Amphiregulin-Dependent Mucous Cell Metaplasia in a Model of Nonallergic Lung Injury

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Proliferation and differentiation of the pulmonary epithelium after injury is a critical process in the defense against the external environment. Defects in this response can result in airway remodeling, such as mucus cell metaplasia (MCM), commonly seen in patients with chronic lung disease. We have previously shown that amphiregulin (AREG), a ligand to the epidermal growth factor receptor (EGFR), is induced during the repair/differentiation process elicited by naphthalene-induced lung injury. Thus, we hypothesized that AREG signaling plays an important role in epithelial proliferation and differentiation of the repairing airway. Mice deficient in AREG and lung epithelial EGFR were used to define roles for AREG-dependent EGFR signaling in airway repair and remodeling. We show that AREG and epithelial EGFR expression is dispensable to pulmonary epithelial repair after naphthalene-induced lung injury, but regulates secretory cell differentiation to a mucus-producing phenotype. We show that the pulmonary epithelium is the source of AREG, suggesting that naphthalene-induced MCM is mediated through an autocrine signaling mechanism. However, induction of MCM resulting from allergen exposure was independent of AREG. Our data demonstrate that AREG-dependent EGFR signaling in airway epithelial cells contributes to MCM in naphthalene-induced lung injury. We conclude that AREG may represent a determinant of nonallergic chronic lung diseases complicated by MCM.

Keywords: Clara cells; epidermal growth factor receptor; amphiregulin; mucus cell metaplasia

The epithelium lining the lungs plays a critical role in defense against inhaled particles, viruses, and xenobiotics. In health, the pulmonary epithelium can adapt to these insults by the induction of defensive mechanisms, which include increased secretory cells and the secretions they produce, such as mucins (1). Even though these changes typically resolve after acute airway injury, in humans with chronic lung diseases, such as asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis, these mechanisms may be compromised, resulting in pathologic remodeling of airways, including mucus cell metaplasia (MCM) and mucus hypersecretion (2–4). Much attention has been placed on understanding roles for T helper cell type 2 cytokines and inflammatory mediators in regulating epithelial remodeling and mucus production. Growth factors, such as epidermal growth factor (EGF) and related ligands for the EGF receptor (EGFR), have been shown to contribute to cellular proliferation and MCM that accompanies exposure to allergens and pneumotropic viruses (5). However, it is not clear what mechanisms drive epithelial remodeling and MCM in nonallergic lung diseases.

CLINICAL RELEVANCE

Collectively, these data highlight the variable contribution made by amphiregulin (AREG) to mucus cell metaplasia (MCM) in different models of lung injury and repair. Our data indicate that any lung disease involving induction of AREG may include a component of AREG-induced MCM.

We previously demonstrated that amphiregulin (AREG), a ligand that binds to EGFR, was significantly up-regulated during repair from naphthalene-induced lung injury (6). Parenterally delivered naphthalene is bioactivated to a toxic naphthalene dihydrodiol by cytochrome P450 mono-oxygenases, which results in depletion of airway Clara cells (7, 8). Clara cell depletion occurs within the first 12–48 hours after naphthalene exposure and is repaired by proliferation of naphthalene-resistant Clara cells (variant Clara cells) residing within neuroepithelial bodies and at the bronchoalveolar duct junction (BADJ) of terminal bronchioles that subsequently differentiate into nascent Clara, ciliated, and mucus-producing cells (7–15).

The EGF family of peptides, and corresponding receptor activation, plays important roles in epithelial growth and differentiation in numerous tissues (16–18). In the lung, the epithelium of both healthy individuals and those with chronic lung diseases produce EGFR-binding ligands, such as EGF, transforming growth factor- α (TGF- α), heparin-binding EGF-like growth factor, and AREG (19–27). Structurally related to EGF, AREG has been shown to regulate morphogenesis of epithelial tissues in the embryo and proliferation of multiple cell types of adult tissues, including the epidermis, mammary gland, intestine, and lung (28–33).

In the present study, we used mice deficient in either *Areg* or lung epithelial-specific *Egfr* to determine roles for these factors in pulmonary epithelial cell proliferation, repair, and differentiation after naphthalene-induced lung injury. We hypothesized that AREG and EGFR signaling would promote lung epithelial repair and regulate epithelial cell differentiation after injury. *Areg*- and *Egfr*-deficient mice were exposed to nonallergic lung injury resulting from the Clara cell-selective toxicant, naphthalene, and subsequently evaluated for their capacity to: (1) activate proliferation and subsequently repair the pulmonary epithelium; and (2) alter the differentiation of the pulmonary epithelium. Similarly, we investigated the ability of AREG to regulate the differentiation of cultured primary lung epithelial cells. Findings from these experiments suggest that AREG is a mediator of pulmonary epithelial mucus cell differentiation, but does not regulate epithelial restitution in the setting of naphthalene-induced lung injury.

MATERIALS AND METHODS

Mouse Strains

AREG-deficient (*Areg*^{-/-}) mice were established by crossing *Areg*^{+/-} mice (kindly provided by David Lee, University of North Carolina, Chapel Hill, NC) to produce *Areg*^{+/+} and *Areg*^{-/-} offspring (32).

Conditional deletion of lung epithelial *Egfr* was generated by crossing *Egfr* floxed mice (*Egfr*^f) (kindly provided by David Threadgill,

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University of North Carolina) to *SFTPC*-Cre mice (34, 35). Heterozygous Cre⁺ female offspring were backcrossed to *Egfr*^f males to establish *SFTPC*-Cre/*Egfr*^{fl} mice (*Egfr*^Δ).

Lung Injury Models

Naphthalene (Fisher Scientific, Pittsburg, PA) was administered intraperitoneally at 225 mg/kg body weight, as previously described (6). Before mice were killed (2 h) 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO) was administered intraperitoneally at 50 mg/kg body weight.

Human neutrophil elastase (hNE; 50 μg) was administered three times via oropharyngeal aspiration, as previously described (36, 37). Bronchoalveolar lavage was performed immediately before harvesting lung tissue for RNA analysis and tissue fixation 11 days after the first exposure as previously described (6).

Immunofluorescence

Immunofluorescence imaging was performed on fixed lung tissue embedded in paraffin and processed as previously described (6). The primary antibodies used were: Clara cell secretory protein (CCSP) (goat anti-CCSP, 1:15,000; in house); 5-bromo-2'-deoxyuridine (BrdU) (rat anti-BrdU, 1:200; BD Biosciences, San Jose, CA); and mucin (MUC)5AC (mouse anti-MUC5AC, 1:100; Thermo Fisher Scientific, Fremont, CA). Sections were incubated with appropriate secondary antibodies conjugated to Alexa Fluors (Invitrogen, Carlsbad, CA), counterstained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich), and immunofluorescence assessed with a Zeiss Axiovert 40 inverted microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY). The numbers of MUC5AC-labeled epithelial cells were counted and presented as the percentage of 4',6-diamidino-2-phenylindole-labeled epithelial cells.

Real-Time RT-PCR

RNA from either flow-sorted/cultured epithelial cells or whole lung tissue was isolated by guanidinium thiocyanate-phenol-chloroform extraction (38). Using gene-specific primers/probes (Applied Biosystems, Foster City, CA), real-time RT-PCR was performed as previously described (6). Differential expression of genes was determined using the 2^{-ΔΔC_T} method (39).

EGFR Protein Expression

Equal amounts of lung protein were electrophoretically separated, transferred to polyvinylidene fluoride, and probed with antibodies to either EGFR (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or β-actin (1:50,000; Sigma-Aldrich). Immunoreactive bands were detected by chemiluminescence (PerkinElmer, Waltham, MA) after incubation with corresponding biotinylated antibodies.

Lung Cell Flow Cytometry and Cell Culture

Single-cell lung suspensions were prepared using a previously published method and fractionated for epithelial, stromal, or hematopoietic/endothelial cells stained with the following antibodies: APC-Cy7 anti-EpCAM (Biolegend, San Diego, CA); AF660(APC) anti-CD34 (eBioscience, San Diego, CA); PE-Cy7 anti-CD45 (eBioscience); PE-Cy7 anti-CD31 (eBioscience); and 7-aminoactinomycin D (40). Epithelial-only populations were positively selecting for EpCAM (PE-Cy7 anti-EpCAM; eBioscience) and negatively selected for 7-aminoactinomycin D and biotin-labeled antibodies to anti-CD34, -CD31, and -CD45 (eBioscience) -labeled with APC-Cy7 anti-biotin (eBioscience). Cells were sorted using a FACS Vantage cell sorter (BD Biosciences).

Flow-sorted epithelial cells (EpCAM⁺; CD34⁻, CD31⁻, and CD45⁻) were cultured with mouse lung fibroblasts and Matrigel (BD Biosciences) seeded onto Transwell filter inserts (BD Biosciences) as previously described (40). Cell cultures were maintained, with and without recombinant murine AREG (50 ng/ml; R&D Systems, Minneapolis, MN), for 14 days at 37°C in a humidified incubator (5% CO₂).

RESULTS

AREG Is Not Necessary for Lung Epithelial Repair

We previously demonstrated that *Areg* gene expression is dramatically up-regulated during repair of naphthalene-injured lungs (6). To define roles for AREG in pulmonary epithelial repair, *Areg*-deficient (*Areg*^{-/-}) mice were exposed to naphthalene-induced lung injury and compared with similarly treated wild-type mice. Immunofluorescence staining for BrdU and CCSP was used to evaluate epithelial cell proliferation and repair, respectively (Figure 1A). A dramatic reduction was observed in the abundance of CCSP-immunoreactive cells in airways of wild-type mice 3 days after naphthalene exposure. At that time, BADJ-associated naphthalene-resistant CCSP-immunoreactive cells frequently contained for nuclear BrdU, indicating that they were actively proliferating. At 10 days after naphthalene injury, large, contiguous stretches of CCSP-immunoreactive cells were observed, indicative of epithelial regeneration. However, no differences were observed between *Areg*^{-/-} and wild-type mice in the extent of naphthalene-induced Clara cell depletion, the appearance of BrdU-labeled epithelial cells, or restoration of CCSP-expressing cells in repairing airways, at either 3- or 10-day recovery times (Figure 1A). Analysis of Clara cell gene expression (*Scgb1a1*) revealed no significant difference between uninjured wild-type and *Areg*^{-/-} mice, or at either 3 or 10 days after naphthalene-induced lung injury (Figure 1B). At 3 days after naphthalene exposure, *Scgb1a1* expression was significantly reduced in wild-type (0.05 ± 0.01 SEM) and *Areg*^{-/-} (0.03 ± 0.01 SEM) mice as compared with untreated wild-type mice. At 10 days after naphthalene exposure, *Scgb1a1* expression increased in wild-type and *Areg*^{-/-} mice, to 0.17 ± 0.03 and 0.39 ± 0.27 SEM, respectively, further indicating reconstitution of CCSP expressing epithelium. These data suggest that *Areg* deficiency has no impact on either naphthalene-induced lung injury or the ensuing repair response to replenish depleted Clara cells.

AREG-Dependent Mucosecretory Cell Differentiation

In addition to epithelial repair and proliferation, the gene expression of differentiation markers for ciliated cells (*Foxj1*), alveolar type II cells (*Sftpc*), and mucin-secreting cells (*Muc5AC*, *Muc5B*, and *Clca3*) was evaluated in naphthalene-exposed *Areg*^{-/-} mice (Figure 2A). Expression of neither *Foxj1* nor *Sftpc* was significantly affected by either loss of *Areg* (*Areg*^{-/-}) or naphthalene-induced lung injury. However, despite very low levels of *Muc5AC*, *Muc5B*, and chloride channel calcium activated-3 (*Clca3*) gene expression in lung tissue of uninjured wild-type and *Areg*^{-/-} mice, naphthalene exposure resulted in the significant up-regulation of all three mucin-related genes. In wild-type mice, the expression of *Muc5AC*, *Muc5B*, and *Clca3* were not significantly altered 3 days after injury, but were dramatically increased (>20-fold, >1.5-fold, and >125-fold, respectively) compared with untreated wild-type mice 10 days after naphthalene exposure. In comparison, in *Areg*^{-/-} mice at the same time point (Day 10), the magnitude of this change was significantly blunted and, in some cases, returned to baseline levels. Immunolocalization of MUC5AC in lung sections from naphthalene-exposed mice collected 10 days after injury revealed a similar response (Figure 2B). Wild-type mice exposed to naphthalene developed MCM, evident by increased MUC5AC-immunoreactive conducting airway epithelium. In contrast, *Areg*^{-/-} mice exhibited decreased MCM, revealed by a decrease in MUC5AC-immunoreactive cells in conducting airway epithelium. Morphometric analysis of MUC5AC immunofluorescence staining showed roughly 50%

Figure 1. Proliferation and repair of airway epithelium in amphiregulin (*Areg*) -deficient mice after naphthalene injury. (A) Immunofluorescence detection of epithelial proliferation (nuclear 5-bromo-2'-deoxyuridine [BrdU]; red) and epithelial reconstitution (Clara cell secretory protein [CCSP]; green) from formalin-fixed lung tissue isolated at steady state and after naphthalene exposure (3 or 10 d) from wild-type and *Areg*-deficient (*Areg*^{-/-}) mice. (B) Real-time PCR expression of CCSP marker, *Scgb1a1*, in naphthalene-exposed *Areg*^{-/-} mice. Total lung RNA was isolated at steady state and 3 or 10 days after naphthalene-induced lung injury from wild-type (open bars) or *Areg*^{-/-} (closed bars) mice. Relative mRNA abundance was normalized to β -glucuronidase and presented as the average fold change ($\pm$ SEM) over untreated wild-type mice. Data were derived from three to six mice per genotype per time point, and are representative of two separate experiments.

of the proximal airway epithelium stained positive for MUC5AC 10 days after naphthalene exposure (Figure 2C). The prevalence of MUC5AC-positive cells in the proximal airway was significantly attenuated in *Areg*^{-/-} mice exposed to naphthalene, with only 20% of the epithelium staining for MUC5AC.

To assess whether AREG dependency of MCM observed after naphthalene exposure was unique to an injury model that lacks an allergic inflammatory component, we compared responses of wild-type and *Areg*^{-/-} mice exposed to hNE (36, 37). Differential assessment of bronchoalveolar lavage fluid cellularity revealed that, 4 days after the last hNE aspiration (Day 11; when MCM and *Muc5AC* gene expression are maximal), wild-type and *Areg*^{-/-} mice both developed robust eosinophilic (wild-type, $29.58 \pm 8.47\%$; *Areg*^{-/-}, $24.42 \pm 6.62\%$) and lymphocytic (wild-type, $5.46 \pm 1.44\%$; *Areg*^{-/-}, $6.07 \pm 1.13\%$) infiltration, with no significant differences between genotypes. Evaluation of *Muc5AC*, *Muc5B*, and *Clca3* gene expression (Figure 2B) and MUC5AC protein expression (Figure 2C) in lung lysates and tissue from wild-type and *Areg*^{-/-} mice were all significantly increased after hNE exposure, yet with no significant differences between genotypes. These data suggest that AREG plays an important role in the regulation of mucosecretory cell differentiation after naphthalene exposure, yet has no role in mucosecretory differentiation in a model of allergic lung inflammation.

AREG Is an Autocrine Mediator of Mucus Cell Differentiation

To gain a better understanding of the mechanisms by which AREG regulates epithelial cell differentiation, we sought to identify the cellular source of AREG after naphthalene-induced lung injury. Increases in *Areg* mRNA expression, relative to the expression of the housekeeping gene, β -glucuronidase, in dissociated and fractionated lung cells from wild-type mice 1 day after naphthalene exposure were compared with unexposed mice (Figure 3). In cell fractions enriching for endothelial/hematopoietic (CD34⁻, CD31⁺, CD45⁺, EpCAM⁻) or resident stromal cells (CD34⁺, CD31⁻, CD45⁻, EpCAM⁻), exposure of mice to naphthalene did not result in significant increases in *Areg* expression when compared with similar fractions from unexposed mice. In contrast, the epithelial cell fraction (CD34⁻, CD31⁻, CD45⁻, EpCAM⁺) resulted in a 5.4-fold increase in *Areg* expression, relative to β -glucuronidase, after naphthalene exposure as compared with a similar fraction from unexposed mice. The change in *Areg* expression is similar to our previously published data that describe a 6.7-fold increase in *Areg* expression from whole-lung homogenates 1 day after naphthalene exposure (6). Together, these data indicate that the pulmonary epithelium responds to naphthalene injury by the induction of *Areg*.

We next sought to determine whether the effects of AREG on pulmonary epithelial cell differentiation were direct, through

Figure 2. Differentiation markers during epithelial injury and repair in naphthalene-exposed *Areg*^{−/−} mice. (A) Real-time PCR expression of ciliated cells (*Foxj1*), alveolar cells (*Sftpc*), and mucus-secreting cells (*Muc5AC*, *Muc5B*, and *Clca3*) in naphthalene- and human neutrophil elastase (hNE)–exposed *Areg*^{−/−} mice. Total lung RNA was isolated at steady state and 3 or 10 days after naphthalene-induced lung injury from wild-type (open bars) or *Areg*^{−/−} (closed bars) mice. Relative mRNA abundance was normalized to β-glucuronidase and presented as the average fold change (±SEM) over untreated wild-type mice. Significance (*P* < 0.05) indicated by: * versus untreated wild-type mice, ** versus wild-type for the respective time point. (B) Immunofluorescence detection of secretory cell marker MUC5AC from formalin-fixed lung tissue from wild-type and *Areg*^{−/−} mice isolated 10 days after naphthalene exposure. (C) Morphometry of MUC5AC-positive airway epithelial cells of wild-type (open bars) and *Areg*^{−/−} (closed bars) mice 10 days after naphthalene exposure. Data are presented as the percentage of MUC5AC-labeled epithelial cells per 4',6-diamidino-2-phenylindole–labeled epithelial cells. Significance (*P* < 0.05) indicated by: * versus untreated wild-type mice, ** versus wild-type for the respective time point.

autocrine signaling, or indirect, through a nonepithelial cell intermediate. Because EGFR is the obligate receptor for AREG-dependent signaling, we used conditional targeted deletion of the *Egfr* gene to abrogate epithelial responses to AREG in the pulmonary epithelium. Mice that were homozygous for an *Egfr*^{fllox} allele and harbored a surfactant protein C (*SFTPC*)–Cre transgene were established for efficient deletion of *Egfr* within pulmonary epithelial cells (*Egfr*^Δ). *SFTPC*–Cre-negative mice that were homozygous for an *Egfr*^{fllox} allele were used as controls (*Egfr*^{WT}). We assessed the efficiency of *Egfr* gene deletion by mRNA and protein expression in total lung homogenates.

Egfr^Δ mice possessed significantly less *Egfr* mRNA (reduced by roughly 50%) and protein as compared with *Egfr*^{WT} mice (Figure 4A). A similar analysis was performed to quantify *Egfr* mRNA abundance within isolated lung epithelial cells (fractionated CD34[−], CD31[−], CD45[−], and EpCAM⁺ cells), which revealed a more than 90% reduction in *Egfr* mRNA abundance within cells isolated from *Egfr*^Δ mice compared with those of *Egfr*^{WT} mice (Figure 4B). These data confirm that gene expression of *Egfr* was effectively reduced in the pulmonary epithelium of *Egfr*^Δ mice.

We used *Egfr*^{WT} and *Egfr*^Δ mice to determine the role of epithelial EGFR signaling in the repair and differentiation of

Figure 3. *Areg* mRNA expression from isolated and fractionated lung cells. Total RNA from isolated and fractionated lung cells of wild-type (open bars) or naphthalene-treated (closed bars) mice was assessed for *Areg* mRNA expression by real-time PCR. Changes in *Areg* mRNA ($\pm$ SEM) were determined relative to the expression of the housekeeping gene, β -glucuronidase. Significance ($P < 0.05$) indicated by: * versus wild-type for the respective fractionated cell population. Data were derived from three mice per exposure, and are representative of two separate experiments.

the pulmonary epithelium of naphthalene-exposed mice (Figure 5). Despite the involvement of EGFR signaling in the proliferation of numerous other tissues, targeted deletion of *Egfr* (*Egfr* $^{\Delta}$) in the pulmonary epithelium did not result in any overt gross changes in lung structure or changes in CCSP or nuclear BrdU immunoreactivity at steady state that would be suggestive of epithelial hypoplasia. At 3 days after naphthalene exposure, the airway epithelium of *Egfr* WT mice possessed significantly fewer CCSP-expressing cells, as well as increased nuclear BrdU staining of naphthalene-resistant CCSP-immunoreactive cells located at the BADJ (Figure 5A). This was subsequently followed by epithelial repair evident by increased CCSP-immunopositive cells 10 days after injury. In comparison, *Egfr* $^{\Delta}$ mice showed no appreciable difference in epithelial injury (loss of CCSP immunoreactivity) or proliferation (nuclear BrdU immunoreactivity) 3 days after injury, and a similar degree of epithelial repair (increases in CCSP immunoreactivity) 10 days after naphthalene exposure (Figure 5A). Likewise, gene expression of the Clara cell differentiation marker, *Scgb1a1*, revealed no significant difference between *Egfr* WT and *Egfr* $^{\Delta}$ mice at baseline or after naphthalene exposure, reducing *Scgb1a1* expression in both groups by 90% 3 days after injury. Epithelial reconstitution was apparent 10 days after naphthalene exposure, as *Scgb1a1* expression increased back toward baseline levels in both *Egfr* WT (0.23 ± 0.05 SEM) and *Egfr* $^{\Delta}$ mice (0.15 ± 0.03 SEM) (Figure 5B).

Further analysis of gene transcript differentiation markers for ciliated cells (*Foxj1*), alveolar type II cells (*Sftpc*), and mucus-secreting cells (*Muc5AC*, *Muc5B*, and *Clca3*) in *Egfr* WT and *Egfr* $^{\Delta}$ mice treated with naphthalene revealed a similar differentiation trend witnessed in the *Areg* $^{-/-}$ mice (Figure 5B). Whereas *Foxj1* and *Sftpc* expression was similar in both *Egfr* WT and *Egfr* $^{\Delta}$ mice at baseline and after naphthalene injury, *Egfr* $^{\Delta}$ mice displayed altered secretory cell differentiation 10 days after naphthalene injury (Figure 5B). At 3 days after naphthalene exposure, both *Egfr* WT and *Egfr* $^{\Delta}$ mice displayed similar changes in *Muc5AC*, *Muc5B*, and *Clca3* although these changes in gene expression were not significantly different among either genotype. At 10 days after naphthalene exposure, *Egfr* WT mice displayed significantly increased *Muc5AC* (>10 -fold), *Muc5B* (>4.6 -fold), and *Clca3* (>35 -fold) gene expression, which was dramatically attenuated in *Egfr* $^{\Delta}$ mice (*Muc5AC*, 4.7 ± 0.9 -fold; *Muc5B*, 2.98 ± 0.4 -fold; *Clca3*, 16.4 ± 6.2 -fold), although still substantially elevate as compared with unexposed *Egfr* WT mice. These results suggest that AREG and EGFR are both necessary for mucus cell differentiation that accompanies repair from naphthalene-induced lung injury.

AREG-Dependent EGFR Signaling Is Sufficient to Drive *Clca3* Gene Expression

To determine whether AREG is sufficient to induce epithelial MCM via EGFR signaling, we treated primary lung epithelial

cells from *Egfr* WT and *Egfr* $^{\Delta}$ mice with exogenous recombinant AREG and assessed *Muc5AC* and *Clca3* mRNA expression (Figure 6). Primary lung epithelial cells (fractionated CD34 $^{-}$, CD31 $^{-}$, CD45 $^{-}$, and EpCAM $^{+}$ cells) were isolated from unexposed *Egfr* WT and *Egfr* $^{\Delta}$ mice, suspended in a matrix plug, and cultured in the presence of feeder fibroblasts at an air-liquid interface. Whereas treatment of *Egfr* WT cells with exogenous AREG for 14 days did not result in altered *Muc5AC* expression (data not shown), the expression of *Clca3*, which showed the most robust response in both prior *in vivo* models, was significantly increased (3.0 ± 0.1 SEM) compared with untreated *Egfr* $^{\Delta}$ cells.

Figure 4. Conditional targeted deletion of epidermal growth factor receptor (*Egfr*) in the pulmonary epithelium. (A) Total lung *Egfr* mRNA and protein (inset) was assessed from tissue homogenates of *Egfr* WT (*SFTPCre* $^{-}$ *Egfr* $^{fl/y}$) and *Egfr* $^{\Delta}$ (*SFTPCre* $^{+}$ *Egfr* $^{fl/y}$) by real-time PCR and Western blot, respectively. *Egfr* mRNA abundance was normalized to β -glucuronidase and presented as the average fold change ($\pm$ SEM) over *Egfr* WT mice. (B) *Egfr* mRNA expression, as assessed by real-time PCR, from isolated and fractionated epithelial cells. mRNA abundance was normalized to β -glucuronidase and presented as the average fold change ($\pm$ SEM) relative to *Egfr* WT mice. Significance ($P < 0.05$) indicated by: * versus *Egfr* WT mice. Data were derived from three mice per genotype, and are representative of two separate experiments.

Figure 5. Epithelial proliferation, repair, and differentiation of naphthalene-exposed *Egfr^Δ* mice. (A) Immunolocalization of CCSP (green) injury and repair via proliferation (BrdU; red) in formalin-fixed lung tissue isolated at steady state and after naphthalene exposure (3 or 10 d) from *Egfr^{WT}* (*SFTPCcre⁻ Egfr^{f/f}*) and *Egfr^Δ* (*SFTPCcre⁺ Egfr^{f/f}*) mice. (B) Real-time PCR expression of differentiation markers during epithelial injury and repair in naphthalene-exposed *Egfr^Δ* mice. Total lung RNA was isolated at steady state and 3 or 10 days after naphthalene-induced lung injury from *Egfr^{WT}* (open bars) or *Egfr^Δ* (closed bars) mice. Relative mRNA abundance was normalized to β -glucuronidase and presented as the average fold change ($\pm$ SEM) over steady state *Egfr^{WT}* mice. Significance ($P < 0.05$) indicated by: * versus untreated *Egfr^{WT}* mice, ** versus *Egfr^{WT}* mice for the respective time point. Data were derived from three to five mice per genotype per time point, and are representative of two separate experiments.

Figure 6. EGFR-dependent expression of *Clca3* mRNA in primary lung epithelial cells treated with AREG. Isolated and fractionated primary epithelial cells from *Egfr*^{WT} (SFTPCre⁻ *Egfr*^{+/+}) and *Egfr*^{-/-} (SFTPCre⁺ *Egfr*^{+/+}) mice were cocultured with mouse lung fibroblast cells in the presence or absence of exogenous AREG. *Clca3* mRNA expression was assessed by real-time PCR from total RNA isolated from the coculture. *Clca3* mRNA abundance was normalized to *EpCAM* and presented as the average fold change ($\pm$ SEM) relative to saline-treated *Egfr*^{WT} epithelial cells. Significance ($P < 0.05$) indicated by: * versus *Egfr*^{WT}, ** versus *Egfr*^{WT} + AREG. Data were derived from epithelial cells obtained from four mouse donors per genotype, and are representative of two separate experiments.

In contrast, primary epithelial cells from *Egfr*^{-/-} mice, cultured and treated with AREG as above and subjected to a similar analysis, displayed significantly reduced AREG-induced *Clca3* expression to levels below that of untreated *Egfr*^{WT} cells (0.26 ± 0.05 SEM). These results suggest that AREG treatment can drive the expression of epithelial *Clca3* expression in an EGFR-dependent mechanism.

DISCUSSION

In this study, we investigated the role of AREG and pulmonary epithelial-specific EGFR signaling in proliferation, repair, and differentiation of airway epithelium after lung injury. Naphthalene-induced lung injury was used to model lung disease that does not include an allergic inflammatory component. Mice exposed to naphthalene developed acute airway epithelial cell injury, leading to repair and aberrant differentiation of pulmonary secretory cells, evident by elevated *Muc5AC*, *Muc5B*, and *Clca3* expression, suggestive of MCM. Genetic loss of *Areg* in mice significantly attenuated the MCM phenotype elicited by naphthalene-induced lung injury, but had no effect on the extent of epithelial injury or proliferation. Lung epithelial cells were the principal source of *Areg* in response to naphthalene-induced lung injury, and MCM of bronchial airways was dependent upon epithelial cell expression of the EGFR. Taken together, our data indicate that AREG is a component of MCM in naphthalene-induced lung injury that acts in an autocrine fashion on the airway epithelium to induce *Muc5AC*, *Muc5B*, and *Clca3* expression.

Epithelial repopulation after pulmonary injury is first preceded by epithelial proliferation. In mice exposed to naphthalene, this is accomplished by naphthalene-resistant variant Clara cells that reside in discrete microenvironments of the lung (neuroepithelial bodies and at the BADJs) (8, 14). The EGF family of ligands has been shown to participate in epithelial proliferation and repair in various cell types (41–46). Studies by Van Winkle and colleagues (7) showed increased EGF, TGF- α , and EGFR at sites

of epithelial injury/repair in mice exposed to naphthalene, suggesting a critical role of EGFR signaling in the proliferation and repair of the pulmonary epithelium after naphthalene-induced lung injury. Likewise, AREG can induce the proliferation of cultured epithelial cells, as well as promote epithelial branching in explanted embryonic lung rudiments (30, 46). However, herein we show *in vivo* that AREG, and pulmonary epithelial-specific EGFR signaling, is not essential for the proliferation of naphthalene-resistant Clara cells, and mice deficient in *Areg*, as well as epithelial *Egfr*, are equally as capable as wild-type mice in repairing the injured epithelium. These results argue against a direct role for AREG in the proliferation of variant Clara cells after naphthalene-induced lung injury.

MCM and mucus hypersecretion typically accompany remodeling of the airway epithelium in chronic lung disease (47). Experimental evidence suggests that the resulting epithelial remodeling and MCM is mediated in large part by the effects of allergic inflammation and the resulting production of T helper cell type 2 cytokines that act in conjunction with EGFR signaling (48–51). For example, TGF- α has been shown to stimulate *Muc5AC* gene expression in both cultured human airway epithelial cells as well as intratracheal installation of rats (52). In addition, animal models of allergic asthma, achieved by antigen inhalation in sensitized mice, have shown AREG immunostaining to be increased in the bronchial airway epithelium (53, 54). Previous work by us and others has shown that AREG, as well as other EGFR ligands, such as EGF and TGF- α , are up-regulated after naphthalene-induced lung injury; however, the role of AREG and its obligate receptor, EGFR, in epithelial cell differentiation was not explored (6, 7). Herein, we describe a role for AREG in the development of mucus-secreting cells after airway epithelial cell injury elicited by naphthalene exposure. Mice exposed to naphthalene developed increased gene expression and protein expression of MUC5AC, MUC5B, and CLCA3, characteristic of MCM. Genetic loss of *Areg* expression significantly blunted this mucosecretory cell differentiation after naphthalene exposure, but had no effect on Clara, ciliated, and alveolar type 2 cell differentiation. Together, these data highlight a contributing role for AREG in the promotion of MCM in this model of nonallergic lung injury.

Within the lung, AREG can be produced by multiple cellular sources, including eosinophils, stromal cells, and the pulmonary epithelium (22, 30, 55, 56). Clinical and experimental evidence suggests that multiple EGF ligands are produced and released by the pulmonary epithelium, and may possibly act in an autocrine fashion in the development of MCM. For example, analysis of sputum and bronchial biopsy samples from patients with asthma have shown that expression of EGF family ligands and their corresponding receptors are significantly increased (20, 46, 57). Likewise, in animal models of allergic asthma, the bronchial airway epithelium stains positive for AREG immunostaining (53, 54). We show here that the pulmonary epithelium is both the source of *Areg* gene expression and the ultimate target for its effects on mucus cell differentiation after naphthalene-induced lung injury. Furthermore, using mice with the genetic deletion of *Egfr* in the pulmonary epithelium, we show *in vivo* that AREG is an autocrine regulator of *Muc5AC*, *Muc5B*, and *Clca3* expression in pulmonary epithelial cells after naphthalene exposure. This was further supported by our complimentary *in vitro* data in which cultured epithelial cells lacking *Egfr* displayed a blunted *Clca3* gene expression in response to exogenous AREG treatment. To our surprise, despite the robust AREG- and EGFR-dependent MUC5AC response observed *in vivo*, *Muc5AC* gene expression was not altered by AREG treatment in *Egfr*-sufficient and -deficient epithelial cells grown in culture. One possible explanation for this lack of response

may relate to the conditions in which the epithelial cells were cultured; more specifically, the addition of SB431542, an inhibitor of activin receptor–like kinase receptors of the TGF- β family of proteins. Although we did not specifically assess, in this investigation, the role of TGF- β signaling in AREG-induced mucin expression, evidence suggests that TGF- β signaling may play an essential role in mucin expression (58, 59). However, despite the lack of *Muc5AC* expression in AREG-treated epithelial cells in culture, the *in vivo* data presented in this article show that *Clca3* induction was the more robust marker of MCM. Similar studies have shown *CLCA3* to be highly up-regulated—in many instances greater than *MUCAC*—in diseased states and animal models complicated with MCM (60). Together, these results suggest that AREG behaves as an autocrine mediator of pulmonary epithelial MCM after naphthalene-induced lung injury, and that this altered mucosecretory differentiation may be dependent upon active TGF- β signaling.

The development of MCM is a complex process in which activation of multiple signaling pathways lead to a common pathological outcome. Our findings, and those of others, show no effect of AREG in the promotion of allergic lung inflammation or the development of airway MCM induced by allergen challenge (61). However, the finding that AREG is elevated in patients with COPD raises the possibility of its potential role in MCM that accompanies nonallergic lung injury. This is further corroborated by *in vitro* and *in vivo* models of COPD involving cigarette smoke exposure that have shown dose-dependent increases in AREG expression and EGFR-dependent MCM (62, 63). Using naphthalene exposure as a model of nonallergic lung injury, our results suggest a clear role for AREG in the development of MCM after nonallergic lung injury. Collectively, these data highlight the variable contribution made by AREG to injury-related MCM. Further studies are needed to determine the contribution made by AREG to MCM seen either in experimental models or in patients with nonallergic lung disease.

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