Keratinocyte growth factor supports pulmonary innate immune defense through maintenance of alveolar antimicrobial protein levels and macrophage function

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Running Title: KGF supports alveolar defense against infection
ABSTRACT

Keratinocyte growth factor (KGF) is an epithelial mitogen that has been reported to protect the lungs from a variety of toxic and infectious insults. In prior studies, we found that recombinant human KGF (rhKGF) accelerates the clearance of bacteria from the murine lung by augmenting the function of alveolar macrophages (AM). In this study we tested the hypothesis that endogenous KGF plays a role in the maintenance of innate pulmonary defense against gram-negative bacterial infections. KGF deficient mice exhibited delayed clearance of *E. coli* from the lungs, attenuated phagocytosis by AM, and decreased antimicrobial activity in bronchoalveolar lavage fluid (BALF), due in part to reductions in the levels of surfactant protein A (SP-A), surfactant protein D (SP-D), and lysozyme. These immune deficits were accompanied by lower alveolar type II epithelial cell (AECII) counts and reduced AECII expression of collectin and lysozyme genes on a per cell basis. There were no significant between group differences detected in selected inflammatory cytokines or BAL inflammatory cell populations at baseline or post bacterial challenge in the wild type and KGF deficient mice. A single intranasal dose of rhKGF reversed defects in bacterial clearance, AM function, and BALF antimicrobial activity. We conclude that KGF supports alveolar innate immune defense through maintenance of alveolar antimicrobial protein levels and functions of AM. Together these data demonstrate a subtle but important role for endogenous KGF in the maintenance of normal pulmonary innate immune function.

KEYWORDS: Keratinocyte Growth Factor, Pneumonia, Innate Defense, Collectins, Lysozyme, Alveolar Macrophages
INTRODUCTION

Pneumonia accounts for over one million hospital discharges each year and for about 5% of all inpatient hospital deaths (8, 17). Although antibiotics typically speed recovery in bacterial pneumonias, even the most aggressive available therapies can fail to control virulent or resistant pathogens. Inadequate antimicrobial treatment is a leading cause of death for patients in the intensive care unit and predicts the development of life threatening acute lung injury in patients with pneumonia (18, 19). The mortality and morbidity of serious respiratory infections could be substantially impacted by the development of broad-spectrum strategies that augment the innate immune defenses of the lung and enhance resistance to tissue injury.

KGF, also known as fibroblast growth factor 7, is an attractive candidate for this role. KGF is a potent epithelial mitogen and differentiation factor, produced almost exclusively by cells of mesenchymal origin, that acts predominantly on epithelial cells through ligation of an alternatively spliced tyrosine kinase receptor called FGFR2-IIIb (7, 12, 30). Exogenously administered KGF has been reported to protect the lungs of animals from a variety of subsequent insults including acid instillation (44), hyperoxia (25), bleomycin (9), radiation exposure (36, 46), LPS instillation (14), bacterial challenge (39) and ventilator induced injury (40). The protective effects of KGF have been linked to stimulation of AECII proliferation and differentiation, inhibition of apoptosis, attenuation of DNA damage and induction of factors that reduce oxidative stress (11). We have recently reported that rhKGF treatment results in the
release of GM-CSF from the alveolar epithelium, leading to STAT5 associated macrophage activation and enhanced clearance of *E. coli*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis* from the murine lung (26, 43). A recent study extended these findings to humans by demonstrating that bronchoalveolar lavage fluid (BALF) isolated from rhKGF challenged individuals enhances phagocytic uptake of bacteria by alveolar macrophages through a GM-CSF dependent mechanism (32). In addition, data from several laboratories indicates that KGF enhances expression of the pulmonary collectins, SP-A and SP-D; proteins which bind and aggregate bacterial, fungal, and viral agents, directly activate macrophages, and enhance the phagocytosis and intracellular killing of a variety of pulmonary pathogens (16, 24). We have found that SP-A and SP-D directly inhibit the growth of gram-negative bacteria by a membrane destabilizing mechanism that is sensitive to pH, calcium concentration, and conditions that are hostile to protein structural integrity (e.g.-heat, or protease digestion) (23, 42). Together, these studies suggest that KGF may have promise as a therapeutic agent that mitigates lung injury and enhances bacterial clearance in patients with pneumonia.

Although multiple laboratories have reported beneficial effects of exogenous KGF supplementation on protection and repair of epithelial tissues, there is little information on the natural role of endogenous KGF in lung injury and healing. Surprisingly, the phenotype described in the original KGF null mouse study revealed only an aberration in hair coat with no gross alterations in lung morphology or epidermal wound healing (13). Subsequent studies in KGF null mice have revealed subtle effects of KGF deficiency including reduced nephron number and kidney size (29), thinned bladder urothelium (37), impaired presynaptic differentiation and reduced seizure threshold (38), reduced thymopoiesis following irradiation despite normal baseline thymic cellularity (2), delayed cutaneous wound contraction in a diabetic
mouse background (27), attenuated angiogenesis in a cutaneous wound model (28), depressed liver progenitor cell expansion and increased mortality in a model of toxin-induced hepatic injury (35) and increased injury with delayed repair of mucosal tissue following dextran sulfate induced colitis (6). Overall these studies suggest endogenous KGF plays a role in regulating epithelial homeostasis and repair. Importantly, to our knowledge no study has described a lung phenotype in the KGF null mouse despite the critical role of respiratory epithelial cells in pulmonary function and host defense.

The purpose of the current study was to determine if endogenous KGF plays a role in the maintenance of innate defense against pulmonary infection. We found that KGF deficient mice exhibit delayed clearance of *E. coli* from the lungs, attenuated phagocytic function of AM, and reduced cell-free BALF antimicrobial activity that was associated with lower levels of alveolar SP-A, SP-D and lysozyme. AECII cell counts and per cell gene expression of antimicrobial proteins were also reduced in KGF deficient mice. These immune deficiencies in KGF KO mice were largely rescued three days following a single intrapulmonary dose of rhKGF. We conclude that KGF supports alveolar innate immune defense through maintenance of alveolar antimicrobial protein levels and AM function.
MATERIALS AND METHODS

Mouse model and KGF treatment

All animal procedures were approved by the University of Cincinnati Institutional Care and Use Committee. Mice were maintained under barrier containment in the vivarium facilities, and appeared healthy and free of infection at the time of study. Age matched female KGF WT and KO mice were generated by backcrossing hybrid B6;129-Fgf7^{tm1Efu}/J with wildtype C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) through ten generations. Heterozygous littermates from generation ten were used to establish breeding pairs for the generation of KGF WT and KO mice used in this study. In experiments in which rhKGF was used in a rescue scenario in mice, rhKGF/Kepivance® (Swedish Orphan Biovitrium, Stockholm, Sweden) was delivered in a single intranasal (i.n.) dose of 5 mg/kg in a 50 µl volume. For terminal experiments, mice were sacrificed by intraperitoneal injection of Fatal-Plus solution (Vortech Pharmaceuticals, Dearborn, MI).

Infection model and quantitative culture

*E. coli* K12 was grown in brain heart infusion (BHI) broth overnight at 37 °C with continuous agitation. The following morning, bacteria were centrifuged, washed, and resuspended in PBS. Isoflurane-anesthetized mice were inoculated i.n. with 5x10^6 cfu of *E. coli* in a 50 µl volume. At 6 hours post infection, lungs were removed, homogenized, serially diluted and plated on trypticase soy agar (TSA). After overnight incubation, manual plate counts were performed to quantify bacterial burden in the lung.
Whole lung and isolated AECII gene expression

Total RNA was isolated from murine lung tissue with RNAzol® RT (Molecular Research Center, Cincinnati, OH) and used in 1 µg aliquots to generate cDNA using the SuperScript® III First-Strand Synthesis System (Life Technologies, Carlsbad, CA). Power SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) was used to amplify DNA fragments through up to 40 cycles at 95 °C for 15 seconds followed by 60 °C for 1 minute. Intron-spanning primers used in this study are detailed in Table 1. Expression was determined relative to the housekeeping gene hypoxanthine guanine phosphoribosyl transferase (Hprt). To quantify relative gene expression in approximately 2x10^6 AECII isolated from individual mice 100 ng of RNA was used for the conversion to cDNA.

Quantitation of AECII

The tracheas of euthanized mice were cannulated and lungs were inflation-fixed with neutral buffered 10% formalin (Fisher Scientific, Fairlawn, NJ) at 25 cm H2O. The lungs were immersed in neutral buffered 10% formalin overnight, embedded in paraffin, cut into 5 µm sections, and mounted on Trubond 380 adhesive microscope slides (Tru Scientific, Bellingham, WA). AECII were identified by staining with an anti-pro-SPC antibody (Seven Hills Bioreagents, Cincinnati, OH). After antigen retrieval in citrate buffer, tissue sections were quenched with 0.3% hydrogen peroxide in methanol, blocked with 15% normal goat serum and incubated with rabbit pro-SPC antibody (1:5000 dilution) overnight at 4 °C. Slides were developed by incubation with biotin labeled goat anti-rabbit IgG secondary antibody (1:500 dilution), and avidin-peroxidase (Vector Laboratories, Burlingame, CA) dependent oxidation of diaminobenzidine (DAB). A blinded
quantitation of pro-SPC positive cells/HPF was performed at 40x magnification on 5 random fields of lung parenchyma that were devoid of large airways or blood vessels.

**Isolation of AECII**

Lungs were perfused with 10 ml of sterile normal saline via the pulmonary artery. The airway was cannulated via tracheostomy with a 20-gauge metallic angiocatheter and 3 ml (50 Caseinolytic Unit/ml) of dispase (Corning, Corning, NY) was instilled followed by 0.5 ml of 1% low-melt agarose (warmed to 45 °C). Lungs were rapidly cooled on ice for 2 min, incubated in 1 ml of dispase for 45 min at room temperature, and transferred to a culture dish containing 100 units/ml of DNase I (Worthington Biochemical, Lakewood, NJ). The parenchymal lung tissue was gently teased from the bronchi and homogenized. Cell suspensions were filtered, collected by centrifugation, and placed on prewashed 100 mm tissue culture plates coated with anti-mouse CD45 and CD32/16 antibodies (BD Biosciences, Franklin Lakes, NJ). After incubation for 60 min at 37 °C in 5% CO2 atmosphere to promote adherence of contaminating macrophages and fibroblasts, the AECII were gently panned from the plate, collected by centrifugation, and counted. Cell viability determined with trypan blue staining was routinely >90%, and cell purity determined by SP-C staining ranged from 75 to 90%.

**BAL cell and fluid collection**

Bronchoalveolar lavage (BAL) was performed by five cycles of infusion and aspiration using 1 ml of HBSS, repeated five times. Recovery was routinely greater than 80% of infused volume. BAL cells were separated from fluid by centrifugation at 400 x g for 5 minutes at 4 °C. Fluid from the first 1 ml lavage was stored in a -80 °C freezer until use, while cells recovered from all
lavage cycles were combined and used immediately for assays. For some assays BALF samples were concentrated using Centricon® 3000MW cut off spin filters (Millipore, Billerica, MA). Routine protein concentrations were determined with a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, Illinois) using bovine serum albumin (BSA) as a standard.

Surfactant protein and cytokine concentration determination in BALF

SP-A and SP-D protein levels were quantified in BALF using a self-sandwich ELISA. Polyclonal rabbit anti-mouse SP-A and SP-D were generated against recombinant antigens. ELISAs were performed by loading equivalent volumes of BALF from each mouse into wells of a microplate precoated with 10 µg/ml of anti-mouse SP-A or SP-D antibody. After incubation and washing, HRP conjugated anti-mouse SP-A or SP-D secondary antibody was added, and the mixture was incubated at room temperature. After washing, the assays were developed by incubation with SureBlue Reserve TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, MD) and measurement at a wavelength of 450 nm. Recombinant mouse SP-A and SP-D were used to generate standard curves. Cytokine and chemokine concentrations were determined in BALF using commercial ELISA’s (R&D Systems, Minneapolis, MN).

Lysozyme activity assay

Lysozyme activity was quantified in BALF according to published methods with minor modifications (21). Briefly, Micrococcus luteus (Sigma-Aldrich, St. Louis, MO) was suspended in 0.4 M potassium phosphate buffer (32% Dibasic, 68% Monobasic, pH 6.5 at 25 °C) containing 0.1% sodium azide and 0.1% BSA. A 20 µl aliquot of BALF was mixed with 180 µl aliquot of the M. luteus suspension pre-titrated to result in an ultimate O.D.450 nm of 1.0 for the
mixture. Changes in absorbance readings were recorded every four minutes during incubation at 37°C in a VICTOR²™ microplate reader (Perkin Elmer, Waltham, MA). Wells containing purified chicken lysozyme (Worthington Biochemical, Lakewood, NJ) or assay buffer alone were used as positive and negative controls, respectively. Lysozyme activity was calculated as the ∆OD450nm = (Buffer OD₄₅₀nm – Sample OD₄₅₀nm) over the linear range of the curve.

**Bacterial killing assay**

*E. coli* K12 was grown overnight in BHI broth at 37 °C with continuous agitation. The following morning, bacteria were subcultured in fresh media for 3 hours and log phase organisms were harvested by centrifugation, washed, and re-suspended in HBSS. BALF antimicrobial activity was assessed by mixing 90 µl BALF and 10 µl (5 x 10³ cfu) of the *E. coli* suspension into wells of a 96 well microtiter plate followed by incubation at 37 °C with constant agitation for 90 minutes. Bacteria were disaggregated by vigorous discharge through a pipette, diluted and plated on TSA plates. After overnight incubation of the plates at 37 °C, cfu were counted.

**Bacterial viability assay**

Bacterial viability was assessed by incubating 2x10⁶ *E. coli* in 100 µl of BALF (200 µg/ml) for 30 minutes followed by addition of the fluorescent probe SYTO 9, which stains the bacterial membrane green regardless of viability state, and propidium iodide, which stains DNA red in permeabilized (dead) cells (*BacLight™;* Life Technologies, Grand Island, NY), as previously described (3). To determine the percent viability, live (green) and dead (red) cells were quantified on a VICTOR²™ fluorescence microplate reader (Perkin Elmer, Waltham, MA) by
comparing fluorescence at 485 and 530 nm with that of a 5 point standard curve generated by mixing varying proportions of ethanol killed *E. coli* and live *E. coli* prior to staining.

**Bacterial permeability assay**

*E. coli* were grown to mid-log phase in BHI broth at 37 °C, washed twice with 5 mM Tris and 150 mM NaCl, and resuspended to an OD of 1.0 at 600 nm. A 50 µl volume of the *E. coli* suspension was aliquoted into a 96-well polystyrene microtiter plate and incubated in concentrated BALF (450 µg/ml) for 15 minutes at 37 °C with constant agitation. The cleavage-activated, alkaline phosphatase substrate ELF 97 (Life Technologies, Grand Island, NY) was added, and fluorescence was measured over time at excitation and emission wavelengths of 355 and 535 nm, respectively (20).

**Phagocytosis assay**

The *E. coli* K12 isolate was labeled with pHrodo™ Red, succinimidyl ester (Life Technologies, Grand Island, NY) according to manufacturer’s instructions. Labeled bacteria were pre-opsonized and incubated for 30 minutes at 37 °C with freshly isolated BAL cells at a ratio of 200 bacteria/cell in HBSS supplemented with 10 mM HEPES and 10% normal mouse serum. Following incubation, cells were washed two times in ice cold HBSS with 10 mM HEPES and 0.2% BSA and placed on ice. Nonspecific binding was blocked with 2 µl of Fc Block (BD Biosciences, Franklin Lakes, NJ) and 5 µl of rat serum per 100 µl sample for ten minutes prior to staining cells with EF660 F4/80 (Clone BM8), EF450 CD11b (Clone M1/70) and Percp EF710 CD11c (Clone N418) for thirty minutes. All antibodies were purchased from Ebioscience (San Diego, CA). After washing, samples were run on a BD LSRII flow cytometer using Fluorescence
Minus One (FMO) controls to correct for spectral overlap. Unlabeled bacteria served as a negative control for pHrodo red fluorescence. Median channel fluorescence of pHrodo red was used as the measure of phagocytosis for F4/80+, CD11c+, and CD11b-/low AM.

**BAL and blood cell composition determination**

BAL cells were suspended in 100 µl ice cold FACS Buffer (PBS, 1% BSA and 0.1% sodium azide). Nonspecific binding was blocked by adding 5 µl of rat serum and 1 µg of anti-mouse CD16/32 (BD biosciences, San Diego, CA) to the suspension for 15 minutes. The cells were then stained with anti-mouse CD45, F4/80, CD11c, CD3, CD45R/B220, and Ly6G (BD biosciences or Affymetrix, San Diego, CA) for 30 minutes. Samples were run on a BD LSRII flow cytometer using FMO controls and analyzed with FCS Express 5 software (Denovo Software, Los Angeles, CA). BAL cells were identified by the profile of antibody staining as alveolar macrophages (CD45+, CD11c+, F4/80+, CD11b-/low), neutrophils (CD45+, CD11b+, Ly6G+), monocytes (CD45+,CD11b+,F4/80+, Ly6G-) or lymphocytes (CD45+, CD3+ or CD45R+). Absolute BAL leukocyte numbers were calculated by multiplying total BAL cell counts by the percentages of cells in each subpopulation (macrophage, monocyte, etc) as determined by flow cytometry. Blood was collected by cardiac puncture in EDTA coated tubes and cellular composition was determined using an automated system (Hemavet® 950, Drew Scientific, Miami Lakes, FL).

**Statistics**

Statistical analysis was performed using GraphPad Prism 5. Significance between two groups was determined with the Student's t-test. In experiments where more than two groups were compared, an ANOVA and Bonferroni posttest comparing selected groups was employed to
correct for multiple comparisons. The term ‘selected groups’ refers to WT versus KO and KO versus KO+rhKGF. Significant differences were determined at \( p \) values of <0.05.

**RESULTS**

Pulmonary bacterial clearance is impaired in KGF knockout mice in association with reduced antimicrobial activity in alveolar lining fluid and reduced phagocytic function of alveolar macrophages.

To test the role of endogenous KGF in the response to pulmonary infectious challenge, KGF WT and KO mice were inoculated i.n. with \( 5 \times 10^6 \) cfu of *E. coli*. The lungs were harvested six hours post challenge, homogenized and plated on agar. The mean bacterial burden in the lungs of KGF KO mice was three fold higher than WT mice (KO \( 1.1 \times 10^7 \) cfu/lung versus WT \( 3.7 \times 10^6 \) cfu/lung, \( p < 0.01 \)) (Fig. 1A). To investigate mechanisms responsible for the bacterial clearance defect in KGF KO mice, we performed BAL on uninfected KGF WT and KO mice to collect BALF and cells for *in vitro* bacterial clearance assays. BALF isolated from KGF KO mice was found to contain reduced antimicrobial activity, allowing approximately twofold more *E. coli* growth than BALF isolated from KGF WT mice (KO \( 3.9 \times 10^4 \) cfu versus WT \( 2.0 \times 10^4 \) cfu, \( p < 0.01 \)) during a 90 minute incubation period (Fig. 1B). Phagocytic function of AM isolated from KGF KO mice was found to be reduced by more than threefold compared to AM isolated from WT mice (KO 922 MFI versus WT 3000 MFI, \( p < 0.01 \)) (Fig. 1C). Together, these results indicate pulmonary bacterial clearance in KGF KO mice is impaired and is associated with reduced alveolar lining fluid antimicrobial activity and AM function.

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Leukocyte recruitment and the inflammatory response are not impaired in KGF deficient mice.

To determine if the bacterial clearance defect in KGF deficient mice was associated with altered inflammatory cell recruitment to the lung, we quantified blood and BAL leukocytes prior to infection and six hours after i.n. inoculation with $5 \times 10^6$ cfu of \textit{E. coli}. Blood leukocyte composition was similar in KGF WT and KO mice prior to infection and neutrophilia occurred in both groups following bacterial challenge, as expected. Although circulating neutrophils following infection tended to be higher in KGF KO mice, the levels achieved did not reach statistical significance (Fig. 2A). BAL leukocyte counts were similar in KGF WT and KO mice prior to infection, and neutrophil counts were marginally higher in KGF deficient mice post challenge (KO $1.4 \times 10^6 \pm 1.7 \times 10^5$ neutrophils versus WT $9.6 \times 10^5 \pm 4.2 \times 10^4$ neutrophils, \( p < 0.05 \)) (Fig. 2B). To assess potential differences in the inflammatory tone of the lungs of KGF WT and KO mice we assessed proinflammatory chemokines and cytokines including CXCL1/KC, GM-CSF, TNF\(\alpha\) and IL-6 in BAL fluid before and after infection. These mediators were below detectable limits in BALF from both groups prior to infection (data not shown) and increased similarly in KGF WT and KO mice following infection (Fig. 2C). Collectively, these data indicate leukocyte recruitment and the inflammatory responses are not significantly altered in KGF deficient mice suggesting that deficits in antimicrobial proteins and alveolar macrophage function are likely the primary mechanisms responsible for the clearance defect in KGF deficient mice.

Whole lung expression of the antimicrobial collectins and lysozyme genes are selectively reduced in KGF knockout mice.
Whole lung gene expression analysis was performed to determine if deficiencies in specific innate immune mediators were associated with the observed bacterial clearance defect in KGF KO mice (Fig. 3). Fgf7 was not detected in the KGF KO mice. Whole lung expression of Csf2 (GM-CSF) was not different between groups. Expression of Sftpc (SP-C), which is restricted to AECII, was reduced by 25% in lungs of KGF KO mice compared to WT mice (KO 393±16 versus WT 527±25, \( p<0.01 \)). Whole lung expression of antimicrobial proteins Sftpa (SP-A), Sftpd (SP-D), Lyz1 (lysozyme 1) and Lyz2 (lysozyme 2) were also found to be reduced in KGF KO compared to WT mice; Sftpa expression by 39% (KO 36±1.4 versus WT 59±2.7, \( p<0.01 \)), Sftpd by 36% (KO 7.3±0.5 versus WT 11.4±0.8, \( p<0.01 \)), Lyz1 by 49% (KO 2.3±0.2 versus WT 4.5±0.4, \( p<0.01 \)), and Lyz2 by 31% (KO 81±3.2 versus WT 116±5.4, \( p<0.01 \)).

Expression of genes for other antimicrobial proteins tested, including Slpi (secretory leukocyte protease inhibitor), Ltf (lactoferrin), Defb1 (β-defensin 1), Camp (cathelicidin), Splunc1 (short plate, lung, and nasal epithelium clone 1), S100a8 (calgranulin A) and S100a9 (calgranulin B), were not different in KGF KO compared to WT mice. Defb2 (β-defensin 2), Defb3 (β-defensin 3) and Defb4 (β-defensin 4) were not detected in either group (data not shown). Together, these results indicate expression of the antimicrobial collectins SP-A and SP-D as well as the two forms of lysozyme found in mice are selectively reduced in the absence of endogenous KGF. The concurrent reduction in Sftpc expression suggests deficits may be attributable to changes in AECII numbers or per cell gene expression or both in the absence of endogenous KGF.

*Antimicrobial collectin and lysozyme protein levels are reduced in alveolar lining fluid of KGF knockout mice.*
Collectin and lysozyme protein concentrations were assessed in BALF isolated from KGF WT and KO mice to determine if the changes in gene expression observed in KGF KO mice were reflected at the protein level. SP-A and SP-D levels were decreased in KGF KO mice by 20% (KO 2.4±0.2 µg/ml versus WT 3.0±0.2 µg/ml, *p*<0.05) and 39% (KO 0.85±0.08 µg/ml versus WT 1.36±0.07 µg/ml, *p*<0.01), respectively (Fig. 4A&B). Lysozyme levels in BALF, quantified by a turbidimetric activity assay using *M. luteus* as the substrate, were also found to be reduced by 18% in KGF KO mice compared to KGF WT mice (KO 0.42±0.01 OD versus WT 0.51±0.02 OD, *p*<0.01) (Fig. 4C). These results demonstrate that impaired bacterial clearance and reduced antimicrobial activity in the alveolar lining fluid of KGF KO mice are associated with reductions in alveolar levels of SP-A, SP-D and lysozyme, suggesting a possible causal relationship.

**AECII counts and antimicrobial gene expression are reduced in KGF deficient mice.**

Reduction of whole lung expression of Sftpc observed in KGF KO mice raises the question of whether the deficiency of antimicrobial collectins and lysozyme in the alveolar lining fluid is due to reduction in AECII cell numbers or per cell gene expression or both. We therefore assessed AECII numbers by morphometric analysis and measured relative gene expression in isolated AECII. A representative image of KGF WT and KO mouse lung sections incubated with anti-pro SP-C IgG linked to HRP shows staining of pro-SPC positive AECII cells following avidin-peroxidase dependent oxidation of DAB (Fig. 5A). Counts of pro-SPC positive cells per high power field were found to be reduced by 17% (KO 11.5±0.5 versus WT 13.8±0.8, *p*<0.01) in KGF KO mice compared to WT mice (Fig. 5B). Relative expression of surfactant protein and lysozyme genes in isolated AECII tended to reflect the magnitude of differences in expression.
that we found in whole lung homogenates (Fig. 5C). Relative expression of Sftpd, Lyz1, and
Lyz2, were found to be reduced by 37% (KO 27.7±2.1 versus WT 43.9±4.4, \(p<0.01\)), 48% (KO
10.9±0.9 versus WT 21.1±4.0, \(p<0.05\)), and 44% (KO 161±7 versus WT 242±17, \(p<0.01\)),
respectively, in AECII isolated from KGF KO mice compared to KGF WT mice. The magnitude
of these differences in gene expression exceed the reductions that would have been predicted
based on 17% lower AECII counts in the KO mice. Trends toward reductions in Sftpc and Sftpa
gene expression on a per cell basis in the KO vs. WT mice did not reach statistical significance.
These data suggest that endogenous KGF supports both maintenance of AECII cell numbers and
differentiated expression of genes that encode antimicrobial proteins.

Reduced levels of antimicrobial collectins and lysozyme in KGF knockout mice are at least
partially restored by rhKGF.

To determine if the deficiency of antimicrobial collectins and lysozyme in KGF knockout
mice could be rescued by administration of exogenous KGF, we treated mice with a single 5
mg/kg i.n. dose of rhKGF. Control KGF WT and KO mice received PBS vehicle alone by the
same route. BALF was collected from each group three days after rhKGF treatment. SP-A levels
were lower in BALF isolated from vehicle treated KGF KO mice compared to vehicle treated
WT mice (KO 0.8±0.1 µg/ml versus WT 2.5±0.5 µg/ml, \(p<0.01\)), and the trend toward partial
correction of BALF SP-A levels in KGF treated KO mice did not reach statistical significance
(Fig. 6A). SP-D levels were also lower in BALF isolated from vehicle treated KGF KO mice
compared to vehicle treated WT mice (KO 0.6±0.04 µg/ml versus WT 1.3±0.08 µg/ml, \(p<0.01\))
and supra-normal SP-D levels were induced following KGF treatment (KO 0.6±0.04 µg/ml
versus KO+KGF 4.0±0.02 µg/ml, \(p<0.01\)) (Fig. 6B). Similar to SP-A, lysozyme levels were
significantly lower in BALF isolated from vehicle treated KGF KO mice compared to vehicle treated WT mice (KO 0.29±0.03 OD versus WT 0.47±0.02 OD, p<0.01), and the treatment of KO mice with KGF resulted in a partial increase in lysozyme levels that did not achieve statistical significance (Fig. 6C). These results demonstrate SP-D deficiency in KGF KO mice can be rescued by exogenous administration of KGF.

The pulmonary bacterial clearance defect in KGF knockout mice is rescued by exogenous KGF.

To determine if the defect in pulmonary bacterial clearance in KGF knockout mice could be rescued by administration of exogenous KGF, we treated mice with a single 5 mg/kg i.n. dose of rhKGF. Control KGF WT and KO mice received PBS vehicle alone by the same route. Three days after treatment, mice were lavaged to separately assess the antimicrobial activities of the soluble and cellular alveolar compartments, or inoculated with bacteria i.n. to assess in vivo clearance. Six hours after intranasal inoculation of 5x10^6 cfu of *E. coli*, the bacterial burden was more than threefold higher in the homogenized lungs of vehicle treated KGF KO mice compared to vehicle treated WT mice (KO 1.7x10^7 cfu versus WT 5.3x10^6 cfu, p<0.01) and the clearance defect was reversed by treatment of KO mice with KGF (KO 1.7x10^7 cfu versus KO+KGF 6.1x10^6 cfu, p<0.01) (Fig. 7A). BALF isolated from vehicle treated KGF KO mice exhibited reduced antimicrobial activity, allowing approximately twofold more growth than BALF isolated from vehicle treated WT mice (KO 2.9x10^4 cfu versus WT 1.5x10^4 cfu, p<0.01) over a 90 minute incubation period, while BALF antimicrobial activity was restored in KO mice treated with KGF (KO 2.9x10^4 cfu versus KO+KGF 9.0x10^3 cfu, p<0.01) (Fig. 7B). To control for the possibility that rhKGF could have intrinsic antimicrobial activity, an experiment was performed in which BALF from KGF KO mice was spiked with 100 µg of rhGKF or vehicle alone prior to
performing the bacterial killing assay. Identical colony counts from rhKGF and vehicle spiked BALF demonstrated that even if 100% of the instilled rhKGF was recovered in lavage three days after instillation it would have no effect on the bacterial killing assay (data not shown). Exposing *E. coli* to BALF isolated from vehicle treated WT mice or KGF treated KO mice resulted in a 30% reduction in bacterial viability relative to BALF isolated from vehicle treated KO mice (WT 71±5% viability or KO+KGF 71±3% viability versus KO 100±7% viability, \( p < 0.01 \)) (Fig. 7C). Bacterial permeabilizing activity was nearly threefold lower in BALF isolated from vehicle treated KGF KO mice compared to vehicle treated WT mice (KO 133±31 arbitrary units versus WT 366±65 arbitrary units), however the partial restoration of permeabilizing activity in BALF isolated from KO mice treated with KGF did not reach statistical significance (Fig. 7D). Finally, phagocytic function was more than threefold lower in AM isolated from vehicle treated KGF KO mice compared to AM isolated from vehicle treated WT mice or KGF treated KO mice (KO 972±199 MFI versus WT 2851±627 MFI or KO+KGF 2837±484 MFI, \( p < 0.05 \)) (Fig. 7E). Together these data demonstrate the pulmonary bacterial clearance defect, the deficiency of alveolar lining fluid antimicrobial activity, and the reduced phagocytic function of AM in KGF KO mice can largely be reversed by exogenously delivered KGF.

**DISCUSSION**

KGF is a well characterized pulmonary epithelial mitogen and differentiation factor but relatively little is known about the role of the growth factor in host defense. Our prior studies demonstrated that rhKGF treatment results in the release of GM-CSF from the alveolar
epithelium, leading to STAT5 associated macrophage activation and enhanced clearance of *E. coli*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis* from the murine lung (26, 43). Exogenously delivered KGF is also known to induce the secretion of the pulmonary collectins (45), surfactant associated proteins that have well characterized host defense properties (24). Based on this information we postulated that endogenous KGF deficiency would result in impaired pulmonary innate immune function.

To explore this hypothesis, we studied KGF KO animals that were developed by breeding the KGF null allele through 10 generations into the C57BL/6 background to eliminate subtle immune differences between littermates attributable to genetic variations. We found that KGF deficiency resulted in a defect in bacterial clearance, an attenuation of AM phagocytic function, and a reduction in the killing and permeabilizing activity of the alveolar lining fluid, which were largely rescued three days following a single intrapulmonary dose of rhKGF. Our data suggest that the reduction of antimicrobial activity in BALF collected from KGF deficient animals is at least partially attributable to decreased pulmonary collectin and lysozyme expression; deficiencies that are likely related to reduction in AECII numbers and differentiated AECII functions. The antimicrobial functions of collectins and lysozyme have been previously validated in models of excess and deficiency (1, 22, 42). Our results also demonstrate bacterial permeabilization activity is reduced in KGF KO BALF, a finding which is consistent with the known antimicrobial mechanism of collectins and lysozyme (15, 42). It is possible and perhaps likely that other antimicrobial proteins are playing a role in the BALF permeabilizing and killing defects, but we did not find any other differences from wild type mice in the expression of the antimicrobial protein genes tested. It is also possible that small reductions in collectins and lysozymes may interfere with cooperative interactions with other antimicrobial proteins.
Synergistic killing of *E. coli* has been demonstrated for lysozyme in combination with other antimicrobial factors including lactoferrin and secretory leukocyte protease inhibitor (33). We conclude that the concurrent reduction of collectins and lysozyme negatively impact antimicrobial activity within alveolar lining fluid, through loss of their individual contributions to bacterial killing, and perhaps through loss of cooperative interactions with other antimicrobial partners.

A limitation of this study is that the relative physiologic importance of the collectins and lysozyme in host defense activities of KGF deficient and exogenously supplemented models remain incompletely defined. In addition, this study cannot exclude the possibility that other antimicrobial peptides such as defensins or cathelicidin are induced by rhKGF and contribute to the rescue of alveolar lining fluid antimicrobial activity in KGF deficient animals. Finally, the relative role of the AM in altered bacterial clearance and the mechanisms of attenuated AM phagocytic function in KGF deficient animals remain to be determined. We demonstrated that other than a mild BAL neutrophilia in the KGF deficient animal after bacterial challenge, there were no meaningful between group differences in selected inflammatory mediators or inflammatory cell numbers or composition, either at baseline or 6 hours after intratracheal E. coli delivery. It is notable that in our prior studies (26, 43) and those of others (32), KGF supplementation enhanced GM-CSF levels. It is curious therefore that we did not detect any difference in whole lung GM-CSF gene expression prior to infection or in post infection BALF GM-CSF protein levels. BALF GM-CSF protein levels in unchallenged animals are at or below the limits of detection by ELISA, rendering it difficult or impossible to measure reductions in BAL GM-CSF protein levels in the KGF null animals using available methods. To overcome these limitations our ongoing studies to explore the mechanism of attenuated AM function in
KGF KO animals are focused on AM transcription. This preliminary data suggests there are no significant between group baseline differences in M1 or M2 macrophage phenotypes, based on expression of markers such as Arg1, iNOS and IL-10, but does provide some evidence of decreased GM-CSF dependent transcript expression (data not shown) in the KGF deficient animals relative to wild type controls. Further study will be required to definitively determine if altered GM-CSF expression plays a role in the immune defects that are present in the KGF deficient mouse model. Despite these limitations, our study validates a role for KGF in the maintenance of AECII pool sizes and differentiated AECII functions that support pulmonary host defense.

Nature often borrows elements of normal developmental processes to execute repair and host defense functions in the mature organism (41). Examples include the key roles of NFkB pathway proteins both in Drosophila morphogenesis and in inflammation in mammalian organisms. KGF is a potent epithelial mitogen with well described cytoprotective and regenerative properties (11). Reports that KGF is upregulated in gingival fibroblasts by LPS and inflammatory cytokines (IL1α, IL1β, IL6, TNFα, TGFα, PDGF-BB) (4, 5, 31), and that KGF stimulates the production of antimicrobial peptides and enhances bactericidal activity of skin grafts by more than 500 fold (10), are consistent with a role for the growth factor in the augmentation of innate immune defenses. Viget et al reported that a single intratracheal instillation of KGF (5 mg/kg) in rats 48 hours prior to infection with Pseudomonas aeruginosa resulted in improved barrier function in vivo and in isolated perfused lungs, enhanced bacterial clearance, and increased survival (39). The documented efficacy of KGF in reducing the severity of mucositis following radiation and chemotherapy illustrates the protective potential of KGF for epithelial and mucosal tissues (34). Although the mechanism of beneficial KGF actions in
mucositis is widely attributed to preservation of epithelial barrier integrity, infection is central to
the pathogenesis of the condition, and we speculate that the antimicrobial actions of KGF might
have also contributed to the favorable outcomes of the mucositis studies that lead to FDA
approval for that indication.

Collectively, these data are consistent with a previously undefined role for endogenous
KGF in the maintenance of alveolar epithelial homeostasis and pulmonary innate immunity. In
conjunction with our prior studies, our most current results suggest that KGF may enhances
bacterial clearance through a dual mechanism that includes augmentation of macrophage
antimicrobial actions and maintenance of antimicrobial protein levels in the alveolar lining fluid.
KGF maintains AECII in vivo and in pharmacological concentrations induces them to
proliferate, and to release antimicrobial proteins and GM-CSF, augmenting the permeabilizing,
aggregating and killing activities of the alveolar lining fluid and enhancing recruitment, oxidant
and killing functions of alveolar macrophages. We submit that pharmacologic augmentation of
antimicrobial protein production and macrophage activation through a KGF/FGFR2-IIIb/GM-
CSF/GM-CSFR paracrine loop might be exploited to advantage, to enhance the host defense
functions in the airspace. Although the magnitude of the effect of KGF on bacterial clearance in
this model is modest relative to standard treatments, the combination of antibiotic therapy with
pharmacologic augmentation of endogenous innate immune defense is an intuitively appealing
treatment approach that may prove to be additive or even synergistic.

ACKNOWLEDGEMENTS
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REFERENCES


FIGURE LEGENDS

Figure 1. Impaired bacterial clearance in lungs of KGF knockout mice is associated with reduced antimicrobial activity in alveolar lining fluid and reduced phagocytic function of alveolar macrophages. (A) In vivo pulmonary bacterial clearance was assessed by quantitative culture of homogenized lung tissue six hours after i.n. inoculation of KGF WT and KO mice with 5x10⁶ cfu of *E. coli*. (B) Antimicrobial activity of alveolar lining fluid was determined by mixing 5x10³ cfu of *E. coli* with BALF collected from KGF WT and KO mice in wells of a microplate. After a 90 minute incubation at 37 °C, the suspensions were plated and cfu/well determined following overnight growth. (C) Phagocytic function of alveolar macrophages was assessed by incubating BAL cells from KGF WT and KO mice with pHrodo™ labeled bacteria in a 1:200 ratio for 30 minutes at 37 °C. The median fluorescence intensity (MFI) of pHrodo in alveolar macrophages was determined by FACS analysis and used as a measure of the number of *E. coli*/cell. Data are plotted for individual mice and lines represent the mean value for each group. n=6-8 mice/group, **p<0.01.

Figure 2. Leukocyte recruitment and the inflammatory response are not impaired in KGF deficient mice. (A) Blood and (B) BAL leukocyte composition were assessed prior to infection and 6 hours after i.n. inoculation of KGF WT and KO mice with 5x10⁶ cfu of *E. coli*. Data are mean ±SEM, n=5-6 mice/group, *p<0.05. (C) BAL cytokine and chemokine levels six hours after i.n. inoculation of KGF WT and KO mice with 5x10⁶ cfu of *E. coli*. Data are plotted for individual mice and lines represent the mean value for each group. n=6 mice/group.
Figure 3. Whole lung expression of the antimicrobial collectins and lysozyme genes are differentially reduced in KGF knockout mice. Lungs were harvested from KGF WT and KO mice and whole lung gene expression for 14 loci was determined on lung homogenates by RT-PCR and normalized to expression of the housekeeping gene Hprt. Data are mean ±SEM, n=5-6 mice/group, **p<0.01. ND indicates expression not detected.

Figure 4. Antimicrobial collectin and lysozyme protein levels are reduced in alveolar lining fluid of KGF knockout mice. BAL was performed on KGF WT and KO mice and levels of antimicrobial proteins were assessed in equivalent volumes of isolated BALF. An ELISA was used to determine (A) surfactant protein A (SP-A) and (B) surfactant protein D (SP-D) concentrations per ml of BALF. (C) Lysozyme activity in BALF was quantified with a turbidimetric assay using M. luteus as the substrate. The rate of substrate clearance is plotted as the ∆OD 450nm = (Buffer OD_{450nm} – Sample OD_{450nm}). The difference in absorbance at the 60 minute time point was used to compare groups. Data are mean ±SEM, n=6-8 mice/group, *p<0.05, **p<0.01.

Figure 5. KGF deficiency is associated with reduced AECII cell counts and antimicrobial protein gene expression. AECII counts and isolated AECII gene expression were assessed in KGF WT and KO mice. (A) Representative images of histological sections from inflation fixed lungs of KGF WT and KO mice stained with anti-pro-SPC antibody. Images were captured at 40x magnification and lines representing 20 µm are shown. (B) Blinded quantitation of pro-SPC positive cells per high power field in KGF WT and KO mice is shown. (C) Relative expression of antimicrobial protein genes in isolated AECII determined by RT-PCR. Data are mean ±SEM, n=5-6 mice/group, *p<0.05, **p<0.01.
Figure 6. Response of collectins and lysozyme levels in KGF knockout mice to a single dose of rhKGF. KGF KO mice were treated with a single 5 mg/kg i.n. dose of rhKGF. Control KGF WT and KO mice received PBS vehicle alone by the same route. Three days after treatments BAL was performed on all mice and levels of antimicrobial proteins were assessed in equivalent volumes of isolated BALF. An ELISA was used to determine (A) surfactant protein A (SP-A) and (B) surfactant protein D (SP-D) concentrations per ml of BALF. (C) Lysozyme activity in BALF was quantified with a turbidimetric assay using *M. luteus* as the substrate. The rate of substrate clearance is plotted as the ΔOD 450nm = (Buffer OD450nm – Sample OD450nm). The difference in absorbance at the 60 minute time point was used to compare groups. Data are mean ±SEM, n=6 mice/group. Significance was determined by ANOVA comparing selected pairs of groups (WT versus KO and KO versus KO+KGF), **p<0.01, ns indicates not significant.

Figure 7. Rescue of the pulmonary bacterial clearance defect in KGF knockout mice following a single dose of rhKGF. KGF KO mice were treated with a single 5 mg/kg i.n. dose of rhKGF. Control KGF WT and KO mice received PBS vehicle alone by the same route. Three days after treatment mice were inoculated with bacteria to assess *in vivo* clearance or BAL was performed to collect cells and fluid for *in vitro* assays. (A) *In vivo* pulmonary bacterial clearance was assessed by quantitative culture of homogenized lung tissue six hours after i.n. inoculation with 5x10^6 cfu of *E. coli*. (B) Antimicrobial activity of alveolar lining fluid was determined by mixing 5x10^3 cfu of *E. coli* with BALF in wells of a microplate. After a 90 minute incubation at 37 °C, the suspensions were plated and cfu/well determined following overnight growth. (C) Bacterial viability was assessed with the BacLight™ Live/Dead staining kit after incubating 2x10^6 *E. coli* in BALF (200 µg/ml) at 37 °C with shaking for 30 minutes. (D) Permeabilizing activity of concentrated BALF (450µg/ml) was assessed by incubating *E. coli* in the presence of
the impermeant alkaline phosphate substrate, ELF97, and using fluorescence expressed as arbitrary units (a.u.) to quantify permeabilization of the bacterial membrane. (E) Phagocytic function of alveolar macrophages was assessed by incubating the harvested BAL cells with pHrodo™ labeled bacteria in a 1:200 ratio for 30 minutes at 37 °C. The median fluorescence intensity (MFI) of pHrodo in alveolar macrophages was determined by FACS analysis and used as a measure of the number of \( E. coli \)/cell. \( *p<0.05, **p<0.01. \)

**TABLES**

**Table I. Primers for quantitative RT-PCR**

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<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<td><strong>Camp</strong></td>
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Figure 1.
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Figure 4.
Figure 5.

A. WT vs KO images showing differences in expression.

B. Bar graph of proSPC+ cells/HPF comparing WT and KO, with * indicating statistical significance.

C. Relative expression levels for Sftpc, Sftpa, Sftpd, Lyz1, and Lyz2, with p-values indicating significance: * for p=0.05, ** for p=0.01.
Figure 6.
Figure 7.