permit determination of whether inflammation occurred before or after the onset of mucus obstruction, and what stimuli trigger early inflammation in the absence of infection.

In previous longitudinal studies in *Scnn1b*-Tg mice with CF-like lung disease, we demonstrated that airway mucus plugging caused by increased absorption of Na^+ and liquid from airway surfaces was associated with cellular hypoxia and

necrosis of a subset of airway Clara cells, as evidenced by *in vivo* labeling with the hypoxia probe pimonidazole hydrochloride and transmission electron microscopy studies showing intracellular glycogen depletion, endoplasmic reticulum (ER) vacuolarization, and pyknotic nuclei as sign of necrosis (20). Necrotic cells were observed from the first days of life before the onset of neutrophilic inflammation, whereas chronic airway neutrophilia with elevated levels of the neutrophil chemoattractant KC, the functional ortholog of IL-8 in humans, was detected from postnatal day 5 onward (20, 21). Interestingly, neutrophilic inflammation was also observed in *Scnn1b*-Tg mice raised in germ-free conditions demonstrating that

inflammation occurred even with greatly reduced levels of pathogen-associated molecular patterns that activate MyD88 signaling via TLR (34).

Based on these results, and reports that activation of MyD88 signaling via IL-1R on nonhematopoietic cells by IL-1 α secreted in response to necrotic cell death constitutes a potent stimulus of sterile neutrophilic inflammation in other organs (12, 13) we investigated the role of this pathway in the development of airway neutrophilia in *Scnn1b*-Tg mice. We found that genetic deletion of IL-1R had no effect on elevated necrotic airway epithelial cells and IL-1 α , but abrogated airway neutrophilia and reduced KC in 5-day-old neonatal *Scnn1b*-Tg mice (Figure 1). Besides IL-1 α that is

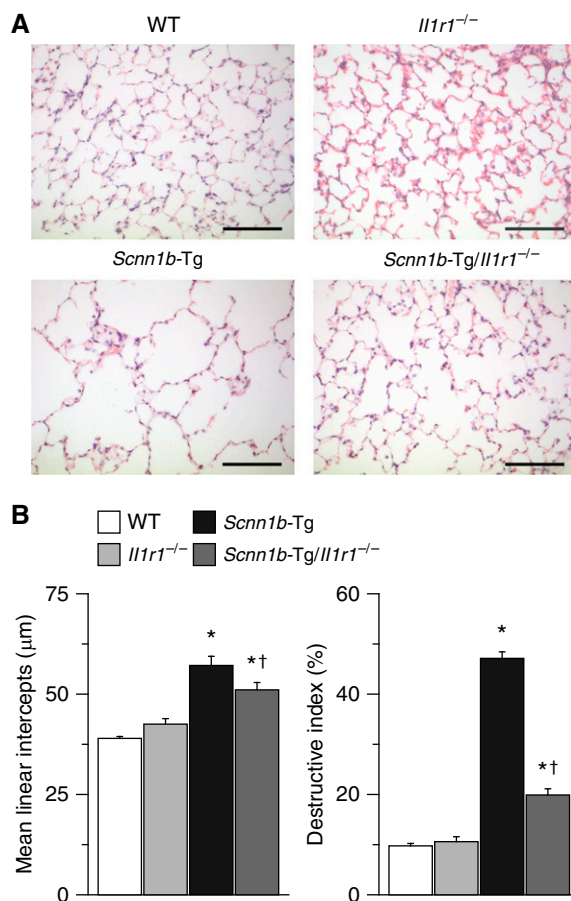


Figure 6. Lack of IL-1R reduces emphysema in *Scnn1b*-Tg mice. Comparison of distal airspace morphology (A) and mean linear intercepts and destructive index (B) in 6-week-old wild-type (WT), *Il1r1*^{-/-}, *Scnn1b*-Tg, and *Scnn1b*-Tg/*Il1r1*^{-/-} mice. (A) Representative lung sections stained with hematoxylin and eosin. Scale bars = 100 μm. (B, left) Summary of mean linear intercepts. n = 17–19 mice per group from 27 litters. **P* ≤ 0.001 compared with WT mice. †*P* < 0.05 compared with *Scnn1b*-Tg mice. (B, right) Destructive index. n = 6 mice per group from 13 litters. **P* ≤ 0.001 compared with WT mice. †*P* ≤ 0.001 compared with *Scnn1b*-Tg mice.

released in a fully functional form, IL-1R is activated by IL-1β that requires caspase-1-dependent activation of its inactive precursor in the inflammasome (15). In addition to neutrophils and KC, IL-1β was elevated in 5-day-old *Scnn1b*-Tg mice, but reduced to near normal levels in *Scnn1b*-Tg/*Il1r1*^{-/-} mice (Figure 1), indicating that secretion of IL-1β occurred secondary to IL-1α-mediated activation of IL-1R-MyD88 signaling and inflammation. Tissue localization by *in situ* hybridization and immunohistochemistry showed distinct signals for IL-1α transcripts and protein in the airway epithelium and to a lesser extent in lung parenchyma, but not in macrophages contained in lung sections from WT and *Scnn1b*-Tg mice (Figure 2; see Figure E1). As observed in neonatal mice, necrotic airway epithelial cells

remained elevated, but airway neutrophilia and KC were significantly reduced in adult *Scnn1b*-Tg/*Il1r1*^{-/-} compared with *Scnn1b*-Tg mice (Figure 3). Collectively, these results identify activation of IL-1R-MyD88 signaling by IL-1α that is released from airway epithelial cells undergoing necrosis, rather than infiltrating macrophages responding to epithelial injury, as an important pathway in the onset and perpetuation of neutrophilic inflammation in mice with CF-like lung disease.

Our studies also demonstrate that IL-1R-MyD88 pathway activation by epithelial necrosis contributes to CF-like airway mucus obstruction in *Scnn1b*-Tg mice. Previous longitudinal studies demonstrated that a subgroup of *Scnn1b*-Tg mice die in the neonatal period because of severe mucus plugging of the trachea and that

adult survivors exhibit chronic mucus obstruction of intrapulmonary airways. Consistent with a role of IL-1R-MyD88 signaling in airway mucus obstruction in the present study, survival was significantly increased and airway mucus content was reduced in adult *Scnn1b*-Tg/*Il1r1*^{-/-} compared with *Scnn1b*-Tg mice (Figure 4). The importance of IL-1R-MyD88 signaling in early mucus obstruction was supported by independent studies using whole-genome expression profiling of lungs from neonatal *Scnn1b*-Tg mice on a mixed genetic background with variable mucus obstruction (20) that identified an inverse relationship between the severity of tracheal mucus plugging and expression of *Il1ra* (Figure 5). Of note, previous studies from various laboratories showed that activation of MAPK and nuclear factor-κB via TLR-MyD88 signaling induces mucin expression in mice and human airway cultures (35–37) establishing a role of this pathway in mucus hypersecretion associated with airway infection and inflammation. Furthermore, it was shown that genetic deletion of the adaptor molecule MyD88 reduced goblet cell metaplasia and airway mucus plugging in neonatal *Scnn1b*-Tg mice in the context of bacterial airway infection. However, whether MyD88 was activated via TLRs or IL-1R remained unknown (34).

We recently demonstrated that lack of NE, another potent stimulus of mucus hypersecretion (10), reduced goblet cell metaplasia and reduced elevated mucin expression. However, in contrast to genetic deletion of *Il1r*, lack of NE was not sufficient to prevent airway mucus obstruction and associated mortality in *Scnn1b*-Tg mice (9). Based on these results, we speculate that activation of the IL-1R-MyD88 pathway by IL-1α released from necrotic epithelial cells provides an alternative stimulus of mucin hypersecretion that may aggravate mucus hyperconcentration and plugging independent of pathogens and NE. In addition, these results suggest that the endogenous inhibitor of this pathway, IL-1Ra, may act not only as a modulator of inflammation, but also as a potential modifier of mucus hypersecretion and obstruction. However, in contrast to previous studies where preventive treatment with the epithelial sodium channel blocker amiloride abrogated airway mucus obstruction almost completely and reduced necrotic cells substantially

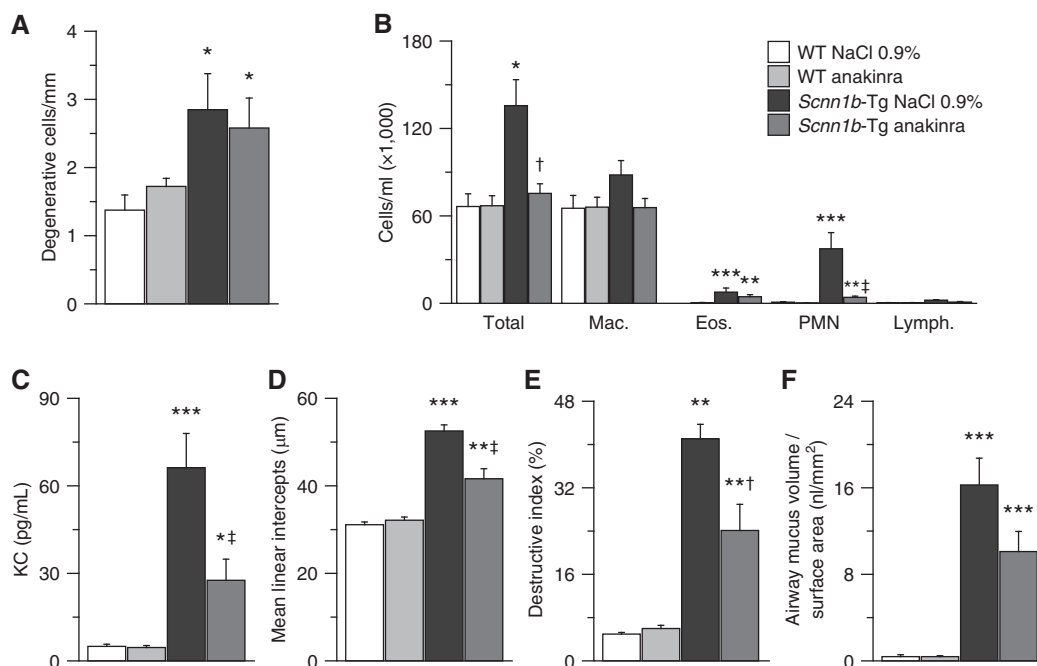


Figure 7. Anakinra treatment reduces neutrophilic airway inflammation and emphysema in *Scnn1b*-Tg mice. Adult (4–6 wk old) wild-type (WT) and *Scnn1b*-Tg mice were treated with subcutaneous injection of anakinra (200 mg/kg body weight) or vehicle (NaCl 0.9%) alone twice per day for 7 days. Comparison of airway epithelial necrosis (A), bronchoalveolar lavage (BAL) inflammatory cell counts (B), and keratinocyte chemoattractant (KC) levels (C). (A) Summary of degenerative epithelial cells per millimeter of basement membrane. $n = 6$ –9 mice per group. (B) Inflammatory cell counts and (C) concentrations of KC in BAL. $n = 11$ –16 mice per group. (D and E) Comparison of mean linear intercepts (D) and destructive index (E). $n = 6$ –9 mice per group. (F) Airway mucus content determined from the presence of alcian blue periodic acid–Schiff–positive material in airways. $n = 14$ –16 mice per group. * $P < 0.05$, ** $P < 0.01$, and *** $P \leq 0.001$ compared with vehicle-treated WT mice. † $P < 0.05$ and ‡ $P < 0.01$ compared with vehicle-treated *Scnn1b*-Tg mice. Data were derived from four independent studies with mice from 14 litters. Eos. = eosinophils; Lymph. = lymphocytes; Mac. = macrophages; PMN = neutrophils.

(~50%) (22), neither genetic deletion nor pharmacologic inhibition of IL-1R signaling had effects on epithelial necrosis in *Scnn1b*-Tg mice (Figures 1, 3, 7). We therefore speculate that the moderate (~40%) reduction in airway mucus content obtained by inhibition of IL-1R signaling was not sufficient to improve luminal O₂ delivery to levels that result in a measurable reduction of epithelial necrosis in the presence of CF-like Na⁺ hyperabsorption that has been reported to increase epithelial O₂ consumption (16, 20).

Neutrophilic airway inflammation constitutes a major risk factor for early onset and progression of bronchiectasis in patients with CF and is associated with early structural damage of distal airspaces in *Scnn1b*-Tg mice (1, 4, 20, 30). The present study demonstrates that genetic deletion of the IL-1R–MyD88 pathway resulted in a significant reduction of structural lung damage in this model of CF lung disease (Figure 6). In recent studies, we demonstrated that proteolytic activities of NE and matrix metalloproteinase 12 released from activated neutrophils and

macrophages in chronic airway inflammation play key roles in emphysema formation in *Scnn1b*-Tg mice (9, 27). We therefore speculate that the abrogation of airway neutrophilia reduced elastolytic activity, thus ameliorating structural lung damage in *Scnn1b*-Tg/*Il1r1*^{-/-} mice (Figure 6). However, infiltration of airspaces by enlarged, morphologically activated macrophages persisted (Figure 1C) suggesting that secretion of matrix metalloproteinase 12 may contribute to residual lung damage observed in *Scnn1b*-Tg/*Il1r1*^{-/-} mice (Figure 6).

Hypoxia is a common feature in CF lungs that is caused by airway mucus plugging and increased epithelial oxygen consumption due to increased epithelial sodium channel–mediated absorption of Na⁺ and fluid from airway surfaces (16, 17). The role of hypoxia in CF has been studied in the context of chronic infection with *Pseudomonas aeruginosa* and anaerobic bacteria (17, 38). However, although it is well established that hypoxic necrosis is a potent stimulus of neutrophilic inflammation in other common diseases,

such as stroke and myocardial infarction (12), the prevalence of necrotic cells in CF airways remains unknown. To determine the relevance of our findings in *Scnn1b*-Tg mice for CF lung disease in humans, we evaluated sections of small airways from patients with CF for the presence of necrotic cells. We show for the first time that hydropic degeneration (i.e., the morphologic correlate of necrotic cell death [39]), was detectable in the airway epithelium and significantly increased (~2.3-fold) in patients with CF compared with control subjects (Figure 8).

Based on recent evidence suggesting that patients with COPD exhibit acquired CF transmembrane conductance regulator (CFTR) dysfunction leading to CF-like dehydration of airway surfaces (40–43) and that small airways mucus plugging also plays an important role in COPD (44), we extended our studies to patients with this common chronic obstructive lung disease. Similar to CF, small airways from patients with COPD exhibited intraluminal mucus obstruction and increased numbers of highly vacuolated degenerative epithelial cells.

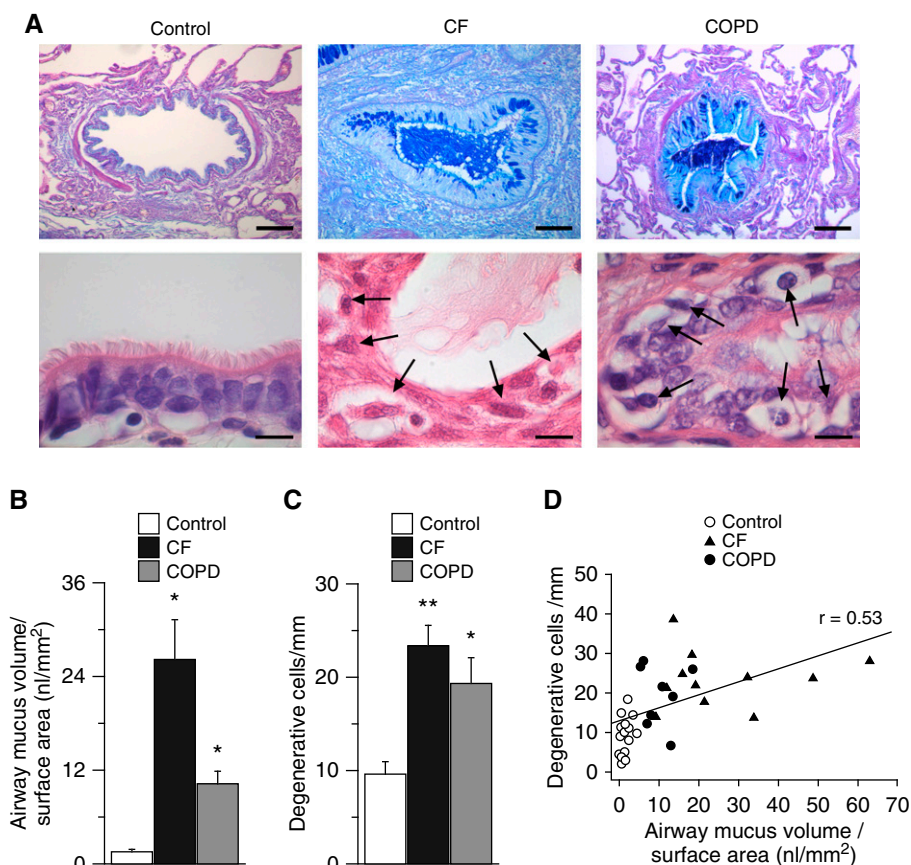


Figure 8. Mucus obstruction is associated with epithelial necrosis in small airways of patients with cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD). (A) Representative airway sections from a control subject and patients with CF and COPD stained with alcian blue periodic acid–Schiff to determine the presence of mucus (top, scale bars = 100 μ m) and hematoxylin and eosin to identify degenerative airway epithelial cells (arrows) (bottom, scale bars = 10 μ m). (B and C) Comparison of airway mucus content determined from the presence of alcian blue periodic acid–Schiff–positive material (B) and numeric densities of degenerative airway epithelial cells in control, CF, and COPD groups (C). $n = 8$ –19 individuals per group. * $P < 0.01$ and ** $P < 0.001$ compared with control subjects. (D) Correlation between numeric densities of degenerative cells and airway mucus content in control subjects and patients with CF and COPD. $n = 8$ –15 individuals per group. Pearson product-moment correlation coefficient $r = 0.53$, $P < 0.01$.

Furthermore, we found that necrotic cell densities correlated with airway mucus content in CF and COPD (Figure 8). Taken together, these results show that small airways of patients with CF and COPD exhibit increased frequencies of necrotic epithelial cells *in situ* suggesting that release of IL-1 α from necrotic cells may activate IL-1R–MyD88 signaling and contribute to neutrophilic airway inflammation characteristic of these chronic obstructive lung diseases.

Our preclinical studies with the IL-1Ra anakinra show that pharmacologic inhibition of IL-1R signaling had significant protective effects in adult *Scnn1b*-Tg mice with chronic lung disease (Figure 7; see

Figure E2). Similar to genetic deletion of IL-1R, anakinra treatment abrogated airway neutrophilia almost completely and reduced KC levels significantly in *Scnn1b*-Tg mice demonstrating potent antiinflammatory effects. Of note, inhibition of airway neutrophilia by anakinra treatment was associated with a significant reduction of distal airspace enlargement and structural damage characteristic of *Scnn1b*-Tg mice (20). These results are consistent with an important role of NE in emphysema formation in *Scnn1b*-Tg mice (9), and work from other laboratories demonstrating substantial neoalveolarization during compensatory lung growth after

pneumonectomy or elastase-induced emphysema in mice (45, 46).

Taken together, these results support inhibition of IL-1R signaling as a promising antiinflammatory strategy. However, therapeutic strategies that inhibit neutrophilic inflammation to prevent lung damage also have the potential risk to further compromise antibacterial host defense and aggravate infection-related exacerbations in CF (47). Because TLR-MyD88 signaling remains intact even when the IL-1R–MyD88 pathway is blocked, therapeutic targeting of IL-1R may provide an attractive strategy to reduce CF airways inflammation without disabling host defense against pathogens. Consistent with this concept, previous studies showed that recruitment of neutrophils by microbial stimuli was not impaired in *Il1r1*^{-/-} mice and patients treated with IL-1 blocking agents for other inflammatory disorders have not shown an increased risk of opportunistic infections (12, 15). Although genetic deletion and pharmacologic inhibition of IL-1R reduced influx of neutrophils, recruitment of and morphologic activation of macrophages remained intact in *Scnn1b*-Tg mice (Figure 1C) and in mice with necrosis-induced inflammation in other organs (12).

We therefore speculate that therapeutic inhibition of IL-1R signaling may have benefits by limiting the damaging effects of airway neutrophils (Figure 7) (4, 8, 9, 31) without compromising antibacterial host defense or macrophage-mediated tissue repair functions in CF airways. In addition to anakinra, several other agents that block IL-1 signaling including soluble decoy receptor and IL-1 β neutralizing antibodies have been approved for the treatment of other chronic inflammatory diseases (15), and these existing drugs may facilitate clinical testing of benefits of IL-1R pathway blockade in CF and potentially other chronic neutrophilic airway diseases.

In addition to the *Scnn1b*-Tg mouse, CFTR-deficient pigs and ferrets have recently become available for *in vivo* studies of the relationship between mucus, inflammation, and infection in early CF lung disease (48, 49). In newborn CF pigs, lack of CFTR was shown to cause impaired mucociliary clearance and a pH-dependent defect in bacterial killing in the absence of detectable airway surface liquid depletion and inflammation (48, 50, 51). In newborn CF ferrets, CFTR malfunction was shown

to produce airway surface liquid depletion and elevated IL-8 levels in BAL even in the absence of detectable bacteria. CF ferrets also showed a defect in bacterial killing following challenge with *P. aeruginosa*. However, this defect was not pH-dependent (52). Despite these distinct differences in initial phenotypes, both models develop a spontaneous lung disease that shares key features of lung disease in infants and young children with CF (1, 2, 53). These include regions with highly tenacious mucus plugs that contain neutrophils and cause obstruction of airways and air trapping in otherwise unaffected lungs, and regions with a more purulent airway exudate and associated pneumonia (48, 49). We expect that these additional animal

models will provide unique opportunities for further elucidation of the relative role of epithelial necrosis and IL-1R signaling in the complex *in vivo* pathogenesis of neutrophilic inflammation at different stages of CF lung disease.

In summary, this study demonstrates for the first time that necrosis of airway epithelial cells is a characteristic feature of CF lung disease in mice and patients that provides a potent stimulus of neutrophilic inflammation. Furthermore, we identified activation of IL-1R–MyD88 signaling by IL-1 α released from necrotic cells as the mechanism underlying necrosis-induced airway neutrophilia. Besides neutrophilic inflammation, we show that activation of the IL-1R–MyD88 pathway is implicated in

mucus obstruction and structural lung damage *in vivo*. These results suggest that hypoxic epithelial necrosis may provide an important trigger for neutrophilic airway inflammation independent of bacterial infection, and support inhibition of IL-1R signaling as a novel antiinflammatory strategy in CF and potentially other mucoobstructive airway diseases. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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