Published in final edited form as:

J Cell Physiol. 2013 February ; 228(2): 371–379. doi:10.1002/jcp.24140.

Impaired Pulmonary Defense Against *Pseudomonas aeruginosa* in VEGF Gene Inactivated Mouse Lung

Ellen C. Breen^{1,*}, Jaret L. Malloy, Kechun Tang¹, Feng Xia¹, Zhenxing Fu¹, Robert E. W. Hancock², Joerg Overhage^{2,3}, Peter D. Wagner¹, and Roger G. Spragg⁴

¹Division of Physiology, Department of Medicine, University of California, San Diego, La Jolla, CA, 92093-0623

²Centre for Microbial Diseases and Immunity Research, Univ. British Columbia, Vancouver, Canada V6T1Z4

³Institute of Functional Interfaces, Karlsruhe Institute of Technology (KIT), Eggenstein-Leopoldshafen, Germany

⁴Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of California, San Diego, La Jolla CA, 92093

Abstract

Repeated bacterial and viral infections are known to contribute to worsening lung function in several respiratory diseases, including asthma, cystic fibrosis and chronic obstructive pulmonary disease (COPD). Previous studies have reported alveolar wall cell apoptosis and parenchymal damage in adult pulmonary VEGF gene ablated mice. We hypothesized that VEGF expressed by type II cells is also necessary to provide an effective host defense against bacteria in part by maintaining surfactant homeostasis. Therefore, *Pseudomonas aeruginosa* (PAO1) levels were evaluated in mice following lung-targeted VEGF gene inactivation, and alterations in VEGF-dependent type II cell function were evaluated by measuring surfactant homeostasis in mouse lungs and isolated type II cells. In VEGF-deficient lungs increased PAO1 levels and pro-inflammatory cytokines, TNF α and IL-6, were detected 24 hours after bacterial instillation compared to control lungs. *In vivo* lung-targeted VEGF gene deletion (57% decrease in total pulmonary VEGF) did not alter alveolar surfactant or tissue disaturated phosphatidylcholine (DSPC) levels. However, sphingomyelin content, choline phosphate cytidyltransferase (CCT) mRNA and SP-D expression were decreased. In isolated type II cells an 80% reduction of VEGF protein resulted in decreases in total phospholipids (PL), DSPC, DSPC synthesis, surfactant associated proteins (SP)-B and -D, and the lipid transporters, ABCA1 and Rab3D. TPA-induced DSPC secretion and apoptosis were elevated in VEGF-deficient type II cells. These results suggest a potential protective role for type II cell-expressed VEGF against bacterial initiated infection.

Keywords

Pseudomonas aeruginosa; Surfactant; TNF α ; ABCA1; apoptosis

Introduction

Host defense against pathogens, pollutants and cigarette smoke is dependent on an intact, functioning epithelium. In patients with emphysema, a loss of alveolar structure is

*Correspondence and requests for materials should be addressed to: Ellen Breen, PhD. UCSD Department of Medicine 0623A, 9500 Gilman Drive, La Jolla, CA, 92093-0623, (858) 822 5859, fax (858)534-4812, ebreen@ucsd.edu.

associated with increased apoptosis of both epithelial and endothelial cells and decreased lung parenchyma VEGF and VEGFR-2 (Kasahara et al., 2001). In addition our laboratory has previously reported that inactivation of the pulmonary VEGF gene, through airway delivery of a *cre* recombinase adeno-associated virus (AAV/Cre) to *VEGFLoxP* mice, reproduces this increase in caspase-3 associated lung cell apoptosis accompanied by a reduction of VEGFR-2 and compromised integrity of the alveolar-capillary barrier (Tang et al., 2004). Alveolar type II cells express high levels of VEGF as well as VEGF receptors (Ng et al., 2001). Thus, this cell type may be important for both producing VEGF and responding to an injury or insult such as cigarette smoke by actively regulating the alveolar epithelium through VEGF signaling.

Type II cells produce surfactant. Alveolar surfactant is well known to lower surface tension at the air-liquid interface and contribute to a protective barrier. In addition, two hydrophilic proteins associated with surfactant, SP-A and SP-D, provide a specific role in the pulmonary innate immune system through their ability to recognize and participate in the phagocytosis of bacteria, apoptotic cells and DNA by alveolar macrophages and epithelial cells (Pastva et al., 2007; Vandivier et al., 2002). The critical role of surfactant is supported by targeted gene deletion studies in mice which reveal emphysema-like changes in airspace size and inflammatory cell influx following gene inactivation of several components of the alveolar surfactant system (SP-D, SP-C, PL_{A2}, ABCA1) (Bates et al., 2005; Botas et al., 1998; Glasser et al., 2003; Korfhagen et al., 1998; Rice et al., 1988; Wert et al., 2000).

Thus, we hypothesized that in adult mice VEGF plays an important role in regulating surfactant homeostasis. Alterations in the surfactant system, in particular reductions in the surfactant associated pulmonary collectins, SP-A and SP-D, were hypothesized to weaken the innate immune system. In this study lung-targeted VEGF-deficient mice were challenged with a bioluminescent strain of *Pseudomonas aeruginosa* (PAO1-Lux) and pulmonary levels monitored by optical imaging. VEGF-dependent type II cell function was determined *in vivo* by measuring several components and regulators of the surfactant system. *In vitro* a direct role of VEGF was tested by inactivating the gene in isolated adult *VEGFLoxP* type II cells and measuring surfactant homeostasis and apoptosis.

Materials and Methods

Animals

The Animal Subjects Committee of University of California, San Diego, approved the animal protocol for this study. The *VEGFLoxP* mouse strain was engineered by Dr. Ferrara's laboratory at Genentech, South San Francisco, CA (Gerber et al., 1999). Male and female mice (age 8–12 week) were delivered either AAV/LacZ (control) or AAV/Cre to inactivate the gene and mice were challenged 5 weeks later with PAO1. There was no change in body weight or BAL fluid volume, protein concentration or cell number following VEGF gene inactivation (Supplement Table 1).

Preparation of Recombinant Cre Recombinase Expressing Virus

For *in vitro* gene deletion experiments: recombinant adenovirus (Adv) expressing *Cre* recombinase or *LacZ* were produced and quantified. Recombinant adenoviruses were constructed using an adenovirus-5 backbone with protein-E1 deletion (Wang et al., 1996). The inserted gene cassette contained a CMV promoter, *cre* recombinase or *LacZ* reading frame, and SV40 poly A. Adv/Cre and Adv/LacZ were packaged in 293 cells and purified by cesium chloride density centrifugation. Viral titers were estimated by determining the number of plaque forming units (pfu) in NIH3T3 cells infected with serial dilutions of adenovirus preparations. For the *in vivo* lung-targeted VEGF gene deletion experiment:

recombinant adeno-associated virus (AAV) which express either LacZ or *Cre* recombinase coding regions were prepared as previously described (Tang et al., 2004). Anesthetized VEGF $LoxP$ mice were instilled via the trachea with 100 μ l of AAV/Creor AAV/LacZ solution ($\sim 2 \times 10^{10}$ total viral particles) followed immediately with a 200- μ l bolus of perflubron (Rimar 101, Miteni, Tissino, Italy). At 5 weeks post-AAV infection, mice were either sacrificed or challenged with PAO1. Based on our previously published results this time point is associated with decreased levels of VEGF and VEGFR-2, increased septal wall cell apoptosis, alveolar damage and loss of lung elastic recoil (Tang et al., 2004).

PAO1 Infection and Optical Imaging

A bioluminescent reporter *P. aeruginosa* PAO1 $_{pgem-Lux}$ was created by inserting a *luxCDABE* gene cassette under the control of the gentamicin resistance gene promoter into a single attTn7 intergenic site within the bacterial chromosome according to the method of Choi (Choi et al., 2005). This strain constitutively expresses the lux gene cassette and does not have an altered PAO1 phenotype based on characteristic growth, motility, biofilm formation and virulence against lettuce (*unpublished data*). Tracheal delivery of PAO1 $_{pgem-Lux}$ [$\sim 2 \times 10^5$ colony forming units (CFU)/30 μ l/mouse] was accomplished by anesthetizing mice with ketamine: xylazine (100 mg: 10 mg), making a small incision above the trachea and directly injecting the bacterial suspension just above the carina. Post-inoculation, mice were anesthetized with isoflurane and imaged using the Spectrum In Vivo Imaging System (IVIS) from Caliper Life Sciences. Bioluminescent PAO1 was quantified from images captured over 15 seconds.

VEGF, TNF α and IL-6 ELISA

VEGF and cytokine levels in type II cells and/or lung homogenates were measured by mouse specific ELISA's (R&D, Minneapolis, MN and Endogen, part of ThermoScientific).

Pulmonary Type II Cell Isolation and VEGF Gene Inactivation

Type II pneumocytes were isolated from VEGF $LoxP$ mice according to the method of Dobbs (Dobbs et al., 1986). Cells were panned over culture dishes coated with anti-CD-32 and anti-CD-45 biotin labeled antibodies (AbCam, Cambridge MA) plus strep-avidin coated magnetic beads (Becton Dickinson, San Diego) and cultured on rat type I collagen dishes (4 mg/ml coating solution, Becton Dickinson, San Diego). Type II pneumocyte purity was greater than 95% according to anti-Lamellar body protein ABCA3 immunohistochemical analysis (Covance, Berkeley, CA). VEGF gene inactivation was accomplished *in vitro* by incubation of type II cells with 5–10 pfu/cell of Adv/Cre and compared to cells infected with a control virus, Adv/LacZ or untreated cells.

Surfactant Measurements

Total Phospholipids (PL) and Disaturated Phosphatidylcholine (DSPC)—Total lipids from type II cells, lung tissue or cell-free bronchoalveolar lavage fluid (BALF) were extracted with chloroform/methanol according to the method of Bligh (Bligh and Dyer, 1959). Disaturated phosphatidylcholine (DSPC) was further isolated by reaction with osmium tetroxide dissolved in carbon tetrachloride and passage over a column of neutral alumina (Alltech, Deerfield, IL) (Mason et al., 1976). Total phospholipids (TPL) and DSPC were measured by phosphorus assay (Rouser et al., 1970) and expressed as nmol/mg protein. BALF cells, isolated after a $150 \times g$ centrifugation spin, were re-suspended in 1 ml of saline, stained with trypan blue and counted in a hemocytometer. Total protein was measured in the cell-free BALF by the Bio-Rad DC Protein Assay using bovine serum albumin as a standard (Bio-Rad, Hercules CA) (Supplement Table 1).

DSPC Synthesis and Secretion Levels in Type II cells—To measure the level of newly synthesized DSPC in type II cells, cells in culture were washed with Dulbecco's Minimal Essential Media (DMEM) and incubated with 2.5 μ Ci [3 H]-choline chloride [NEN, PerkinElmer Life And Analytical Sciences, Waltham, MA] for 3 h in 1 ml DMEM (containing 28.5 nmol choline/ml) and 0.1% fatty acid-free BSA according to the previously published method of Spragg (Spragg and Li, 2000). At the end of the three hour labeling period, cells were again washed 3 X with DMEM and incubated for an additional 2 hours in DMEM without radiolabel. Cells were collected and assayed for DSPC levels and the level for 3 H choline determined by scintillation counting. The data are expressed as dpm/nm DSPC. To measure the amount of secreted DSPC, type II cells were again labeled with [3 H]-choline chloride for 3 hours before the addition 1 mM of TPA [phorbol-12-myristate-13-acetate (CalBiochem, San Diego, CA)] for 90 minutes (Scott, 1994). The DSPC fraction was isolated from both media and cell layers and the level of radioactivity determined by scintillation counting. The data are expressed as DSPC dpms (media)/DSPC dpms (media + cells).

Alveolar Phospholipid Composition—Alveolar surfactant phospholipid composition was determined by measuring phosphorous levels following separation by thin layer chromatography (TLC). Phospholipids from 2 ml aliquots of cell-free BALF were extracted with chloroform-methanol (Bligh and Dyer, 1959). Each extract was dried under nitrogen, re-suspended in chloroform, and separated on a silica gel TLC plate using triethylamine-ethanol-chloroform-distilled water (35:34:30:8) as the solvent (model LK5D, Whatman, Clifton, NJ). Phospholipid standards (Avanti Polar Lipids, Alabaster, AL) were run on the same plate and detected with Molybdenum Blue Phosphorous Spray (Sigma, St. Louis MO). The separated BALF phospholipids were quantified using the Rouser phosphorus assay as described earlier (Rouser et al., 1970).

Real Time RT-PCR—Total cellular RNA was isolated from lung parenchyma using Tripure Isolation Reagent (Roche Molecular Biochemicals, Indianapolis, IN) followed by further purification over RNeasy Mini Kit columns including DNase I treatment (Qiagen Inc. Valencia, CA). RNA quantity and purity was determined by spectrophotometer and confirmed by formaldehyde-agarose electrophoresis. 300 ng of total RNA was reversed transcribed using the Thermoscript RT-PCR System (Invitrogen Corp., Carlsbad, CA). The RT product was amplified in a reaction volume containing 2X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), HPLC purified forward and reverse primers (Supplement Table 2) and the reference dye, ROX. Reactions were run on a MX3000P Real-Time PCR System (Stratagene) at 95°C, 10 min, 40 cycles of 95°C, 30 s; 55°C, 60 s; and 72°C for 30 s, followed by a 30 minute dissociation curve to check for product purity. Relative mRNA levels were quantified and normalized to GAPDH using the $2\Delta\Delta C_t$ method.

Western Blot Analysis—Surfactant-associated proteins and lipid transport regulatory proteins, ABCA1, ABCA3 and Rab3D, were measured in type II cells, BALF and lung tissue by Western blot. Type II cell protein extracts were prepared by freeze-thaw in lysis buffer [50 mM Tris, 150 mM NaCl, 0.5% Triton X-100 plus a Complete Protease Inhibitor Cocktail Tablet plus EDTA (Roche Diagnostics, Mannheim, Germany)]. Lung tissue was homogenized in 2 ml of homogenization buffer (150mM NaCl, 50 mM Tris pH 7.5, 1% Triton-X-100, complete protease inhibitor tablet with EDTA). Cell protein extracts (50 μ g), BALF volume (15 μ l) or lung homogenate protein (100 μ g) were electrophoresed on a 15% SDS-PAGE gel. SP-A and SP-D were electrophoresed under reducing conditions. SP-B was analyzed under non-reducing conditions. For type II cells blots were incubated with polyclonal rabbit primary antibodies specific for SP-A, SP-B, Pro-SP-C, SP-D (Santa Cruz

Biotech CA) at dilutions of 1:500. For western blot analysis of BALF and lung homogenates, rabbit anti-sheep SP-A and rabbit anti-rat SP-D antibodies, which cross react with mouse antigens and have a high specificity for detection of tissue samples, were used. These antibodies were generously given to us by Dr. Jo Rae Wright, Duke University Medical Center, NC. Lipid transporter molecules for all samples were detected with anti-ABCA1 (Novus, Littleton, CO), anti-ABCA3 (Covance, Berkeley, CA) or anti-Rab3D (Abcam, Cambridge, MA). Signals were detected with an HRP conjugated anti-IgG secondary antibody and detected by chemiluminescence using an ECL Kit (Amersham, Piscataway, NJ). Densitometry was performed with Gel Pro Analyzer software (Mediacybernetics, Silver Springs, MD).

Type II Cell Apoptotic Index—Type II cells were fixed with 2% paraformaldehyde and the number of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) positive nuclei detected using an In Situ Cell Death Detection Kit, POD (Roche Applied Science, Indianapolis, IN). TUNEL positive nuclei were identified by light microscope and expressed as the number of positive nuclei per one hundred total nuclei in 10 randomly selected fields per culture dish in three separate cultures/experimental group.

Statistical Analyses—The probability of a significant difference between control (uninfected), Adv/LacZ, and Adv/Cre infected type II cell cultures was calculated using a one-way ANOVA and Tukey post-hoc tests for individual differences. An unpaired Student's T test was used to determine differences in PAO-lux, cell number, phospholipid content and gene expression between mouse lungs infected with AAV/LacZ or AAV/Cre. Data were expressed as mean $\pm$ SEM and $p < 0.05$ was considered significant.

Results

In vivo detection of bioluminescent *Pseudomonas aeruginosa* (PAO1-Lux) in VEGF gene inactivated lungs

In *VEGFLoxP* mice instilled with AAV/Cre, lung homogenate VEGF levels were reduced by 57% at 5 weeks compared to mice instilled with AAV/LacZ (Figure 1A). At this post-AAV time point, mice were instilled with PAO1-Lux and imaged *in vivo*. At 6 hours there was no difference in the amount of PAO1-Lux detected in the lungs of AAV/Cre infected mice compared to the AAV/LacZ group (LacZ $1.6 \pm 0.5 \times 10^5$; Cre $1.3 \pm 0.4 \times 10^5$). Twenty-four hours later pulmonary PAO1-Lux levels were increased in both mouse groups but were 3.25 fold higher in the AAV/Cre group compared to LacZ (LacZ $4.9 \pm 1.5 \times 10^6$, Cre $1.6 \pm 0.5 \times 10^7$, $p = 0.046$) (Figure 2). PAO1-Lux was detected in liver isolated at 24 hours in 5 out of 13 LacZ mice and 5 out of 16 Cre mice. PAO1-Lux was not detected in blood samples.

Cytokines

Comparison of inflammatory cytokines in the AAV/LacZ and AAV/Cre groups, both challenged with PAO1-Lux, revealed increased lung levels of IL-6 (LacZ 366 ± 93 pg/mg, Cre 1013 ± 140 pg/mg, $p = 0.01$) and TNF α (LacZ 86 ± 10 pg/mg, Cre 382 ± 39 pg/mg, $p = 0.04$) in the VEGF gene inactivated lungs compared to controls (Figure 3). Lungs from both AAV/LacZ and AAV/Cre groups challenged with PAO1-Lux contained numerous neutrophils (Supplement Figure 2).

Surfactant-associated protein D (SP-D) in lungs and cultured alveolar type II cells

In vivo—SP-D levels from lung BALF (Figure 4A) and tissue homogenates (Figure 4B) before PAO1 challenge were reduced by 54% in BALF ($p < 0.05$) and by 53% ($p < 0.05$) in lung tissue in VEGF-deficient lungs compared to the AAV/LacZ group (Figure 4C & D). No

significant differences between AAV/Cre and AAV/LacZ lungs were detected for SP-A or SP-B (Figure 4AD). SP mRNA levels measured in lung homogenates revealed a 56% ($p<0.05$) lower SP-D mRNA level in VEGF-deficient lungs (Figure 4H).

In vitro—Type II cells, isolated from adult VEGF $LoxP$ mice revealed a 69% decrease ($p=0.014$) in VEGF by day 2 post-Adv/Cre infection compared to uninfected or Adv/LacZ-infected cells. VEGF levels were further reduced to 20% of the control values ($p=0.0024$) on day 3 and remained decreased at 23% of control values ($p=0.0002$ and $p=0.0003$) on days 4 and 5 (Figure 1B). Western analysis of cellular extracts from VEGF-inactivated and control type II cultures revealed a 58% ($p=0.015$) decrease in SP-B levels and a 46% ($p=0.008$) decrease in SP-D levels by Western analysis compared to uninfected and Adv/LacZ infected cultures (Figure 4I, K & M). SP-A and pro-SP-C were not different between the cell culture groups (Figure 4I, J & L).

Surfactant phospholipids in VEGF-inactivated lung and cultured type II cells

In vivo—Alveolar surfactant levels, assessed by measuring phospholipids in BALF and total DSPC levels in lung homogenates, were not altered at 5 weeks post-AAV/Cre delivery compared to AAV/LacZ lungs (Figure 5A & B). Further analysis of the alveolar phospholipid composition by thin layer chromatography revealed a 23% decrease in the sphingomyelin fraction in AAV/Cre-infected lungs compared to control AAV/LacZ lungs (Table 1, $p=0.029$).

In vitro—Three days post Adv/Cre infection, type II cells revealed a 33% ($p=0.041$) decrease in total phospholipid levels and a 45% ($p=0.023$) decrease in DSPC levels compared to uninfected and Adv/LacZ-infected control cells (Figure 5C). Newly synthesized DSPC decreased by 62% ($p=0.007$) after VEGF inactivation (Figure 5D). However, when VEGF-deficient type II cells were stimulated to secrete surfactant with TPA, DSPC secreted into the cell media was increased by 55% ($p=0.031$) compared to uninfected or Adv/LacZ infected control cells (Figure 5E).

Lipid transport regulators in VEGF deficient lungs and cultured type II cells

In vivo—Five weeks post infection, ABCA1 mRNA and protein levels and Rab3D mRNA levels were similar in AAV/Cre and AAV/LacZ infected lungs (Figure 6, A–D). However, Rab3D protein levels were significantly decreased in AAV/Cre infected lungs compared to AAV/LacZ infected lungs (Figure 6E & F). Type II cell and lamellar body morphology appeared normal (Supplement, Figure 2).

In vitro—In VEGF-deficient type II cells, ABCA1 protein levels decreased by 64% ($p=0.02$) and Rab3D protein levels decreased by 41% ($p=0.03$) compared to the control groups (Figure 6, G–I). ABCA3 protein levels were unaltered in Adv/Cre infected type II cells (Figure 6G, J).

Apoptosis in VEGF-deficient type II cells

In vivo—CTP:phosphocholine cytidyltransferase (CCT) mRNA was decreased by 48% in VEGF-deficient lungs at 5 weeks post-infection compared to AAV/LacZ lungs ($p<0.05$) (Figure 7A).

In vitro—*TUNEL-positive* apoptotic type II cell number was increased 4.2 fold ($p=0.003$) in VEGF-deficient cells compared to control groups (Figure 7B).

Discussion

Our laboratory, as well as others, has previously shown that maintaining adequate pulmonary VEGF or VEGFR-2 signaling is essential for preserving an intact alveolar-capillary structure (Kasahara et al., 2000; Tang et al., 2004). The aim of the present study was to determine whether adult type II cells, which express high levels of VEGF, VEGFR-1 and VEGFR-2, also regulate surfactant homeostasis and innate immunity through a VEGF-dependent pathway (Fehrenbach et al., 1999). The primary findings of this study are 1) while VEGF has the potential to regulate surfactant homeostasis in isolated type II cells, compensatory mechanisms exist *in vivo* to ensure that both the normal amount and composition of surfactant lipids are maintained on the alveolar surface. 2) However, decreased pulmonary VEGF expression *in vivo* results in the down regulation of the surfactant associated pulmonary collectin, SP-D, and a compromised defense against *Pseudomonas aeruginosa*.

VEGF has the potential to regulate the production of surfactant by type II cells

Inhibiting VEGF expression by 80% in isolated type II cells resulted in a decrease in total phospholipids and the level of mature disaturated phosphatidylcholine (DSPC) found in surfactant. Interestingly, while DSPC synthesis was down regulated, secretion of DSPC in response to TPA was augmented. This later observation suggests that signaling mechanisms were still present to localize surfactant lipids to the extracellular space under the appropriate conditions. *In vivo* increased stretch or mechanical tension in an over inflated emphysema-like lung may provide the signal (Wirtz and Dobbs, 1990). In addition several lipid trafficking molecules were down regulated including the intracellular hydrophobic surfactant associated protein, SP-B, the reverse transporter, ABCA1, and a GTPase involved in exocytosis of surfactant lipids, Rab3D. The extracellular hydrophilic surfactant associate protein SP-D was decreased by 50% in VEGF gene inactivated type II cells. One of the functions of SP-D is to regulate the reuptake of lipid aggregates that are recycled and reorganized into surfactant within type II cells (Ikegami et al., 2000).

Alveolar surfactant is preserved in vivo

In contrast to the down regulation of surfactant lipids in isolated type II cells, a 57% reduction in pulmonary cell VEGF *in vivo* was not associated with altered alveolar or tissue DSPC levels. This maintenance of surfactant lipids occurred despite a decrease in CCT mRNA and SP-D expression. CCT is the rate-limiting enzyme for phosphatidyl choline synthesis. However, not all phosphatidyl choline is converted to the mature disaturated form and organized into surfactant. Phosphatidyl choline is also a major component of membrane lipids and a decrease in CCT expression can be indicative of ongoing apoptosis (Henderson et al., 2006). SP-D also has several cellular functions, but its specific role in surfactant homeostasis, as mentioned above, is to facilitate the reuptake of surfactant lipid aggregates that are recycled and reorganized into mature surfactant. In SP-D (−/−) null mice surfactant lipids accumulate in numerous foamy, multinucleated macrophages and hyperplastic type II cells with large lamellar bodies. However, heterozygous SP-D (+/−) mice do not display any obvious phenotypic abnormalities or accumulate lipid despite approximately 50% less SP-D (Botas et al., 1998; Korfhagen et al., 1998). Thus, our observation of unaltered surfactant lipid levels in lung-targeted VEGF inactivated mice, with 50% less SP-D, is consistent with the response in heterozygous SP-D (+/−) mice. Taken together with the data collected in our *in vitro* system, the results from this *in vivo* model of pulmonary VEGF gene inactivation suggest cell-cell interactions and/or signals generated in an *in vivo* environment compensate for reduced pulmonary VEGF expression and maintain alveolar surfactant levels (Tang et al., 2004).

Potential compensatory mechanism for maintaining alveolar surfactant

There are several possible mechanisms that may account for the differences in surfactant lipid levels in VEGF gene ablated cultured type II cells and pulmonary cells *in vivo*. 1] Adaptive changes in the reuptake and/or secretion of surfactant lipids in lung cells targeted *in vivo* may allow alveolar surfactant levels to be restored over a longer 5-week period than the 3 days type II cells were cultured *in vitro*. A reduced reuptake compensatory mechanism has been demonstrated in the lungs of transgenic mice that chronically over express TNF- α . In contrast acute TNF- α treatment of lungs or type II cells decreases phosphatidylcholine synthesis and increases secretion. This latter response to acute TNF- α is similar to the decreased DSPC synthesis and increased secretion in VEGF-deficient isolated type II cells (Carroll et al., 2002; Salome et al., 2000). 2] Another possible *in vivo* compensatory mechanism may involve the interaction of type II cells with other pulmonary phagocytes. Phagocytosis of phosphatidyl serine presenting apoptotic cells by macrophages interacting with the remaining 50% SP-D, or other lung collectins (C1q or SP-A), and calreticlin/CD91 may be sufficient to increase or maintain ABCA1 levels, and allow ABCA1-selective efflux of unsaturated phospholipids and cholesterol from engulfed apoptotic cells while at the same time salvaging saturated PC for incorporation into surfactant (Gardai et al., 2003; Zhou et al., 2004) 3] The one minor surfactant lipid component that was altered in VEGF-deficient lungs was sphingomyelin. The observed 23% decrease in sphingomyelin suggests that it has been hydrolyzed by sphingomyelinases to ceramide and sphingosine, and these sphingomyelin breakdown products have been reported to inhibit CCT expression and activity, stimulate caspase-3 dependent apoptosis, decrease apoptotic cell clearance and increase ABCA1 levels, (Baburina and Jackowski, 1998; Filosto et al.; Lagace et al., 2002; Petrache et al., 2005; Petrusca et al.; Witting et al., 2003). The finding that ABCA1 was down regulated in isolated type II cells but maintained in our *in vivo* model also supports a possible mechanism in which sphingomyelin-induced ABCA1 plays a role in recycling DSPC from phagocytosed apoptotic cells. More experiments will be needed to verify and elucidate the steps involved in potential compensatory pathways for preserving alveolar surfactant *in vivo*.

Maintenance of alveolar surfactant at the cost of inefficient efferocytosis

Consistent in both the *in vitro* and *in vivo* VEGF inactivation models was a decrease in SP-D and ongoing apoptosis (Tang et al., 2004). Decreased levels of CCT mRNA, sphingomyelin and Rab3D in VEGF deficient lungs also indicate persistent or ongoing apoptosis (Baburina and Jackowski, 1998; Cartel and Post, 2005; Petrache et al., 2005; Vandivier et al., 2002). However, *in vivo* decreases in both VEGF and SP-D might suggest not only an increase in the number of cells undergoing apoptosis but also a delay in the clearance of apoptotic cells. In a study by Vandivier *et al.* (Vandivier et al., 2002) comparing SP-D, C1q and SP-A in naïve mouse lungs, SP-D was the most efficient *in vivo* pulmonary collectin for the recognition and uptake of apoptotic cells by resident macrophages. Furthermore, enhancing the expression of VEGF in the lung promotes the clearance of apoptotic cells by alveolar macrophages (Kearns et al., 2012).

VEGF-dependent clearance of *Pseudomonas aeruginosa*

Unresolved apoptosis and lower levels of the innate immune molecule, SP-D, are also potential factors in the ability to efficiently clear bacteria from the lung. In this study, we have demonstrated that an SP-D recognized pathogen, *Pseudomonas aeruginosa*, accumulates in VEGF-deficient mouse lungs and signals amplified IL-6 and TNF α responses. Previous studies have reported SP-D can recognize and stimulate the phagocytosis of *Pseudomonas aeruginosa* by alveolar macrophages (Restrepo et al., 1999) as well as enhance clearance *in vivo* (Giannoni et al., 2006). In addition to poor phagocytosis, SP-D null mice infected with group B streptococcus or *Haemophilus influenzae* demonstrate

increased inflammatory cell recruitment and cytokine production (LeVine et al., 2000). Likewise VEGF pre-treatment has been reported to reduce respiratory syncytial virus replication in neonatal lambs in part by maintaining SP-D levels and attenuating neutrophil recruitment (Meyerholz et al., 2007). In the present study, *P. aeruginosa* infection in VEGF inhibited mice led to enhanced inflammatory cytokines, IL-6 and TNF α , but neutrophil recruitment was unaffected. Furthermore, *P. aeruginosa* (PAO1) levels did not differ early (6 hours) after infection. This observation would suggest that altered lung mechanics due to an emphysema-like lung structure is not likely to be a major factor. However, less available SP-D, due to decreased expression, as well as competition with an abundance of apoptotic cells, would suggest that innate immune mechanisms to defend against and/or clear PAO1 and possibly other SP-D recognized bacteria are compromised.

Conclusion

VEGF has the potential to regulate type II cell function in the adult murine lung. Inhibition of VEGF expression in isolated adult type II cells leads to increased apoptosis and decreases in surfactant lipid and expression of SP-B and SP-D (Clark et al., 1995; Wang et al., 2007). However, *in vivo* alveolar surfactant lipid and associated proteins are preserved with the exception of SP-D. VEGF deficient lungs are more susceptible to an SP-D recognized bacteria, *Pseudomonas aeruginosa*, and exhibit an exaggerated cytokine response. These observations suggest that VEGF may be important for maintaining an effective defense against certain types of bacteria and an inefficient VEGF-dependent mechanism could be an underlying factor in the prevalence of repeated bacterial infections that occur in several chronic lung diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by grants TRDRP 12RT-0062 and NIH PO1 HL 17731-28. JLM was the recipient of the Parker B Francis Foundation Fellowship. REWH was supported by the Canadian Institute for Health Research. We would also like to acknowledge Randolph Hastings for instructing us on the method to isolate alveolar type II cells from mice.

References

- Baburina I, Jackowski S. Apoptosis triggered by 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine is prevented by increased expression of CTP:phosphocholine cytidyltransferase. *The Journal of biological chemistry*. 1998; 273(4):2169–2173. [PubMed: 9442058]
- Bates SR, Tao JQ, Collins HL, Francone OL, Rothblat GH. Pulmonary Abnormalities Due to ABCA1 Deficiency in Mice. *American journal of physiology Lung cellular and molecular physiology*. 2005
- Benarafa C, Priebe GP, Remold-O'Donnell E. The neutrophil serine protease inhibitor serpinb1 preserves lung defense functions in *Pseudomonas aeruginosa* infection. *The Journal of experimental medicine*. 2007; 204(8):1901–1909. [PubMed: 17664292]
- Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol*. 1959; 37(8):911–917. [PubMed: 13671378]
- Botas C, Poulain F, Akiyama J, Brown C, Allen L, Goerke J, Clements J, Carlson E, Gillespie AM, Epstein C, Hawgood S. Altered surfactant homeostasis and alveolar type II cell morphology in mice lacking surfactant protein D. *Proceedings of the National Academy of Sciences of the United States of America*. 1998; 95(20):11869–11874. [PubMed: 9751757]
- Carroll JL Jr, McCoy DM, McGowan SE, Salome RG, Ryan AJ, Mallampalli RK. Pulmonary-specific expression of tumor necrosis factor- α alters surfactant lipid metabolism. *American journal of physiology Lung cellular and molecular physiology*. 2002; 282(4):L735–742. [PubMed: 11880299]

- Cartel NJ, Post M. Abrogation of apoptosis through PDGF-BB-induced sulfated glycosaminoglycan synthesis and secretion. *American journal of physiology Lung cellular and molecular physiology*. 2005; 288(2):L285–293. [PubMed: 15466249]
- Choi KH, Gaynor JB, White KG, Lopez C, Bosio CM, Karkhoff-Schweizer RR, Schweizer HP. A Tn7-based broad-range bacterial cloning and expression system. *Nat Methods*. 2005; 2(6):443–448. [PubMed: 15908923]
- Clark JC, Wert SE, Bachurski CJ, Stahlman MT, Stripp BR, Weaver TE, Whitsett JA. Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice. *Proceedings of the National Academy of Sciences of the United States of America*. 1995; 92(17):7794–7798. [PubMed: 7644495]
- Dobbs LG, Gonzalez R, Williams MC. An improved method for isolating type II cells in high yield and purity. *Am Rev Respir Dis*. 1986; 134(1):141–145. [PubMed: 3637065]
- Fehrenbach H, Haase M, Kasper M, Koslowski R, Schuh D, Muller M. Alterations in the immunohistochemical distribution patterns of vascular endothelial growth factor receptors Flk1 and Flt1 in bleomycin-induced rat lung fibrosis. *Virchows Arch*. 1999; 435(1):20–31. [PubMed: 10431842]
- Filosto S, Castillo S, Danielson A, Franzi L, Khan E, Kenyon N, Last J, Pinkerton K, Tudor R, Goldkorn T. Neutral sphingomyelinase 2: a novel target in cigarette smoke-induced apoptosis and lung injury. *Am J Respir Cell Mol Biol*. 44(3):350–360. [PubMed: 20448054]
- Gardai SJ, Xiao YQ, Dickinson M, Nick JA, Voelker DR, Greene KE, Henson PM. By binding SIRPalpha or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation. *Cell*. 2003; 115(1):13–23. [PubMed: 14531999]
- Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat Med*. 1999; 5(6):623–628. [PubMed: 10371499]
- Giannoni E, Sawa T, Allen L, Wiener-Kronish J, Hawgood S. Surfactant proteins A and D enhance pulmonary clearance of *Pseudomonas aeruginosa*. *Am J Respir Cell Mol Biol*. 2006; 34(6):704–710. [PubMed: 16456184]
- Glasser SW, Detmer EA, Ikegami M, Na CL, Stahlman MT, Whitsett JA. Pneumonitis and emphysema in sp-C gene targeted mice. *The Journal of biological chemistry*. 2003; 278(16):14291–14298. [PubMed: 12519727]
- Henderson FC, Miakotina OL, Mallampalli RK. Proapoptotic effects of *P. aeruginosa* involve inhibition of surfactant phosphatidylcholine synthesis. *J Lipid Res*. 2006; 47(10):2314–2324. [PubMed: 16868337]
- Ikegami M, Whitsett JA, Jobe A, Ross G, Fisher J, Korfhagen T. Surfactant metabolism in SP-D gene-targeted mice. *American journal of physiology Lung cellular and molecular physiology*. 2000; 279(3):L468–476. [PubMed: 10956621]
- Kasahara Y, Tudor RM, Cool CD, Lynch DA, Flores SC, Voelkel NF. Endothelial cell death and decreased expression of vascular endothelial growth factor and vascular endothelial growth factor receptor 2 in emphysema. *American journal of respiratory and critical care medicine*. 2001; 163(3 Pt 1):737–744. [PubMed: 11254533]
- Kasahara Y, Tudor RM, Taraseviciene-Stewart L, Le Cras TD, Abman S, Hirth PK, Waltenberger J, Voelkel NF. Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *The Journal of clinical investigation*. 2000; 106(11):1311–1319. [PubMed: 11104784]
- Kearns MT, Dalal S, Horstmann SA, Richens TR, Tanaka T, Doe JM, Boe DM, Voelkel NF, Taraseviciene-Stewart L, Janssen WJ, Lee CG, Elias JA, Bratton D, Tudor RM, Henson PM, Vandivier RW. Vascular endothelial growth factor enhances macrophage clearance of apoptotic cells. *American journal of physiology Lung cellular and molecular physiology*. 2012; 302(7):L711–718. [PubMed: 22307908]
- Korfhagen TR, Sheftelyevich V, Burhans MS, Bruno MD, Ross GF, Wert SE, Stahlman MT, Jobe AH, Ikegami M, Whitsett JA, Fisher JH. Surfactant protein-D regulates surfactant phospholipid homeostasis in vivo. *The Journal of biological chemistry*. 1998; 273(43):28438–28443. [PubMed: 9774472]

- Lagace TA, Miller JR, Ridgway ND. Caspase processing and nuclear export of CTP:phosphocholine cytidyltransferase alpha during farnesol-induced apoptosis. *Mol Cell Biol.* 2002; 22(13):4851–4862. [PubMed: 12052891]
- LeVine AM, Whitsett JA, Gwozdz JA, Richardson TR, Fisher JH, Burhans MS, Korfhagen TR. Distinct effects of surfactant protein A or D deficiency during bacterial infection on the lung. *J Immunol.* 2000; 165(7):3934–3940. [PubMed: 11034401]
- Mason RJ, Nellenbogen J, Clements JA. Isolation of disaturated phosphatidylcholine with osmium tetroxide. *J Lipid Res.* 1976; 17(3):281–284. [PubMed: 932560]
- Meyerholz DK, Gallup JM, Lazic T, de Macedo MM, Lehmkuhl HD, Ackermann MR. Pretreatment with recombinant human vascular endothelial growth factor reduces virus replication and inflammation in a perinatal lamb model of respiratory syncytial virus infection. *Viral Immunol.* 2007; 20(1):188–196. [PubMed: 17425433]
- Ng YS, Rohan R, Sunday ME, Demello DE, D'Amore PA. Differential expression of VEGF isoforms in mouse during development and in the adult. *Dev Dyn.* 2001; 220(2):112–121. [PubMed: 11169844]
- Pastva AM, Wright JR, Williams KL. Immunomodulatory roles of surfactant proteins A and D: implications in lung disease. *Proc Am Thorac Soc.* 2007; 4(3):252–257. [PubMed: 17607008]
- Petrache I, Natarajan V, Zhen L, Medler TR, Richter AT, Cho C, Hubbard WC, Berdyshev EV, Tuder RM. Ceramide upregulation causes pulmonary cell apoptosis and emphysema-like disease in mice. *Nat Med.* 2005; 11(5):491–498. [PubMed: 15852018]
- Petrusca DN, Gu Y, Adamowicz JJ, Rush NI, Hubbard WC, Smith PA, Berdyshev EV, Birukov KG, Lee CH, Tuder RM, Twigg HL 3rd, Vandivier RW, Petrache I. Sphingolipid-mediated inhibition of apoptotic cell clearance by alveolar macrophages. *The Journal of biological chemistry.* 285(51):40322–40332. [PubMed: 20956540]
- Restrepo CI, Dong Q, Savov J, Mariencheck WI, Wright JR. Surfactant protein D stimulates phagocytosis of *Pseudomonas aeruginosa* by alveolar macrophages. *Am J Respir Cell Mol Biol.* 1999; 21(5):576–585. [PubMed: 10536117]
- Rice KL, Duane PG, Niewoehner DE. Effects of phospholipase A2 and its hydrolytic products on alveolar epithelial permeability and elastase-induced emphysema. *Am Rev Respir Dis.* 1988; 138(5):1196–1200. [PubMed: 3264481]
- Rouser G, Fkeischer S, Yamamoto A. Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids.* 1970; 5(5):494–496. [PubMed: 5483450]
- Salome RG, McCoy DM, Ryan AJ, Mallampalli RK. Effects of intratracheal instillation of TNF-alpha on surfactant metabolism. *J Appl Physiol.* 2000; 88(1):10–16. [PubMed: 10642356]
- Scott JE. Influence of protein kinase C activation by 4 beta-phorbol ester or 1-oleoyl-2-acetyl glycerol on disaturated phosphatidylcholine synthesis and secretion, and protein phosphorylation in differentiating fetal rabbit type II alveolar cells. *Biochim Biophys Acta.* 1994; 1221(3):297–306. [PubMed: 8167152]
- Spragg RG, Li J. Effect of phosphocholine cytidyltransferase overexpression on phosphatidylcholine synthesis in alveolar type II cells and related cell lines. *Am J Respir Cell Mol Biol.* 2000; 22(1):116–124. [PubMed: 10615073]
- Tang K, Rossiter HB, Wagner PD, Breen EC. Lung-targeted VEGF inactivation leads to an emphysema phenotype in mice. *J Appl Physiol.* 2004; 97(4):1559–1566. discussion 1549. [PubMed: 15208295]
- Vandivier RW, Ogden CA, Fadok VA, Hoffmann PR, Brown KK, Botto M, Walport MJ, Fisher JH, Henson PM, Greene KE. Role of surfactant proteins A, D, and C1q in the clearance of apoptotic cells in vivo and in vitro: calreticulin and CD91 as a common collectin receptor complex. *J Immunol.* 2002; 169(7):3978–3986. [PubMed: 12244199]
- Wang J, Edeen K, Manzer R, Chang Y, Wang S, Chen X, Funk CJ, Cosgrove GP, Fang X, Mason RJ. Differentiated human alveolar epithelial cells and reversibility of their phenotype in vitro. *Am J Respir Cell Mol Biol.* 2007; 36(6):661–668. [PubMed: 17255555]

- Wang Y, Krushel LA, Edelman GM. Targeted DNA recombination in vivo using an adenovirus carrying the cre recombinase gene. *Proceedings of the National Academy of Sciences of the United States of America*. 1996; 93(9):3932–3936. [PubMed: 8632992]
- Wert SE, Yoshida M, LeVine AM, Ikegami M, Jones T, Ross GF, Fisher JH, Korfhausen TR, Whitsett JA. Increased metalloproteinase activity, oxidant production, and emphysema in surfactant protein D gene-inactivated mice. *Proceedings of the National Academy of Sciences of the United States of America*. 2000; 97(11):5972–5977. [PubMed: 10801980]
- Wirtz HR, Dobbs LG. Calcium mobilization and exocytosis after one mechanical stretch of lung epithelial cells. *Science*. 1990; 250(4985):1266–1269. [PubMed: 2173861]
- Witting SR, Maiorano JN, Davidson WS. Ceramide enhances cholesterol efflux to apolipoprotein A-I by increasing the cell surface presence of ATP-binding cassette transporter A1. *The Journal of biological chemistry*. 2003; 278(41):40121–40127. [PubMed: 12890677]
- Zhou J, You Y, Ryan AJ, Mallampalli RK. Upregulation of surfactant synthesis triggers ABCA1-mediated basolateral phospholipid efflux. *J Lipid Res*. 2004; 45(9):1758–1767. [PubMed: 15210848]

A. Lung

B. Type II Cells

Figure 1. Decreased VEGF levels in VEGF $LoxP$ mouse lungs and isolated type II cells delivered *cre* recombinase

A) VEGF levels in lungs from VEGF $LoxP$ mice infected *in vivo* with AAV/LacZ or AAV/Cre at 5 weeks post-infection. Values are the mean $\pm$ the SD, n=3 for LacZ and n=4 for Cre.

B) VEGF levels in VEGF $LoxP$ type II cells cultured with adenovirus expressing LacZ (LacZ, $\blacktriangledown$), *cre* recombinase (Cre, $\square$) or media alone (Con, $\blacklozenge$). Values represent the mean $\pm$ SD, n=6. * indicates a significant difference ($P < 0.05$) compared to control, uninfected (Con) type II cell cultures.

Figure 2. Impaired clearance of PAO1-Lux

Five weeks post-infection with either AAV/LacZ (**LacZ**) or AAV/Cre (**Cre**) mice were challenged with PAO1-Lux. **(A)** PAO1-Lux mice bioluminescence detected by optical imaging. **(B)** PAO1 levels quantified from the collected images. Graphs represent the mean $\pm$ SEM. * indicates a significant difference from control, $p < 0.05$. (n= 11 LacZ and 13 Cre mice).

Figure 3. Increased production of cytokines TNF α and IL-6 in PAO1 challenged VEGF gene inactivated mice

TNF α and IL-6 levels 24 hours after PAO1 challenge of AAV/LacZ and (AAV/Cre) mouse lungs. Graphs represent the $\bar{x} \pm \text{SEM}$, * indicates a significant difference from control, $p < 0.05$. (n= 4–7 LacZ and 7–10 Cre mice).

Figure 4. Surfactant protein D (SP-D) mRNA and protein levels are decreased in VEGF-deficient mouse lung and isolated type II cells

Five weeks post-infection with either AAV/LacZ (**LacZ**) or AAV/Cre (**Cre**), surfactant associated protein levels were measured by western analysis and quantified by densitometry in BAL (**A**, **C**) and lung tissue (**B**, **D**). Graphs represent the mean $\pm$ SEM ($n = 3-4$ mouse lungs; $p < 0.05$). SP-A (**E**), SP-B (**F**), SP-C (**G**) and SP-D (**H**) mRNA levels were measured in AAV/LacZ (**LacZ**) and AAV/Cre (**Cre**) infected lungs. Values represent the mean relative mRNA levels normalized to GAPDH $\pm$ SEM, $n = 6$ mouse lungs. * indicates a significant difference between LacZ and Cre lung, $P < 0.05$. VEGF LoxP type II cells were cultured in the presence of Adv/LacZ (**LacZ**), Adv/Cre (**Cre**) or media without adenovirus (**Con**) for 72 hours. **I**) Representative western blot of type II cell extracts probed for SP-A, SP-B, ProSP-C and SP-D. Surfactant protein levels were quantified by densitometry and normalized to β -actin levels **J**) SP-A, **K**) SP-B, **L**) ProSP-C, **M**) SP-D. Values represent the mean $\pm$ SD, $n = 6$ separate type II cell cultures dishes. * indicates a significant difference from uninfected (**Con**) type II cells with a confidence of $p < 0.05$.

Figure 5. Surfactant lipid metabolism is altered in VEGF gene inactivated pulmonary type II cells but not lungs *in vivo*. A) **Unaltered Alveolar Surfactant Levels**

Alveolar surfactant phospholipid pool sizes were similar in the bronchoalveolar lavage fluid (BALF) recovered from VEGF $LoxP$ mice instilled 5 weeks prior with either AAV/LacZ (LacZ; n=7) or AAV/Cre recombinase virus (Cre; n=7). **B) Unaltered Tissue Surfactant Lipids.** The levels of tissue associated surfactant phospholipids, measured as disaturated phosphatidylcholine (DSPC), were also similar in the experimental Cre group (n=7) and the control LacZ group (n=7).

C) VEGF gene inactivation resulted in a decrease in total PL and DSPC levels. Reduced VEGF expression 72 hours post- Adv/Cre infection (Cre) was accompanied by a decrease in type II cell total phospholipid (PL) and disaturated phosphatidylcholine (Sat-PC) levels compared to both uninfected cells (Con) and Adv/LacZ infected cultures (Adv/LacZ) n= 6 cultures, * indicates a significant difference from the uninfected control group (Con) p<0.05. **D) Decreased DSPC synthesis in VEGF-deficient type II cells.** ³H choline incorporation into the DSPC fraction. Values represent the mean $\pm$ SD, n = 6 type II cell cultures. * indicates a significant difference from uninfected, control cells (Con).

E) Increased surfactant secretion in VEGF gene inactivated type II cells. TPA-induced DSPC secretion in uninfected, Adv/LacZ and Adv/Cre infected cells. Values represent the mean level of secreted DSPC $\pm$ SD, n= 6 cultures, * indicates a significant difference from control, uninfected (Con) cells with a confidence of p< 0.05.

Figure 6. Gene expression of the lipid transport molecules, ABCA1 and Rab3D, in VEGF deficient mouse lung

Five weeks post-infection with either AAV/LacZ (**LacZ**) or AAV/Cre (**Cre**) the gene expression of ABCA1 was measured at the mRNA level by real time RT-PCR (**A**) and protein levels were measured by western analysis of lung tissue (**B**) and analyzed by densitometry (**C**). ABCA1 relative mRNA levels normalized to GAPDH represent the $x \pm$ SEM $n=6$ mouse lungs, No significant difference (NS) was detected between LacZ and Cre lung. ABCA1 tissue levels were normalized to β -actin levels. Graphs represent the $x \pm$ SEM, * indicates a significant difference from control, $p < 0.05$. ($n=3-4$ mouse lungs).

Decreased ABCA1 and Rab3D in VEGF gene inactivated type II cells compared to control cells. Type II cells infected with either Adv/LacZ (**LacZ**) or Adv/Cre (**Cre**) or cultured without adenovirus (**Con**) were probed by Western analyses. (**G**) Representative western blots. Densitometry values for (**H**) ABCA1, (**I**) ABCA3 and (**J**) Rab3D each normalized to β actin. Values are the mean $\pm$ SD, $n=6$ type II cell culture dishes. * indicates a significant difference from control, $p < 0.05$.

Figure 7. Increased apoptosis VEGF-inactivated type II cells and decreased CCT mRNA levels in VEGF-deficient lungs

A) CCT relative mRNA levels from five week post-infection AAV/LacZ (**LacZ**) or AAV/Cre (**Cre**) lungs. Values normalized to GAPDH represent the mean $\pm$ SEM $n = 6$ mouse lungs. * indicates a significant difference from control, $p < 0.05$. **B)** The percent TUNEL positive nuclei in 72 hour Adv/LacZ (**LacZ**) or Adv/Cre (**Cre**) post-infection and uninfected type II cells (**Con**). $N = 6$ separate type II cell cultures. * indicates a significant difference ($p < 0.05$) compared to uninfected (**Con**) type II cells.

Table 1

Phospholipid species in BALF from mice 5 weeks post-infection with AAV/LacZ or AAV/Cre

Species	Percent of Total Phospholipids	
	LacZ	Cre
<i>Phosphatidylcholine</i>	76.0 ± 1.3	78.1 ± 0.5
<i>Phosphatidylglycerol</i>	11.4 ± 0.2	12.1 ± 0.5
<i>Sphingomyelin</i>	4.8 ± 0.3	3.7 ± 0.4 *
<i>Phosphatidylethanolamine</i>	3.1 ± 0.1	3.0 ± 0.1
<i>Lysophosphatidylcholine</i>	2.9 ± 0.9	1.7 ± 0.2
<i>Phosphatidylinositol + Phosphatidylserine</i>	1.7 ± 0.2	1.4 ± 0.2

Results represent the percent of total isolated phospholipids (PL).

Values are the mean ± SE, n= mice 4,

*
p< 0.05