Abrogation of Anti-Inflammatory Transcription Factor LKLF in Neutrophil-Dominated Airways

Milene T. Saavedra¹, Abby D. Patterson¹, James West¹, Scott H. Randell², David W. Riches³, Ken C. Malcolm³, Carlyne D. Cool¹, Jerry A. Nick³, and Charles A. Dinarello⁴

¹Division of Pulmonary Sciences and Critical Care Medicine, and ⁴Division of Infectious Diseases, University of Colorado School of Medicine, Denver, Colorado; ³Department of Medicine, National Jewish Medical and Research Center, Denver, Colorado; ²Cystic Fibrosis/Pulmonary Research and Treatment Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

This is the first report to describe a role for Lung Kruppel-like Factor (LKLF or KLF2) in inflammatory airways diseases. In the present study, we identify that LKLF is constitutively expressed in the small airways of normal lungs; however, its expression disappears in severe airway diseases, such as cystic fibrosis (CF) and chronic obstructive pulmonary disease. LKLF from primary airway epithelial cells inhibits NF- κ B-driven transcription induced by *Pseudomonas aeruginosa* 7-fold, but is down-regulated in the presence of TNF- α and activated human neutrophils. As a constitutively expressed protein, LKLF inhibits release of a key pro-inflammatory chemokine, IL-8, from airway epithelia. Its expression by lung epithelial cells is enhanced in the presence of TNF blockade. Thus, cytokine-mediated inhibition of LKLF by neutrophils may contribute to ongoing recruitment by promoting IL-8 release from airway epithelia. We conclude that, in neutrophil-dominated airway environments, such as that seen in CF, reduced LKLF activity releases a brake on pro-inflammatory cytokine production and thereby may contribute to the persistent inflammatory responses seen in CF airway disease.

Keywords: Lung Kruppel-like Factor; cystic fibrosis; bronchial epithelium; human neutrophils; airway inflammation

As the interface between the environment and the lung, airway epithelia occupy a unique biological position. An appropriate balance of host defense and tolerance is required such that sustained epithelial activation does not occur. Inhaled pathogens and inflammatory stimuli are managed via the constitutive and inducible responses of innate immunity. The inducible response includes synthesis and secretion of chemokines and cytokines into the submucosa, driving recruitment of phagocytes to eliminate invaders (1). Dysregulation of this process may contribute to the pathological airway injury seen in diseases such as cystic fibrosis (CF), diffuse panbronchiolitis, chronic bronchitis, and asthma, due to persistent recruitment of inflammatory cells (2). Thus, understanding regulation of cellular homeostasis in the airway is essential to approaching diseases in which chronic immune activation leads to devastating consequences.

In the CF airway, sustained activation of airway epithelium contributes to the persistent recruitment of large quantities of neutrophils to small and medium-sized airways, in response to chronic *Pseudomonas aeruginosa* infection. Although controversy exists over whether excessive inflammation may even

CLINICAL RELEVANCE

This is the first report describing the absence of anti-inflammatory transcription factor Lung Kruppel-like Factor from the lungs of patients suffering from cystic fibrosis (CF), contributing new data supporting the pathophysiology of constitutive inflammation in CF lung disease.

precede infection in early CF airways disease (4), there is no question that decades of intense neutrophilic inflammation contribute to airway damage and disease progression (3).

The Kruppel-like transcription factor family proteins play ubiquitous roles in cellular functions of growth, differentiation and apoptosis (5). Lung Kruppel-like Factor (LKLF or KLF2) is widely known for its critical role in blood vessel development during embryogenesis (6). In vascular endothelium, expression of LKLF programs an atheroprotective phenotype, as an *inhibitor* of endothelial cell adhesive and thrombotic proteins, vascular cell adhesion molecule (VCAM-1) and tissue factor, as well as of pro-inflammatory transcription factor, NF- κ B (7). SenBanerjee and coworkers recently made an important observation regarding the expression of LKLF in the presence of inflammatory cytokines. Although cytokines, in particular TNF- α and IL-1, usually induce hundreds of genes, they potently inhibit LKLF in endothelial cells (7, 8). As an activated endothelium sets the stage for vascular disease, down-regulation of LKLF may serve as one mechanism by which cytokines can maintain a pro-inflammatory endovascular environment, so that pro-adhesive and pro-thrombotic processes may occur (7, 8). LKLF plays a pivotal role in maintenance of T lymphocyte quiescence (9). It has been implicated in naive T cell survival, and its expression is rapidly down-regulated upon T cell stimulation. Its role signifies an active process by which T cells remain quiescent, avoiding inappropriate immune activation, unless the appropriate inflammatory signals appear (9).

The present studies suggest a parallel role for LKLF in airway epithelium. We show here that LKLF suppresses *P. aeruginosa*-induced activation of NF- κ B and subsequent IL-8 release from airway cells, but, in turn, its expression is inhibited by pro-inflammatory cytokine TNF- α . Furthermore, we provide evidence that LKLF is abundantly present in normal small airway human tissue sections, but loses airway signal as inflammation worsens, and that the presence of activated human neutrophils "switches off" LKLF in airway epithelial cells. Our data establishes a counter-inflammatory role for LKLF in airway epithelium, and provides evidence for cytokine regulation of LKLF in a TNF- α -dependent fashion, suggesting that LKLF down-regulation may be a mechanism by which the presence of neutrophil secreted cytokines in the airway lumen contribute to continuous activation of airway epithelium in CF lung disease.

(Received in original form July 24, 2007 and in final form October 29, 2007)

This work was supported by National Institutes of Health grants K08HL74512 (M.T.S.), HL080322 (S.H.R.), and the Cystic Fibrosis Foundation.

Correspondence and requests for reprints should be addressed to Milene T. Saavedra, M.D., University of Colorado Health Sciences Center, 4200 E. Ninth Avenue, Box C272, Denver, CO 80262. E-mail: milene.saavedra@uchsc.edu

Am J Respir Cell Mol Biol Vol 38, pp 679–688, 2008

Originally Published in Press as DOI: 10.1165/rcmb.2007-0282OC on January 24, 2008

Internet address: www.atsjournals.org

MATERIALS AND METHODS

Cell Culture and Reagents

Primary airway epithelial cells, derived from bronchial tissue from normal lung donors, were grown on plastic with bronchial epithelial growth media (10) to confluence. Tissues were obtained with consent from patients using an Institutional Review Board–approved protocol for harvest of lung tissue specimens at the time of lung transplant or lung resection. The 2,122 non–small cell lung cancer cell line was obtained from R. Winn (University of Colorado, Denver, CO). Human TNF- α (R&D Systems, Minneapolis, MN) was added to epithelial cultures at a concentration of 10 ng/ml. Antibodies for LKLF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Plasmids were generated or obtained from the following generous sources: LKLF (Laurie Glimcher, Harvard University, Boston, MA), LKLF–2-kb promoter–reporter construct (Jerry Lingrel, University of Cincinnati, Cincinnati, Ohio), Ad5CMV-EGFP and Ad5CMV-LKLF (gift of Mukesh Jain, Harvard University, Boston, MA and generated at the University of Iowa Viral Vector Core, Iowa City, IA), and full-length iNOS promoter luciferase reporter construct (Ed Chan, National Jewish Medical and Research Center, Denver, CO). A multiplicity of infection of 10 plaque-forming units/cell was used to infect 98% of cells growing on plastic in submerged culture. Recombinant human TNF-binding protein (p55 soluble TNF receptor) was used at 10 μ g/ml (Amgen, Thousand Oaks, CA). The well-characterized *P. aeruginosa* strain PAO1 was used, and LPS purified from both *Pseudomonas* and *Escherichia coli* as previously described (11).

Western Blotting

A549 epithelial cells were exposed to TNF- α (10 ng/ml). After 4 hours of exposure, control and TNF-treated cells were washed with cold PBS, lysed in Radioimmunoprecipitation buffer (PBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, PMSF [10 mg/ml], aprotinin [30 μ l/ml], and sodium orthovanadate [1 mM]), pelleted at 14,000 rpm, and the protein concentration of the lysate determined by Bradford assay. Equal amounts of protein were separated by gel electrophoresis under reducing conditions (1% β -mercaptoethanol) using 8 to 16% gels. The proteins were then transferred to a PVDF membrane, and the membrane was blocked in 1% nonfat milk and 0.1% Tween-20. Immunodetection was done with goat polyclonal anti-LKLF (Santa Cruz Biotechnologies). Anti-goat secondary antibodies conjugated to horseradish peroxidase (HRP) at 1:10,000 dilution were used and detected using ECL-plus (Amersham, Buckinghamshire, UK) (12). These experiments were also performed with BEAS2B normal human bronchial epithelial cell line (ATCC, Manassas, VA), with identical results. Epithelial cells were infected with Ad5CMV-EGFP or Ad5CMV-LKLF at a multiplicity of infection (MOI) of 10 and assessed for inducible nitric oxide synthase (iNOS) protein expression at 24 hours in nuclear and cytoplasmic fractions. Immunodetection was done with anti-iNOS (Calbiochem, San Diego, CA) and ECL-plus as described above. Nuclear and cytoplasmic fractionation was performed as per previously published methods (13). Anti-phospho NF- κ B p65 (ser 536) was purchased from Cell Signaling (Danvers, MA).

Transient Transfection Assays

Primary human airway epithelial cells, passage 2, were grown in 6-well plates to 80% confluence. In addition, the following cell lines were used for this experiment: 16 HBE 14O– (from D. Gruenert, University of Vermont, Burlington, VT), Nu-Li1 (from J. Zabner, University of Iowa, Iowa City, IA) (14), and 2,122 and H157 non–small cell lung cancer cell lines. Cells were transiently transfected with the following using Lipofectamine and PLUS reagent (Life Technologies, Gaithersburg, MD): 1 μ g LKLF or pCDNA empty vector control, 1 μ g of pNF- κ B luciferase plasmid (Stratagene, La Jolla, CA), containing five NF- κ B–binding sites upstream of a luciferase reporter, and 1 μ g of β -galactosidase to normalize results for transfection efficiency. After 24 hours, cells were exposed to *Pseudomonas* strain PAO1 at an MOI of 10:1 for 2 hours. Each condition was done in triplicate. At the time of harvest, cells were lysed with luciferase cell culture lysis reagent (Promega, Madison, WI). Luciferase assays were performed using the Promega protocol for the dual luciferase reporter assay system with a Monolight 2,010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). All

results are expressed as luciferase units normalized to milliunits of β -galactosidase, represent triplicate samples, and are representative of at least three experiments. To evaluate the effect of TNF- α on NF- κ B reporter activity in the presence of LKLF, cells were transfected as described above, then exposed 24 hours later to TNF- α (10 ng/ml) for 6 to 24 hours. For the LKLF reporter experiment, A549 cells were plated 1 day before transfection and transient transfection performed with Lipofectamine and PLUS reagent according to manufacturer protocol. One microgram of each plasmid was used for transfections and total DNA kept constant. At 24 hours cells were exposed to TNF- α or control carrier for 6 hours and then assayed for luciferase activity. For the dose–response experiment, 2,122 epithelial cells were transiently transfected with 1 μ g pNF- κ B luc, 1 μ g β -galactosidase, and varying amounts of LKLF vector, from 0 to 3 μ g. Total amount of DNA was kept constant. Luciferase assay was performed 24 hours after transfection. All other constructs, including TRAF2, TRUSS, and IKK β , were made in the Riches laboratory. In those transfections, the total amount of DNA was kept constant, and lipofectamine transfection performed as described above with pNF- κ B luc and β -galactosidase for transfection efficiency. For the iNOS reporter experiments, A549 cells were plated and transfected as described above, using 1 μ g iNOS luc, 1 μ g β -galactosidase, and 1 μ g of LKLF vector versus control vector. Luciferase activity was measured at 24 hours and experiments done in triplicate.

Electrophoretic Mobility Shift Assay

Nuclear extracts of 2,122 epithelial cells transduced with Ad5CMV-LKLF or GFP control adenovirus were harvested 24 hours after infection at an MOI of 10. Nuclear extracts were prepared by lysing cell pellets with lysis buffer containing 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 mM PMSF, and nuclei extracted with buffer containing 20 mM Hepes, 10 mM NaCl, 1.5 mM MgCl₂, 0.5 mM EDTA, 20% glycerol, and 0.5 mM DTT. Biotin end-labeled oligonucleotides 5'-biotin-AGTTGAGGGGACTTTCCAGGC-3' and 5'-biotin-GCCTGGGAAAGTCCCCTCAACT-3' as well as nonlabeled oligonucleotide containing NF- κ B consensus binding sequences were purchased from Gene Link (Hawthorne, NY). The binding reactions contained 0.5 μ g nuclear extract protein, buffer (10 mM Tris, pH 7.5 40 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 0.05% Nonidet P-40, and 2.5% glycerol), 1 μ g/ μ l poly dI/dC, and 2 nM biotin-labeled DNA. The reactions were incubated at room temperature for 20 minutes. Competition reactions were performed by adding 200-fold excess of unlabeled consensus oligonucleotide to the reaction mixture. The binding reactions were electrophoresed on a 6% DNA denaturing gel, transferred to a nylon membrane and then treated with ultraviolet cross-linking of DNA to the membrane. The biotin end-labeled NF- κ B was detected using streptavidin-HRP conjugate and LightShift Chemiluminescent Substrate (Pierce, Rockford, IL) (15). The supershift analyses were performed by incubating the DNA-binding reactions with anti-NF- κ Bp65 (Santa Cruz Biotechnologies) for an additional 20 minutes on ice before electrophoresis.

Enzyme-Linked Immunosorbent Assay for IL-8

IL-8 was measured by enzyme-linked immunosorbent assay (R&D Systems) 24 hours after infection with adenoviruses: Ad5CMV-LKLF and control adenovirus, Ad5CMV-EGFP.

Immunohistochemistry Analysis

Human lung histopathologic material from adults with CF (with documented sweat chloride and genotyping) at the time of lung transplant ($n = 3$) was obtained. Control samples include specimens from non-CF organ donors whose lungs were not used for transplant, usually due to lack of match in area, age, or smoking history. Samples included: control large airway ($n = 3$); control distal lung ($n = 3$); CF large airway ($n = 3$); CF distal lung ($n = 3$); non-CF bronchiectasis surgical wedge resections, infected with *Mycobacterium avium* intracellulare (MAI) ($n = 3$); and severe chronic obstructive pulmonary disease (COPD) at the time of lung transplantation ($n = 3$). Collection of lung specimens at the time of transplant is approved by the University of Colorado Combined Institutional Review Board (protocol 01–906). Using standard procedures, tissue specimens were

incubated with LKLF primary antibody overnight or IgG staining control at 4°C using a 1:250 dilution in PBS/4% horse serum/0.1% Triton-X100. Slides were rinsed and placed in Alexa-Fluor 594 (Molecular Probes) anti-goat secondary antibody at 1:1,000 dilution for 1 hour at room temperature. For confocal images, images were viewed through the TRITC filter of an Olympus IX81 spinning disc confocal microscope (Olympus, Center Valley, PA) and captured with a Hamamatsu camera (Hamamatsu, Bridgewater, NJ), utilizing Intelligent Imaging Slidebook v. 4.067 acquisition software (Intelligent Imaging Innovations, Denver, CO). A standard S-curve was applied to all darkfield images to set contrast, and a scale bar was placed digitally (16). In addition, normal human primary epithelial cells, passage 2, grown on slides, were fixed in formalin and then incubated with primary antibody overnight or IgG control as described above. After 2 hours of incubation with secondary antibody as above, slides were washed with PBS and DAPI mounting media added, and images captured with a Zeiss microscope with a scanning digital camera using the fluorescein isothiocyanate filter and Axiovision software (Zeiss, Thornwood, NY).

Human Neutrophil Stimulation Experiments

Normal human neutrophils were isolated from healthy volunteers by the plasma percoll method (Institutional Review Board Protocol -HS1285) (17). Isolated neutrophils were left unstimulated or were stimulated with *P. aeruginosa*-derived LPS (PA-LPS), 1,000 ng/ml, for 15 minutes at room temperature. To analyze the effects of dead neutrophils on epithelial cells, neutrophils were activated before cell lysis, as neutrophil activation is required to achieve accumulation in the airway. After stimulation, neutrophils were snap-frozen in liquid nitrogen for 18 hours. Neutrophil death was defined by the loss of cell membrane integrity and confirmed by trypan blue staining. Cellular contents of 10^6 neutrophils were incubated with A549 cells, which were confluent on a 10-cm plate. At specified time points, plates were washed three times with PBS and epithelial cell lysates collected for Western blot. For studies using viable neutrophils, A549 cells were exposed to LPS alone, unstimulated neutrophils (10^6), or PA-LPS-stimulated human neutrophils (10^6) over a time course. Plates were washed three times with PBS and neutrophils counted in the washes to ensure against neutrophil protein contamination. For supernatant experiments, 10^6 neutrophils, which were either unstimulated or stimulated with LPS, were added in RPMI to 10-cm plates containing no epithelial cells for 4 hours. At 4 hours, media was carefully removed and quickly spun at 14,000 rpm for 1 minute to pellet neutrophils. This conditioned media was then added to confluent 10-cm plates of A549 cells for 4 hours and epithelial cells lysates collected and blotted for LKLF. All epithelial cell media was supplemented with polymyxin B (10 µg/ml) on the date of the experiment, in order to neutralize LPS, and neutralization of LPS was confirmed by Limulus assay. The neutrophil/epithelial co-incubation experiments described above were repeated, under conditions of TNF neutralization with recombinant human TNF-binding protein (10 µg/ml). TNF neutralization by TNFBP was confirmed by ECL assay as previously described (18). TNF-binding protein was added both to human neutrophil media, as well as to epithelial cells before co-incubation with human neutrophils. Isolated neutrophils were left unstimulated or were stimulated with LPS (100 ng/ml) for 15 minutes at room temperature. Neutrophils were then coincubated with epithelial cells as described above, and epithelial lysates collected at 24 hours for Western blot, after serial washes to clear neutrophil presence. Each experiment was done a minimum of three times.

Statistical Analysis

Results are represented as mean $\pm$ SD. Quantitative differences between control and test conditions were assessed statistically by ANOVA, unpaired *t* tests, or correlation testing. $P \leq 0.05$ was considered statistically significant.

RESULTS

LKLF Is Absent in Diseases Characterized by Severe Airway Inflammation

We analyzed lung tissue from normal donors to determine a site for LKLF expression. In addition, we performed immunohisto-

chemistry for LKLF expression in multiple lung tissue specimens representing three different diseases. The site of highest expression in normal human lung, the small airways, demonstrated punctate red signal (Figure 1A, *top panel*). In each of three normal lung samples, a signal was present to the greatest degree at the luminal aspect of bronchiolar cells. Comparisons were made to lung tissues with varying degrees of airway inflammation. In non-CF bronchiectasis, infected with non-tuberculous mycobacteria, airway epithelia demonstrate translocation of LKLF from the apical cytoplasm to the nuclear compartment. The area closer to normal lung (at the edges of the surgical specimens) clearly demonstrates that LKLF has translocated to epithelial nuclei. These peripheral areas of non-CF bronchiectasis specimens demonstrate much stronger LKLF signal than central areas, where active chromatin is less condensed, and signal diminished adjacent to luminal cellular debris. End-stage airway diseases were represented by sections taken from patients with severe COPD and CF, at the time of lung transplantation ($n = 3$ each). Both COPD and CF tissue sections were characterized by epithelial hyperplasia and chronic inflammatory cell infiltration. Punctate red signal for LKLF seen in normal and, to a lesser extent, in MAI lung, was completely absent in all COPD and CF airway specimens (Figure 1A, *bottom two panels*).

LKLF Localizes to the Cytoplasmic Compartment of Resting Primary Airway Epithelial Cells

To characterize a site for LKLF expression in primary airway epithelial cells from healthy human lungs, immunohistochemical staining was performed on unstimulated primary airway cells, passage 2, from healthy donors, demonstrating cytoplasmic localization (Figure 1B). In addition, nuclear and cytoplasmic fractions were isolated, and by Western blot, LKLF is primarily present in cytoplasmic fractions in unstimulated airway cells (Figure 1C). The faint signal present in nuclear fraction at a slightly lower molecular weight may reflect processing which occurs after transport to the nucleus.

LKLF Expression Inhibits NF- κ B-Mediated Signaling in the Presence of *Pseudomonas*

Given its role in regulating immune activation in T cells and in endothelial cells, we hypothesized that LKLF may regulate epithelial inflammatory cascades involved in the response to pathogens. Primary airway cells were transfected with an NF- κ B-Luc reporter plasmid before exposure to *Pseudomonas* strain PAO1. As shown in Figure 2A, the ability of *Pseudomonas* to induce NF- κ B luciferase activity was reduced 7-fold in LKLF transfected airway cells. In primary airway cells, LKLF expression reduced NF- κ B reporter activity to levels present in uninfected airway cells (Figure 2A). NF- κ B activity was inhibited by LKLF in each cell type studied: 2-fold in 16 HBE 14O-, 4-fold in Nu-Li1 cells, 3- to 6-fold in 2,122 and H157 lines, and finally 7-fold in primary human airway cells (Figure 2A). LKLF reduction of NF- κ B reporter activity occurs in a dose-dependent manner (Figure 2B).

We next used electrophoretic mobility shift assay (EMSA) analysis to assess NF- κ B nuclear binding in LKLF + airway cells. Briefly, nuclear extracts were prepared from 2,122 cells transfected with either control Ad5CMV-EGFP or Ad5CMV-LKLF on three separate occasions. As shown in Figure 2C, binding of biotinylated probes to nuclear NF- κ B was significantly diminished in Ad5CMV-LKLF-infected cells. Supershift analysis of NF- κ B complexes performed with anti-p65 antibodies confirmed the specificities of the binding complex. The reduction in p65 binding by EMSA appears to be secondary to sequestration of

Figure 1. *In vivo* Lung Kruppel-like Factor (LKLf) expression in a spectrum of airway diseases. (A) Confocal images demonstrate LKLf positivity as red punctate staining in the apical portions of ciliated columnar epithelial cells of normal bronchiolar epithelium in lung tissue sections at $\times 630$ magnification using an Olympus IX81 spinning disk confocal microscope. Bright cytoplasmic red signal is most visible in normal controls. In *M. avium* intracellular bronchiectasis, LKLf has translocated to the nucleus in a region representing the surgical margin ($n = 3$). In the center of the non-cystic fibrosis (CF) bronchiectasis tissue sections, in areas of greater inflammation, chromatin has become less condensed and signal more faint. In the most severe representations of airway inflammation, chronic obstructive pulmonary disease and CF, at the time of lung transplantation, signal is completely absent ($n = 3$ each). An IgG antibody was used as a staining control. (B) LKLf immunostaining in cytoplasm from normal primary human airway epithelial cells grown in culture, passage 2, versus IgG antibody control ($\times 400$). (C) LKLf localizes to cytoplasmic cell compartments in passage 2 primary airway epithelial cells blotted for LKLf.

activated p65 (phosphorylated at serine 536) by LKLf in the cytoplasm (Figure 2D). As shown in Figure 2E, downstream effects of LKLf inhibition of NF- κ B resulted in a reduction in secreted neutrophil chemokine, IL-8. Measurement of IL-8 in cell supernatants from GFP control versus Ad5CMV-LKLf-transduced cells revealed reduced IL-8 release from LKLf + cells.

LKLf Expression Activates iNOS

LKLf overexpression in umbilical vein endothelial cells induces endothelial nitric oxide synthase (eNOS) expression, an anti-

inflammatory regulator of vascular tone (7). In lung epithelia, the mediator of nitric oxide (NO) release is iNOS. iNOS is expressed constitutively by upper and lower airway epithelia, induced in various airway inflammatory states, and dramatically reduced in CF airways (19, 20). We evaluated LKLf effects on iNOS expression. Ad5CMV-LKLf-transduced A549 cells up-regulate iNOS in both nuclear and cytoplasmic compartments compared with controls (Figure 3A). As shown in Figure 3B, a 2.5-fold increase in iNOS promoter reporter activity was seen in LKLf-transfected A549 cells compared with empty vector transfected cells at 24 hours.

Figure 2. Inhibition of NF-κB activation by LKLF. (A) Primary airway cells were transiently transfected with pNF-κB luc and either control or wild-type LKLF plasmid for 24 hours, stimulated with PAO1 at an MOI of 10:1 for 1 hour, and luciferase activity measured. Ctrl (includes pcDNA vector and pNF-κB luc), LKLF+PA (includes LKLF vector, pNF-κB luc, and PAO1), PA (includes pcDNA vector, pNF-κB luc, and PAO1) **P* < 0.001 versus +PAO1 by unpaired *t* test (*n* = 3). (B) 2,122 cells were transiently transfected with pNF-κB luc and concentrations of LKLF vector ranging between 0 and 3 μg were used. Total amount of DNA was kept constant with control vector. Luciferase assay was performed 24 hours after transfection. *P* = 0.007 by correlation *z* test (*n* = 3). All luciferase results shown as fold increase in relative light units and normalized for transfection efficiency to milliunits of β-galactosidase. Data are means ± SEM from three independent experiments. (C) Nuclear extracts were harvested from 2,122 epithelial cells expressing Ad5CMV-EGFP or Ad5CMV-LKLF. NF-κB band and supershift p65 band are shown. (D) A Western blot of cell fractions shows predominance of activated p65 is sequestered in the cytoplasm of LKLF+ cells, while demonstrating total p65. (E) Supernatants of 2,122 cells overexpressing control Adv or Ad5CMV-LKLF were assayed for IL-8 by ELISA. **P* = 0.05 versus control via unpaired *t* test. Data expressed as mean ± SD.

LKLF Expression by Airway Epithelial Cells Is Inhibited by LPS-Stimulated Human Neutrophils

Neutrophil influx into CF airways is the pathological hallmark of the severe persistent inflammation seen in CF lung disease. We tested lung epithelial expression of LKLF in co-incubation experiments, in the presence of stimulated and unstimulated human neutrophils. A549 cells were treated with either: 1×10^6 unstimulated neutrophils or 1×10^6 neutrophils stimulated with LPS. At 4 hours, stimulated neutrophils inhibited expression of LKLF in A549 epithelial cells (Figure 4A). Epithelial cell cytotoxicity was not observed with these quantities of neutrophils. A key question that arises is whether neutrophils must be alive for this effect to occur. This distinction would imply whether the effect is secondary to preformed elements or to the neutrophil functional response. We subsequently exposed A549 cells to dead human neutrophils, in quantities identical to those used in the live human neutrophil experiments (Figure 4B). LKLF was expressed by epithelial cells co-incubated with dead neutrophils. Its inhibition in epithelia requires co-incubation with live stimulated human neutrophils, implying

that inhibition is a result of neutrophil functional activity. To verify this hypothesis, we treated A549 cells with supernatants of both unstimulated and LPS-stimulated neutrophils and confirmed that LPS-stimulated supernatant inhibits LKLF expression (Figure 4C).

TNF-α Inhibits LKLF Expression and Promoter Activity in A549 Cells

While the list of neutrophil-secreted products is extensive, we reasoned that LKLF, as a homeostatic protein expressed by lung epithelia, may be regulated by local concentrations of cytokines, indicating a possible mechanism by which neutrophils regulate LKLF expression. Others have reported that TNF-α inhibits LKLF expression in endothelium (8). We sought to establish whether this inhibition could be demonstrated in lung epithelium. Human TNF-α was added to A549 epithelial cultures for 6 hours. The effect of TNF-α upon LKLF reporter activity, using a proximal -2 kb region of the LKLF promoter, was assessed, and TNF-α diminished reporter activity by 50% at this time point (Figure 5A). A549 cells were treated with TNF-α

Figure 3. Induction of iNOS by LKLF. (A) LKLF induces iNOS expression. A549 cells were infected with control (GFPAdv) or LKLF adenovirus (Ad5CMV-LKLF) at an MOI of 10 for 24 hours and Western blot performed for iNOS expression. (B) LKLF induces iNOS promoter activity. A549 cells were transiently co-transfected with a full-length iNOS promoter-luc plasmid and either empty vector pcDNA3 or LKLF wild-type plasmid and luciferase activity measured at 24 hours. * $P = 0.02$ by unpaired t test. All luciferase results are shown as fold increase in relative light units and normalized for transfection efficiency. Data are means $\pm$ SEM from three independent experiments.

and expression of LKLF at 4 hours evaluated by Western blot. As shown in Figure 5B, reduction of LKLF protein by TNF- α is seen at 4 hours, which was seen at the identical time point in Beas2B cells as well (data not shown). These data demonstrate that TNF- α inhibits LKLF promoter activity, as well as protein expression, in A549 and Beas2B epithelial cells.

LKLF Inhibition of NF- κ B-Driven Transcription Is Regulated by TNF Pathway Proteins

LKLF inhibits NF- κ B in airway epithelial cells; however, this effect is dependent upon activation of adaptor proteins of the TNF receptor 1 (TNF-R1). Given its anti-inflammatory effects in endothelium (7), we examined the effect of LKLF on NF- κ B activity in epithelial cell lines and primary airway cells. LKLF markedly reduces NF- κ B to one third of luciferase activity seen in empty vector controls (Figure 6A). However, when co-transfected with TNF signaling molecules TRAF2 and TRUSS, LKLF could not reduce NF- κ B reporter activity as effectively. We did not observe direct binding between LKLF and TRAF2 by coimmunoprecipitation assay (data not shown). Within minutes of TNF- α stimulation of TNF-R1, recruitment of effector molecules TRAF2, TRUSS, and RIP (receptor-interacting protein), followed by recruitment of IKK β to the TNF-R1 complex, occurs, leading to downstream activation of NF- κ B (21). Given its essential role in TNF-induced NF- κ B activation, we tested the effect of IKK β in LKLF-mediated inhibition of NF- κ B reporter activity. As shown in Figure 6B, we found that LKLF once again inhibited NF- κ B reporter activity once IKK β was overexpressed in the presence of LKLF.

Sustained Loss of LKLF Inhibition of NF- κ B in the Presence of TNF

Studies have demonstrated that in endothelial cells, inhibition of LKLF by TNF- α is a sustained effect, lasting for up to 48 hours (8). To demonstrate that TNF- α inhibits LKLF's functional effects, in particular its ability to inhibit NF- κ B in epithelial cells, we transiently transfected wild-type LKLF plasmid versus empty vector into A549 cells and subsequently treated transfected cells with TNF- α for up to 24 hours. As shown in Figure 7A, LKLF no longer inhibits NF- κ B in the presence of TNF- α at 24 hours. Using Western blot, we demonstrate that A549 cells lose LKLF expression under other pro-inflammatory conditions, specifically in the presence

of LPS-stimulated neutrophils at 24 hours. However, this loss is TNF dependent, and expression is restored when TNF is neutralized by p55 soluble TNF receptor (Figure 7B).

Figure 4. Inhibition of LKLF expression in epithelial cells by stimulated neutrophils. (A) A549 cells were treated with either LPS, 10^6 unstimulated neutrophils, or 10^6 LPS-stimulated neutrophils. Western blot for LKLF expression at 1 hour up to 4 hours ($n = 3$ for each experiment). (B) A549 cells incubated with dead neutrophils (stimulated with LPS before death) for 4 hours, followed by Western blot for LKLF expression. (C) A549 cells treated with four hour supernatants from LPS controls, unstimulated neutrophils, or LPS-stimulated neutrophils followed by Western blot for LKLF expression. β -actin is shown as a loading control. PMN = neutrophil.

Figure 5. LKLF is inhibited by TNF- α . (A) Inhibition of LKLF promoter by TNF- α . A549 cells were transiently transfected with a 2.0-kb LKLF promoter and, after 24 hours of transfection, treated with TNF- α (10 ng/ml) for 6 hours. Luciferase assay was performed. * $P < 0.001$ by unpaired *t* test. (B) Inhibition of LKLF expression in the presence of TNF- α . Western blot of lysates from A549 cells treated with TNF- α for 4 hours. β -actin is shown as a loading control.

DISCUSSION

A role for LKLF in maintaining airway homeostasis has not been defined; however, its presence in uninfected airways free from neutrophilic inflammation, down-regulation by TNF- α , and the ability to reduce NF- κ B reporter activity and increase iNOS each suggest a function in the maintenance of lung epithelia.

Individual TNF scaffolding and adaptor proteins differ markedly in their influence on the ability of LKLF to inhibit NF- κ B reporter activity. Previous investigations have explored TRAF2 regulation of LKLF expression. In TRAF2-null fibroblasts, expression of LKLF is absent, but is restored when TRAF2 is reintroduced into the cells (22). As TRAF2 is a cytoplasmic protein that does not shuttle to the nucleus and hence does not act as a transcription factor, its regulation of LKLF in fibroblasts has been described as indirect and p38 MAP kinase pathway dependent (22). However, based on the results of immunohistochemistry and cellular fractionation studies, LKLF appears to be expressed in the cytoplasm of airway cells. Although we did not detect a direct interaction by LKLF and TRAF2 or RIP by coimmunoprecipitation (data not shown), luciferase cotransfections revealed that LKLF-mediated inhibition of NF- κ B reporter activity is regulated by TNF receptor scaffolding and adaptor proteins, each critical for the crosslinking that leads to IKK activation. TRUSS is a TNF-R1 scaffolding protein, which is constitutively associated with unligated TNF-R1, as well as directly interacting with TNF-R1 signaling proteins, TRADD and TRAF2 (23). LKLF-mediated inhibition of NF- κ B reporter activity is diminished when TRAF2 and TRUSS are expressed but unchanged in the presence of wild-type IKK β , whose activity in the tripartite IKK complex is critical in the activation of the classical NF- κ B pathway (24). Although the mechanisms of LKLF interaction with TNF receptor scaffolding proteins are incompletely understood, the present data establish that, as a transcription factor, LKLF is present in the cytoplasm, and that its NF- κ B inhibitory properties are dependent upon the proximal TNF signaling proteins involved in TNF-R1 crosslinking upstream of IKK activation (23). Demonstrating this cytoplasmic

Figure 6. TRUSS and TRAF2, but not IKK β , reverse LKLF-mediated inhibition of NF- κ B. A549 cells were transiently transfected with pNF- κ B luciferase, as well as control vector or LKLF plasmid in the presence or absence of TRAF2, TRUSS, or IKK β . Luciferase assay was performed 24 hours after transfection. (A) NF- κ B activity is inhibited by transfection with wild-type LKLF plasmid (* $P < 0.001$ versus empty vector); this is rescued by cotransfection with either TRUSS or TRAF2 ($P < 0.001$ for difference from LKLF only by ANOVA). Co-transfection of TRUSS and TRAF2 plasmids with wild-type LKLF significantly decrease NF- κ B activity from levels reached with TRUSS and TRAF2 plasmids alone (**** $P = 0.001$ and **** $P = 0.0003$, respectively, by ANOVA). Though clearly reduced, NF- κ B activity still remains significantly higher than that seen with LKLF plasmid alone (** $P = 0.0015$ for LKLF and TRUSS versus LKLF alone, *** $P = 0.001$ for LKLF and TRAF2 versus LKLF alone). (B) NF- κ B activity is again inhibited by transfection with wild-type LKLF plasmid (* $P < 0.001$). A significant increase in NF- κ B activity occurs with IKK β ($P < 0.0001$). Cotransfection of wild-type IKK β plasmid with wild-type LKLF reduces NF- κ B activity to that seen with LKLF plasmid alone (** $P = < 0.001$ for LKLF and IKK β versus IKK β alone, $P = 0.22$ for LKLF and IKK β versus LKLF alone, by ANOVA).

presence for LKLF supports its role as a protein which, though it actively regulates inflammatory signaling from the nucleus (8), is poised to be extinguished when appropriate extracellular inflammatory signals appear.

The present findings demonstrate that, similar to HUVECs, epithelial cell LKLF is simultaneously inhibited by TNF- α and able to inhibit NF- κ B-driven signaling. This supports a role in the maintenance of a resting epithelial cell phenotype, where a tight rein on immune activation must exist, given the quantities of aeroantigens and microorganisms present in the 10,000 liters of air inhaled each day (1). Data from endothelial cell studies demonstrate that LKLF expression is a means by which the microvasculature regulates attachment and subsequent trans-

Figure 7. Loss of LKLF mediated NF- κ B inhibition in the presence of TNF- α . (A) Primary airway cells were transiently transfected with pNF- κ B luc and either control or wild-type LKLF plasmid for 24 hours, stimulated with TNF- α (10 ng/ml) for up to 24 hours and luciferase activity measured. Ctrl (includes pcDNA vector and pNF- κ B luc), LKLF (includes LKLF vector and pNF- κ B luc) ($P < 0.01$ for LKLF versus control in absence of TNF, $P < 0.001$ for LKLF in presence versus absence of TNF). No statistical difference between LKLF and pcDNA in presence of TNF, by ANOVA ($n = 3$). (B) A549 cells were co-incubated with human neutrophils for 24 hours, either in the presence or absence of TNF-binding protein. A Western blot depicts LKLF expression in the above conditions. Unstim PMN indicates unstimulated neutrophils and stim PMN indicates LPS-stimulated neutrophils. TNFBP refers to TNF-binding protein ($n = 3$).

migration of leukocytes into adjacent tissues, specifically by inhibiting VCAM-1 and E-selectin (7). The present data suggest a comparable role in lung epithelia, and particularly that anti-NF- κ B properties are significantly reduced when inflammatory cytokines are present. Kumar and colleagues demonstrated a nuclear mechanism for this reduction in HUVECs, where p65 cooperates with histone deacetylase 4 to inhibit MEF2 induction of the LKLF promoter (8). In epithelial cells, LKLF sequesters activated p65 in the cytoplasm, sharply reducing nuclear binding as seen by EMSA, though direct interaction was not demonstrated by co-immunoprecipitation.

A key neutrophil chemokine downstream of NF- κ B activation is IL-8, and induction of LKLF leads to diminished IL-8 release into epithelial cell supernatants, indicating a role in the regulation of neutrophil recruitment.

Furthermore, LKLF increases both expression and promoter activity of iNOS, which is produced by neutrophils, macrophages, and epithelial cells in response to LPS and bacterial and cytokine stimulation (25). Although CF airways are populated with millions of bacteria, explanted CF bronchial epithelium expresses less iNOS mRNA compared with non-CF bronchiectasis tissue (26). Immunocytochemistry reveals little iNOS expression in CF epithelia, stimulated or unstimulated, in the presence or absence of neutrophils (27). Exhaled breath studies of patients with CF show decreased NO concentrations compared with nonsmoking control subjects (28). In animal studies, inhaled NO reduces pulmonary sequestration of activated neutrophils, and mice lacking iNOS have increased airway neutrophil populations (29, 30). Thus, while the mechanism of LKLF up-regulation of iNOS expression in epithelial cells is unknown, the paucity of both in CF airways establishes multiple levels upon which neutrophil recruitment to the airways may be increased in their absence.

The present study establishes specific sites in the lung for LKLF expression. In humans, LKLF is most highly expressed in T lymphocytes, followed by heart, lung, and skeletal muscle (31). LKLF has recently been shown to be a key transcriptional regulator at yet another important interface, that between blood and tissues. Its expression in endothelial cells is associated with up-regulation of anti-inflammatory and anti-thrombotic eNOS while inhibiting VCAM-1 and E-selectin adhesion molecules in the presence of cytokines (7). SenBanerjee and coworkers emphasize the association between LKLF function and its anatomical location. LKLF suppresses NF- κ B-mediated VCAM-1 expression and its absence at branch points in the vascular tree, where atherosclerosis is most likely to occur, posits a role in the inflammatory processes of early atherosclerotic disease. The immunohistochemistry images in this report establish anatomic sites of LKLF activity in normal human lung tissue and its waning expression as local inflammation intensifies. LKLF appears to be most highly expressed in human small airways, with less signal generated in alveolar tissue, and minimal expression seen in large airways. Since LKLF localizes to bronchioles, its expression in CF is of interest, as CF shares features with another inflammatory airways disease, namely diffuse panbronchiolitis. Both conditions manifest chronic airways bacterial colonization and marked neutrophilic infiltration ultimately obliterating the small airways (32). The absence of LKLF signal in CF small airways, along with its absence in atherosclerotic disease sites, create a plausible role for this protein in inflammatory diseases affecting two organs—the lung and the vasculature. Given their marked presence in the CF airway, we examined how neutrophils affect the induction of LKLF in epithelial cells.

An unexpected finding of our study was that LPS-stimulated neutrophils clearly inhibited LKLF release from epithelial cells.

Since inhibition did not occur in the presence of dead LPS-stimulated neutrophils, preformed neutrophil products are not implicated. However, LKLF expression from A549 cells is clearly inhibited in the presence of LPS-stimulated neutrophil supernatants, and this suggests the involvement of a secreted factor. Neutrophil functional responses are characterized by release of superoxide anion, cytokines, and granule proteins. Since TNF regulates LKLF expression, we reasoned that TNF- α , coming from neutrophils, or from epithelial cells as a response to neutrophil products, may be a mechanism by which neutrophils inhibit LKLF and can continuously activate the epithelium. This inhibition implies that TNF-rich environments may interfere with the ability of the epithelial cell to reestablish homeostasis once an inflammatory response has occurred. The concentration of neutrophils (10^6 per 5 million epithelial cells) used in this analysis was based on bronchoalveolar lavage sampling studies quantitating inflammatory cells before persistent *P. aeruginosa* infection. In the absence of detectable infection or endotoxin, bronchoalveolar lavage studies have recovered neutrophils ranging from 10^4 to 10^6 per ml from the airways of children, with an estimated recovery rate of approximately 1 to 2% (4, 33, 34). An even broader range of quantity of neutrophils has been isolated from children with CF during early infection (33, 34).

The recruitment of neutrophils to small and medium-sized airways in response to chronic *P. aeruginosa* infection is emblematic of CF lung disease. By their teenage years, the neutrophils in the lungs of patients with CF no longer eradicate bacteria from their airways, but continue to accumulate. We have identified LKLF as a transcription factor, present in human bronchioles, that regulates NF- κ B and IL-8 and in turn is inhibited by neutrophils and TNF- α . Data from the present study raise the possibility that, by inhibiting LKLF in airway epithelial cells, neutrophils contribute to their own excessive recruitment by promoting IL-8 release. Regulation of LKLF by neutrophils provides a novel hypothesis as to how local CF epithelial environments set the stage for an excessive and prolonged primary inflammatory response. The current findings suggest that LKLF may mark neutrophil airways activity and their effects on epithelial anti-inflammatory responses, helping to stratify patient populations in which anti-inflammatory therapies may be more beneficial.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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