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RESEARCH ARTICLE

Docosahexaenoic acid enhances amphiregulin-mediated bronchial epithelial cell repair processes following organic dust exposure

Tara M. Nordgren,[2,3] Art J. Heires,[2] Kristina L. Bailey,[1,2] Dawn M. Katafiasz,[2] Myron L. Toews,[4] Christopher S. Wichman,[5] and Debra J. Romberger[1,2]

[1]*Veterans Affairs Nebraska-Western Iowa Health Care System, Omaha, Nebraska;* [2]*Pulmonary, Critical Care, Sleep and Allergy Division, Department of Internal Medicine, University of Nebraska Medical Center, Omaha, Nebraska;* [3]*Division of Biomedical Sciences, School of Medicine, University of California Riverside, Riverside, California;* [4]*Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, Nebraska; and* [5]*Department of Biostatistics, University of Nebraska Medical Center, Omaha, Nebraska*

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Nordgren TM, Heires AJ, Bailey KL, Katafiasz DM, Toews ML, Wichman CS, Romberger DJ. Docosahexaenoic acid enhances amphiregulin-mediated bronchial epithelial cell repair processes following organic dust exposure. *Am J Physiol Lung Cell Mol Physiol* 314: L421–L431, 2018. First published November 2, 2017; doi: 10.1152/ajplung.00273.2017.—Injurious dust exposures in the agricultural workplace involve the release of inflammatory mediators and activation of epidermal growth factor receptor (EGFR) in the respiratory epithelium. Amphiregulin (AREG), an EGFR ligand, mediates tissue repair and wound healing in the lung epithelium. Omega-3 fatty acids such as docosahexaenoic acid (DHA) are also known modulators of repair and resolution of inflammatory injury. This study investigated how AREG, DHA, and EGFR modulate lung repair processes following dust-induced injury. Primary human bronchial epithelial (BEC) and BEAS-2B cells were treated with an aqueous extract of swine confinement facility dust (DE) in the presence of DHA and AREG or EGFR inhibitors. Mice were exposed to DE intranasally with or without EGFR inhibition and DHA. Using a decellularized lung scaffolding tissue repair model, BEC recolonization of human lung scaffolds was analyzed in the context of DE, DHA, and AREG treatments. Through these investigations, we identified an important role for AREG in mediating BEC repair processes. DE-induced AREG release from BEC, and DHA treatment following DE exposure, enhanced this release. Both DHA and AREG also enhanced BEC repair capacities and rescued DE-induced recellularization deficits. In vivo, DHA treatment enhanced AREG production following DE exposure, whereas EGFR inhibitor-treated mice exhibited reduced AREG in their lung homogenates. These data indicate a role for AREG in the process of tissue repair after inflammatory lung injury caused by environmental dust exposure and implicate a role for DHA in regulating AREG-mediated repair signaling in BEC.

amphiregulin; bronchial epithelial cells; inflammation; organic dust; repair

INTRODUCTION

In agricultural environments, inhaled organic dust leads to robust respiratory acute inflammatory responses (20, 22, 49, 50). Workers who are recurrently exposed to such environments are at heightened risk for lung diseases, including chronic bronchitis and chronic obstructive pulmonary disease (COPD) (50). Dysregulation of lung repair processes is a key feature of numerous chronic lung diseases (8, 43, 54), but the mechanisms underlying dysfunctional repair following lung inflammation and injury are unclear. Additional research is required to identify how improving lung repair mechanisms might protect against debilitating lung disease, particularly in the context of occupational exposures.

The epidermal growth factor receptor (EGFR) ligand amphiregulin (AREG) is an important mediator of epithelial proliferation and repair processes and also governs immune cell activities, including promoting T-regulatory cell polarization and other pro-resolution effector cell functions (5, 18, 21, 52, 61). Together, these coordinated and appropriately controlled responses aid in the repair/recovery processes following inflammation and injury (18, 21, 61). Dysregulation of these processes, however, may lead to insufficient repair or fibrotic remodeling; for this reason, AREG has been characterized as both protective and injurious in the progression of various lung diseases (5, 18, 21, 31, 36, 46, 48, 61).

Our previous preclinical investigations have identified that tumor necrosis factor-α (TNFα)-converting enzyme (TACE) and EGFR signaling activities are required for the proinflammatory responses of bronchial epithelial cells (BEC) following exposure to extracts of organic dusts derived from swine confinement facilities (DE) (14, 38, 44). While a number of reports identify AREG production by BEC exposed to cigarette smoke (36), particulate matter (48), and diesel exhaust particles (31), investigations into the role of AREG in promoting repair and recovery following occupational agriculture exposures are lacking.

Furthermore, studies reveal diets high in omega-3 polyunsaturated fatty acids (n-3 PUFA) may be beneficial in inflammatory lung conditions, including asthma and COPD (17). The mechanistic roles of n-3 PUFA in the processes of lung repair and protection from lung disease are unclear. A number of studies indicate that PUFA-derived lipid mediators regulate the resolution of lung inflammation and promote the production of AREG (4, 13, 21). Specifically, we have demonstrated that the n-3 PUFA docosahexaenoic acid (DHA) and its lipid mediator

Address for reprint requests and other correspondence: D. J. Romberger, Pulmonary, Critical Care, Sleep and Allergy Division, Dept. of Internal Medicine, Univ. of Nebraska Medical Center, Omaha, NE 68198 (e-mail: dromberg@unmc.edu).

derivative maresin-1 are able to limit BEC and lung inflammatory responses to DE in vitro and in vivo (26–28).

To this end, we sought to determine the effects of organic dust exposure on AREG production by BEC and examine the role and regulation of AREG in lung repair processes following DE exposure. Utilizing primary isolated human BEC and a human, decellularized lung scaffolding model of lung epithelial repair, we found that DE exposure induced AREG production in human BEC and enhanced wound repair processes ex vivo. Furthermore, the n-3 PUFA DHA enhanced BEC AREG production and lung scaffolding recellularization. Together, these investigations identify a role for AREG in mediating BEC lung repair processes following organic dust exposure and establish a cooperative function for DHA in enhancing these lung repair processes.

MATERIALS AND METHODS

Reagents. Cell culture medium LHC basal was purchased from Invitrogen/Life Technologies (Carlsbad, CA), primary cell growth medium BEGM from Lonza (Walkersville, MD), and RPMI from Millipore/Sigma (St. Louis, MO). Human and murine AREG ELISA kits (Duoset development kits), recombinant human AREG, and AREG neutralizing antibody were from R&D Systems (Minneapolis, MN). Erlotinib and DHA were obtained from Cayman Chemical (Ann Arbor, MI). Inhibitors TAPI-1 and AG1478 were purchased from Millipore/Sigma (St. Louis, MO), and the *Vybrant* MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] cell proliferation kit was from Molecular Probes (Eugene, OR). All other reagents not specified were from Millipore/Sigma.

Preparation of DE. Organic dust extracts were prepared as previously described (23). Settled dust was collected from surfaces >1 meter above the floor in commercial swine confinement facilities in Nebraska, housing 500–800 animals. Dust was extracted in Hanks' Balanced Salt Solution (100 mg/ml) for 1 h and centrifuged, and the supernate was centrifuged again and then filter sterilized through a 0.22-μm pore membrane. The resulting saturated extract (100% DE) contained 26–40 mg of total protein/ml, 500–975 EU/ml endotoxin, and included only ultrafine particulates. The bacterial composition of the dust has been characterized (6). Batches of sterile DE were frozen in aliquots and diluted to 5% (vol/vol) for in vitro experiments and 12.5% for animal intranasal instillation studies. No measurable cytotoxicity was observed at these concentrations. DE from three different preparations were used in these experiments.

Human BEC. Primary human cells were prepared from deidentified human lungs obtained from the International Institute for the Advancement of Medicine (IIAM), National Disease Research Interchange (NDRI), or via the Nebraska Organ Retrieval System (NORS), following a previously published protocol (3) and in accordance with the University of Nebraska Institutional Review Board guidelines. Primary cells (BEC) were maintained in serum-free growth medium (BEGM Singlequot kit, Lonza) supplemented with the growth factors supplied with the kit, and passaged not more than four times. Cell isolates from three or more donors were used in these studies. The immortalized, SV-40 transformed cell line BEAS-2B was purchased from American Type Culture Collaction (Manassas, VA) and cultured in LHC9/RPMI (50:50) as previously described (23). All cultures were maintained at 37°C in incubators supplied with a 5% CO_2 humidified atmosphere.

Decellularized human lung mesenchymal scaffolding. Human lungs were obtained from IIAM or NORS (as above). Following a modification of published scaffolding preparation methods (7, 16, 51), lung lobes were carefully separated and decellularized by serial inflation and lavage with deionized water (2 washes), followed by inflation and incubation in 0.1% Triton X solution for 2 days at 4°C. Lungs were deflated and then reinflated with 2% sodium deoxycholate for 2 days at 4°C, washed, and reinflated with 2% sodium deoxycholate for 2 additional days at 4°C. Following these incubations, decellularized lungs were washed in PBS to remove all detergent. Decellularized lungs were inflated with a warmed 2% solution of low-melting-point agarose in PBS and allowed to solidify at 4°C overnight. Tissue cores were made with a 10-mm diameter cylindrical punch, embedded in a 1.5% solution of Type IB low EEO agarose, and sectioned to a thickness of 300 μm using a vibratome (Compresstome, Precisionary Instruments, Greenville, NC). The resulting disks of lung tissue (scaffolds) were washed in PBS and stored in a 30% ethanol-PBS solution at −20°C until use. For use in experiments, scaffolds were warmed, rinsed twice in PBS, and equilibrated in BEGM culture medium for at least 30 min at 37°C before being seeded with epithelial cells.

Ex vivo wound repair model. Scaffolds were treated with 5% DE, 10 ng/ml rhAREG, or 1 μM DHA for 30 min before being seeded with 1×10^5 primary BEC per scaffold in BEGM in 12-well cluster plates. Scaffold cultures were refed every third day with fresh mediators, and on *day 3*, scaffolds were removed to new plates to exclude cells adhering to plastic. In certain experiments, recellularized scaffolds were stained with hematoxylin (Shandon Instant Hematoxylin; Thermo Scientific, Pittsburgh, PA) according to the manufacturer's instructions to visualize scaffold cellularity. For experiments using inhibitors, the TACE inhibitor TAPI-1 (0.02–20 μM), the tyrosine kinase/EGFR inhibitors AG1478 (2.5 μM) or erlotinib (0.1–50 μM), and an anti-AREG neutralizing antibody (1 μg/ml) were added concurrently with DE or DHA. Recellularization of BEC within the scaffold matrices was quantified using the MTT method on *days 5, 7*, and *12* of culture. A nontargeting isotype control antibody (goat IgG, 1 μg/ml) was used to control for nonspecific Ig effects, and an inactive structural analog of AG1478 (AG-9, 2.5 μM) was used as a control for the active tyrosine kinase inhibitor.

MTT cell proliferation assay. At the termination of scaffold culture incubation, scaffolds were removed to fresh plates and washed twice in PBS to remove nonadherent cells and phenol red indicator. Scaffolds were labeled with 30 μl of 12 mM MTT in 200 μl of PBS following the kit manufacturer's directions, and incubated at 37°C for 3 h. MTT is readily taken up by epithelial cells and converted to an insoluble formazan. The scaffolds were again rinsed, and the dye was liberated from the cells by extraction with 100 μl DMSO. Extracts were transferred to 96-well microtiter plates, and the absorbance was determined at 540 nm. Cell numbers in each scaffold were interpolated from a standard curve generated using 1×10^5 labeled cells.

Animals. Male wild-type C57BL/6J mice 5–8 wk of age were purchased from The Jackson Laboratory (Bar Harbor, ME) and were group-housed in micro-isolator cages with free access to standard rodent chow and filtered water. Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center and conform to National Institutes of Health guidelines for the use of rodents. Mice were acclimatized for 1 wk before the onset of experiments.

Murine model of dust exposure. For analysis of bronchoalveolar lavage (BAL) AREG levels, mice were instilled with a 12.5% solution of DE or saline (50 μl each) under light sedation (isofluorane) as previously described (2). The 12.5% DE concentration was chosen based on empirical data demonstrating maximal immune response and negligible toxicity at this dose (34). Mice were euthanized 5 or 24 h after DE exposure, and BAL was performed using 3× 1-ml aliquots of sterile saline. BAL fluid (BALF) recovered from the first lavage fraction was stored for murine AREG analysis by ELISA. For the EGFR inhibitor study, mice were randomly assigned to the following treatment groups: saline-only control, DE only, erlo (erlotinib alone), vehicle (0.1% DMSO), erlo + DE, vehicle + DE, and vehicle + saline. Five mice per treatment group were intranasally instilled with DE. For erlotinib experiments, mice were given two doses of 1.25 mg of erlotinib in 200 μl of 0.1% DMSO, or with vehicle only (0.1% DMSO) by oral gavage at 18 h and 1 h before 12.5% intranasal DE

challenge. Mice were euthanized 5 h post-instillation, BAL was performed as described above, and lung lobes were flash-frozen in liquid nitrogen for subsequent homogenization and AREG measurement. Mice were weighed during the course of the experiment, and no changes in weight gain were observed for any condition. For analysis of AREG lung tissue protein levels in DHA-treated mice, lung tissues from mice fed a high-DHA diet or a control diet containing no DHA followed by a single intranasal 12.5% DE challenge were utilized. Diets were prepared as previously described (33). Rodent chow diets were prepared by Envigo (Madison, WI) using a base AIN-93G diet. The modification of AIN-93G to obtain the high-DHA diet replaced soybean oil with DHASCO oil (DSM Nutritional Products, Kingstree, SC), DHA oil, containing 39.2% DHA, and high-oleic safflower oil. The diet was formulated to contain 5.88 DHA/kg to provide ~1.4% of total caloric intake (TCI) from DHA. The modification of AIN-93G to obtain the control diet replaced soybean oil with high-oleic safflower oil. Both diets were formulated to provide ~18.8% TCI from protein (17.7% by weight), 63.9% TCI from carbohydrates (60.1% by weight), and 17.2% TCI from fat (7.2% by weight). The mice were provided free access to both food and water. Mice were housed in micro-isolator cages with five mice per cage. Food was changed weekly by the investigators, while water was changed by the University of Nebraska Medical Center Comparative Medicine staff. Cages were changed every 2 wk. Mice were fed the defined diets for 4 wk before challenge with the DE instillation. Mice were weighed weekly, and watched for any signs of distress. Mice were euthanized 5 h following the DE exposure, lungs were lavaged, and lung tissues were collected and stored in RNAlater (ThermoFisher, Waltham, MA).

Measurement of soluble AREG. Both human and murine AREG were measured using Duoset ELISA development kits (R&D Systems) according to manufacturer's directions. The assays are based on biotinylated antibody detection of captured sample antigen, with horseradish peroxidase-avidin conversion of tetramethylbenzidine substrate to a chromogen detected at 450 nm (OD). Samples were quantified by interpolation from a calibration curve generated using recombinant AREG as the standard. Limits of detectability for human and murine AREG were 9.4 and 11.6 pg/ml, respectively.

Statistical analyses. Statistical calculations were performed using one-way ANOVA with Tukey's post hoc analysis for multiple comparisons (GraphPad Prism, San Diego, CA). For boxplots, quartiles and medians are displayed with minimum and maximum data points represented by whiskers. For line graphs and scatter plots, data shown are means ± SE. Differences between treatments were considered significant if $P < 0.05$ (95% CI).

RESULTS

DE treatment temporally upregulates AREG production by human BEC. Previous investigations have shown that certain environmental exposures stimulate AREG production by BEC (31, 36, 48). We sought to characterize the magnitude and timing of organic dust-mediated AREG production by human BEC. Primary isolated human BEC and immortalized BEAS-2B cells grown in submerged cultures were treated with 5% DE for 1–24 h. We have previously published cell viability measurements at this DE concentration indicating no significant decreases in cell viability (38). Supernatant cell culture fractions were collected and assayed for AREG release. In both primary BEC and BEAS-2B, AREG production was significantly upregulated in a temporal manner following DE treatment compared with the modest increase in AREG released by control cultures ($P < 0.05$; Fig. 1*A*). Furthermore, a single inhaled 12.5% DE challenge in mice led to a significant ($P < 0.05$) temporal increase in lavage fluid AREG levels at 5 and 24 h post-instillation (Fig. 1*B*). These findings identify DE as an

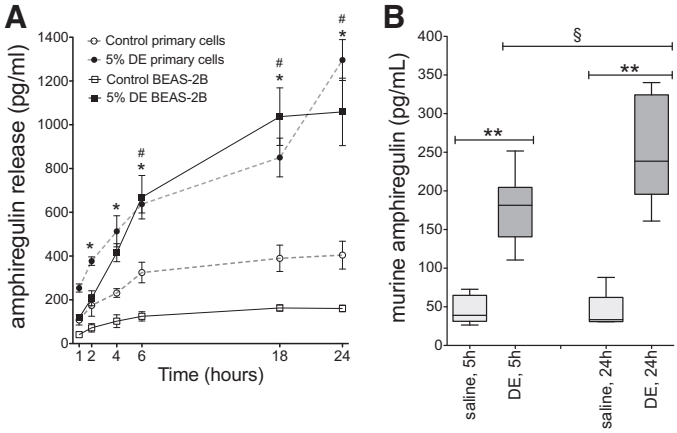

Fig. 1. Swine confinement facility dust extract (DE) exposure stimulates release of amphiregulin (AREG) in vitro and in vivo. *A*: AREG release is induced by DE in both primary bronchial epithelial cells (BEC) and immortalized cells (BEAS-2B) over time. *B*: mice exposed to a single intranasal DE challenge demonstrated a significant increase in lavage fluid AREG vs. saline-treated animals at both 5 and 24 h after DE exposure. Data shown represent means ± SE; $n = 3$ independent experiments, with 12 technical replicates per condition. *A*: $*P < 0.05$ vs. corresponding BEAS-2B control time points; $\#P < 0.05$ vs. BEC time points. *B*: plots represent the median AREG for a minimum of 5 animals per condition. $\S P < 0.01$; $**P < 0.001$ for indicated comparisons.

inducer of AREG production by BEC in vitro and in the lung following inhaled dust extract challenge in vivo.

AREG enhances human BEC wound repair capacity and rescues DE-induced repair deficit ex vivo. AREG is a well-known participant in the promotion of proliferation and wound repair programs in epithelial cells following injury in the lung (5, 18). Our previous work has demonstrated that DE exposure inhibits epithelial migration/wound repair processes in vitro (44). We sought to determine how AREG modulates BEC wound repair processes in the context of DE exposure using a model of lung epithelial wound repair. Thin sections of decellularized human lung lobes were used as scaffolding for human BEC adherence and growth. This model was adapted from several published reports using lung parenchymal scaffolds for tissue engineering and regeneration applications (7, 11, 16, 39, 51). As seen in Fig. 2*A*, the decellularization and sectioning process yields lung scaffolding disks devoid of cells while retaining typical lung tissue structural characteristics. When primary BEC are seeded onto the decellularized lung scaffold, the cells adhere and proliferate across the scaffolding over time, ultimately colonizing much of the denuded alveolar parenchyma (Fig. 2, *B–E*) and airways (Fig. 2*F*). Using this model, we found that continuous exposure to 5% DE during a 12-day course of recellularization resulted in a significant suppression of the recellularization of BEC on scaffold matrices as quantified by epithelial cell incorporation of MTT (Fig. 3). These data confirm our previous observation that DE reduces BEC migration/wound repair capacity in vitro, and validate the ex vivo decellularized lung scaffolding wound repair model. Furthermore, we found that exogenous AREG treatment of the cultures significantly enhanced scaffold recellularization in both the absence and presence of 5% DE at *days 5, 7*, and *12* (Fig. 4*A*). Because EGF is a component of complete BEGM medium, experiments were also performed using reduced

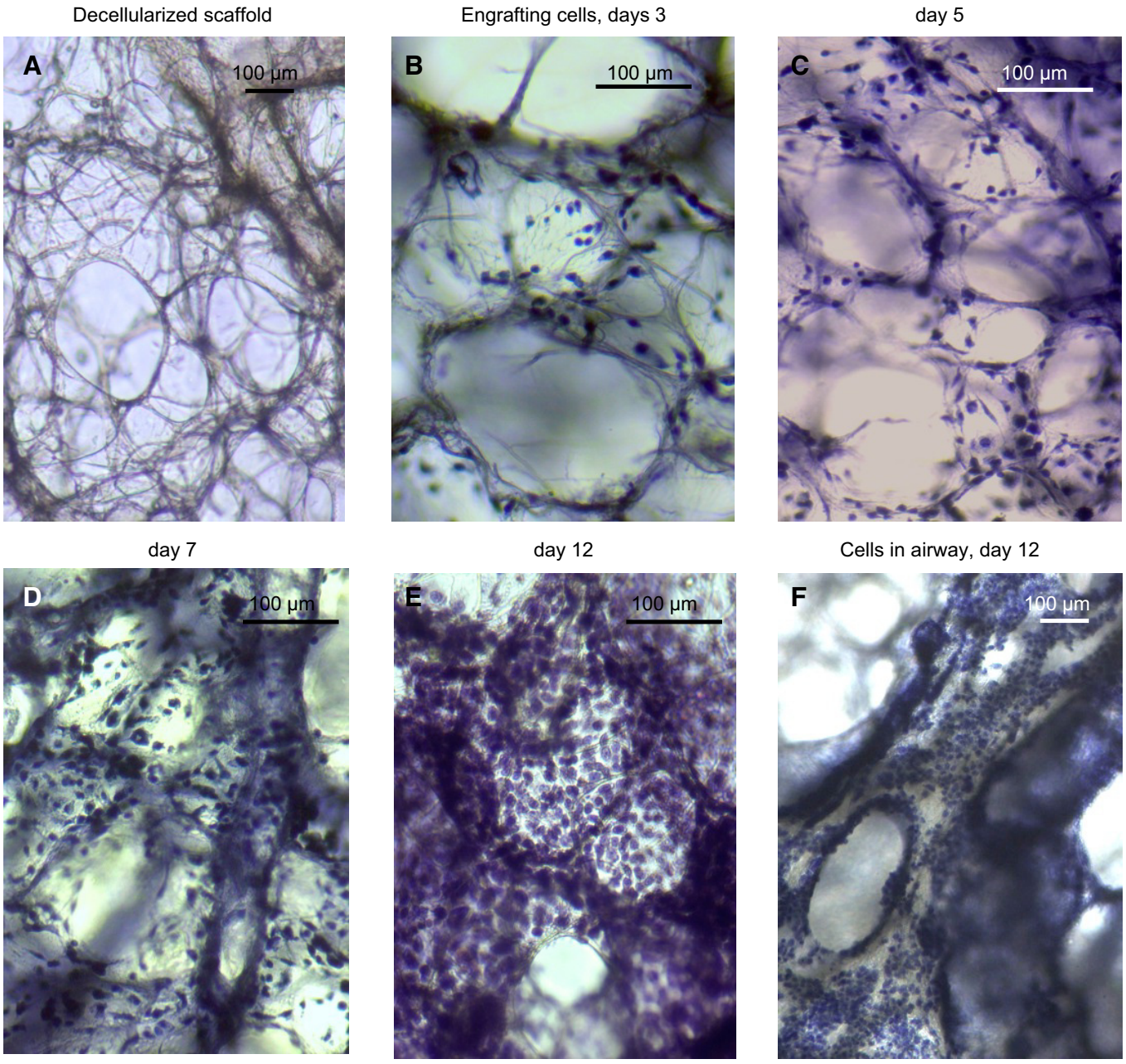

Fig. 2. Primary bronchial epithelial cells (BEC) repopulate denuded human lung scaffolds over time. *A*: normal human lung tissue was decellularized by detergent lavage and inflated with agarose before sectioning with a microtome. *B–E*: primary BEC (100,000 per section) were seeded onto stripped scaffolds and allowed to attach and proliferate in BEC growth medium for 12 days. *F*: although cells most readily adhered to alveolar parenchyma, airway engraftment was also observed. Scaffolds were stained with hematoxylin and photographed at ×100 (*A* and *F*); ×200 (*B–E*). Scale bars indicate image magnification.

EGF-containing BEGM, and similar trends were found (Fig. 5). When BEC were seeded onto decellularized lung scaffolding in the presence of a neutralizing anti-AREG antibody (1 μg/ml), recellularization was significantly impaired in control cell cultures ($P < 0.01$), and DE-induced recellularization deficits were more pronounced than cultures treated with DE alone ($P < 0.05$), or with a nonspecific isotype antibody (Fig. 4*B*). These data establish the importance of AREG in regulating and enhancing the recellularization/pro-repair process of lung epithelial recovery under normative conditions and following DE exposure.

TACE and EGFR are required for DE-induced AREG production in BEC. We have previously identified important roles for TACE and EGFR activities in the promotion of proinflammatory signaling by DE-stimulated BEC (14, 60). Interestingly, TACE is also involved in the cleavage of pro-AREG from cell membranes allowing full paracrine and autocrine signaling by the soluble AREG ligand, which in turn binds and activates EGFR (5, 9, 12, 46). Thus, we assessed the role of DE-stimulated TACE and EGFR activities in the promotion of AREG release and function in human BEC. As shown in Fig. 6, primary BEC pretreated with the TACE inhibitor TAPI-1

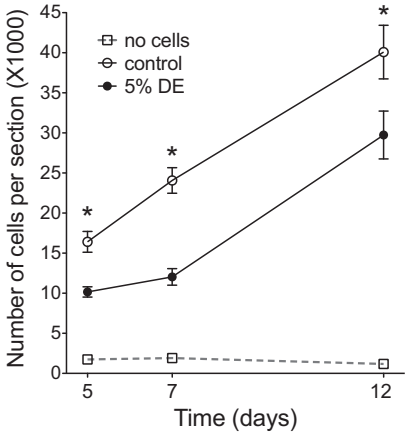

Fig. 3. Dust extract (DE) inhibits recellularization of lung scaffold sections over time. Human lung scaffolds were seeded with 1×10^5 primary bronchial epithelial cells (BEC) and maintained in serum-free BEC growth medium with or without 5% DE for 5, 7, or 12 days. DE exposure significantly diminished the recellularization of BEC within the scaffolds, as quantified by MTT uptake at all time points. Baseline levels of MTT in scaffolds alone were negligible (no cells). Data symbols represent mean ± SE for a minimum of 3 independent experiments with ≥10 scaffolds measured per condition..*$P < 0.05$ vs. 5% DE at corresponding time points.

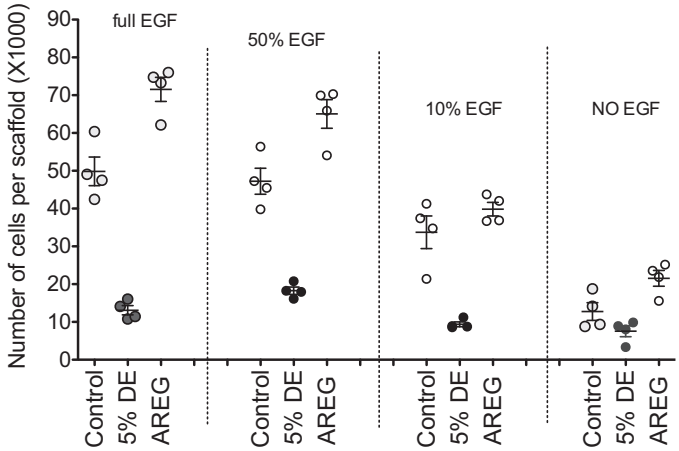

Fig. 5. Restriction of epidermal growth factor (EGF) in growth medium generally suppresses growth of bronchial epithelial cells (BEC) on scaffolds while the effects of dust extract (DE) and amphiregulin (AREG) persist. To assess the affect of EGF as a growth factor in the medium, primary BEC were seeded onto lung scaffold matrices and maintained in BEGM growth medium containing various concentrations of the growth factor EGF ("full" = 2 ng/ml, 50% = 1 ng/ml, 10% = 0.2 ng/ml) or devoid of EGF. Cultures were exposed to 5% DE or AREG (10 ng/ml) continuously for 7 days, and recellularization was assessed. Recolonization of the scaffolds was severely impeded in medium without EGF and markedly hindered in the 10% EGF formulation. Effects of DE and exogenous AREG persisted at all EGF concentrations tested. Each symbol represents one lung scaffold culture.

exhibited a significant and dose-dependent decrease in AREG release following DE treatment compared with cells treated with 5% DE alone (Fig. 6*A*). Similarly, BEC pretreated with the EGFR inhibitors erlotinib (Fig. 6*B*) or AG1478 (Figs. 6*D* and 7) also exhibited significant reductions in DE-stimulated AREG release compared with cells exposed to DE alone. Murine AREG in whole lung tissue homogenates from mice challenged with a single 12.5% DE intranasal instillation was significantly elevated, whereas mice pretreated with erlotinib before DE challenge had significantly reduced AREG levels (Fig. 6*C*). Furthermore, when primary BEC were seeded onto decellularized lung scaffolds in the presence of TAPI-1 or AG1478, the AREG-enhanced recellularization was prevented, and exogenous AREG treatment was ineffective in rescuing the DE-induced recellularization deficit (Fig. 6*D*). These findings demonstrate the necessity for DE-induced TACE and EGFR activation in the production/release of AREG from BEC and illustrate the pro-repair properties of AREG during and after dust-induced inflammatory insult.

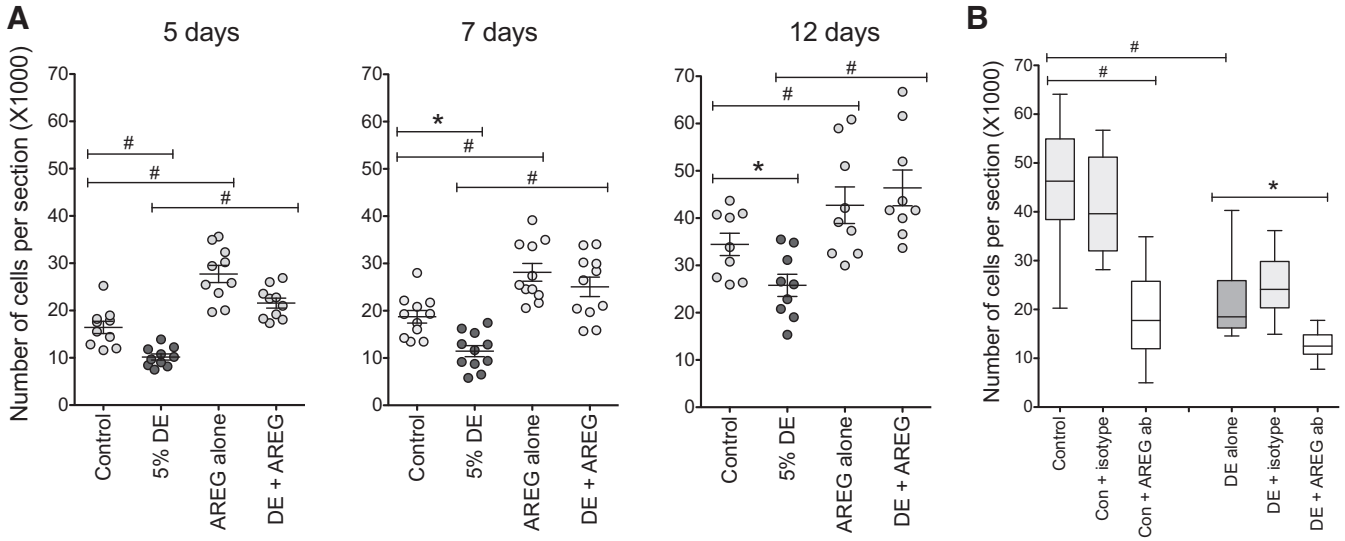

Fig. 4. Amphiregulin (AREG) promotes repopulation of scaffolds, and reverses dust extract (DE)-induced suppression, whereas AREG neutralization impedes recellularization. Lung scaffolds were seeded with primary bronchial epithelial cells as above (Fig. 3). Scaffold cultures were exposed to AREG alone for 2 h, before challenge with 5% DE ± AREG for 5, 7, or 12 days. *A*: at each time point, DE inhibited recellularization, but AREG treatment stimulated recolonization, and reversed the DE-induced deficit. *B*: pretreatment of scaffolds with an AREG-neutralizing antibody significantly inhibited recellularization on both control and DE-exposed cultures by *day 7*, whereas a nontargeting control antibody did not. Experiments were repeated 3 times; each symbol depicts one scaffold section. Box plots represent medians with min. and max. shown. *$P < 0.05$; #$P < 0.01$ for indicated comparisons.

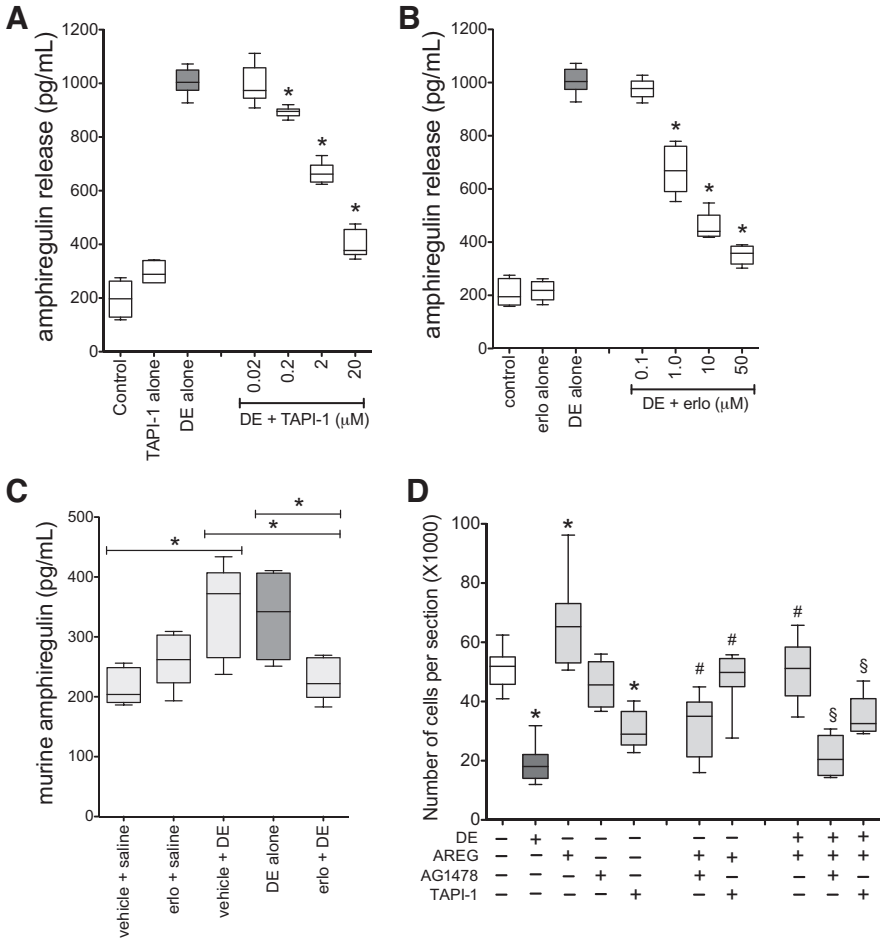

Fig. 6. Amphiregulin release by epithelial cells is partially dependent on TACE and epidermal growth factor receptor (EGFR) function. *A*: the dust extract (DE)-induced augmentation of AREG release by bronchial epithelial cells is dose-responsively inhibited by the TNFα converting enzyme (TACE) inhibitor TAPI-1. *B*: EGFR inhibitor erlotinib also dose-dependently attenuated the DE-mediated response. *C*: lung homogenates from mice ($n = 5$ per condition) pretreated with 2 doses of erlotinib before a single challenge with DE contain significantly less AREG than those from mice exposed to DE alone, or vehicle + DE. *D*: AREG-mediated recellularization of scaffolds in the presence of TAPI-1 or AG1478 was blunted, and AREG was unable to restore the DE-mediated deficit. Boxplots for BEC experiments represent medians for 2 separate experiments (8 technical replicates) and for scaffolding experiments, boxes indicate medians and quartiles for 10 scaffolds per condition. *$P < 0.05$ vs. DE alone (*A–C*). For *D*, *$P < 0.05$ vs. Control; #$P < 0.05$ vs. AREG alone; §$P < 0.05$ vs. DE + AREG.

DHA enhances DE-induced AREG production by human BEC and promotes ex vivo wound repair processes. We have previously found that supplementation with the omega-3 fatty acid DHA reduces the DE-stimulated proinflammatory response of BEC in vitro and lung inflammatory responses to acute DE exposure in vivo (27). To assess the role of DHA on BEC AREG production and pro-repair pathways in vitro, we gave 1 μM DHA as a pretreatment 18 h before or 2 h following a 5% DE challenge on BEAS-2B. Cells pretreated with DHA before DE exposure exhibited reduced AREG production after 6 and 12 h compared with cells treated with DE alone (Fig. 8*A*). Conversely, BEAS-2B posttreated with DHA after 5% DE in vitro exhibited significantly enhanced production of AREG at 12 h following exposure (Fig. 8*B*). A similar effect was likewise observed in human primary BEC (data not shown). Lung tissues from mice on a high-DHA diet for 4 wk before a single inhaled 12.5% DE challenge were assayed for protein expression of AREG. As in the DHA posttreatment in vitro studies, we identified a significant increase in AREG protein levels in DHA-supplemented, DE-challenged mouse lung homogenates (Fig. 9). Additionally, when primary human BEC were seeded onto decellularized lung scaffold matrices, cultures treated with 1 μM DHA alone exhibited significantly enhanced recellularization of lung scaffolds compared with control cultures at *day 7*, and DHA treatment rescued the 5% DE-induced recellularization deficit (Fig. 10*A*). Of interest, in the presence of an anti-AREG neutralizing antibody, DHA-mediated enhancement of BEC recellularization on the scaffold matrices was completely abrogated, and baseline recellularization was also hindered (Fig. 10*B*). These findings implicate a role for DHA in enhancing human BEC pro-repair pathways mediated by an AREG-dependent signaling mechanism.

DISCUSSION

Occupational exposure to inhaled irritants can lead to lung inflammation and injury (1), but it is incompletely understood how inflammatory responses are resolved in some individuals while others develop chronic lung disease. Our findings identify a role for AREG in promoting lung epithelial repair programs following inflammatory organic dust exposure and indicate that n-3 PUFA DHA may enhance these pro-repair pathways during an inflammatory response. Together, these studies identify an omega-3 fatty acid-regulated mechanism modulating AREG-mediated recovery/repair following organic dust-induced airway inflammatory response and injury. Targeting this pathway may lead to improved intervention designs to ameliorate the deleterious effects of occupational dust exposures.

Regulation of AREG production and activity occurs as several different steps. AREG is bound to the plasma membrane in the "pro-AREG" form. In this form, AREG can function as a juxtacrine-signaling molecule (5). Although, release from the membrane, in response to TACE function, for

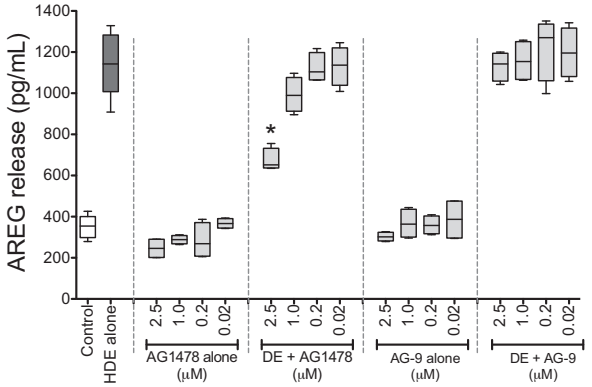

Fig. 7. Tyrphostin AG1478 exhibits specificity for the inhibition of dust extract induced amphiregulin (AREG) release. To assess the effective dose of epithelial growth factor receptor (EGFR) inhibitor tyrphostin AG1478, and to verify its specificity, primary bronchial epithelial cells grown in submerged culture were pretreated with various concentrations of AG1478 or its inactive analog AG-9 for 2 h before being exposed to 5% DE for an additional 24 h. AREG was measured in supernates by ELISA. Neither AG1478 nor AG-9 significantly affected unstimulated AREG release, and AG-9 had no effect on DE-induced AREG at any concentration. Only the 2.5 μM dose of AG1478 significantly dampened the DE response. *$P < 0.05$ vs. DE alone. Boxplots represent medians with minimum and maximum values indicated by whiskers for 2 independent experiments (6 technical replicates per condition).

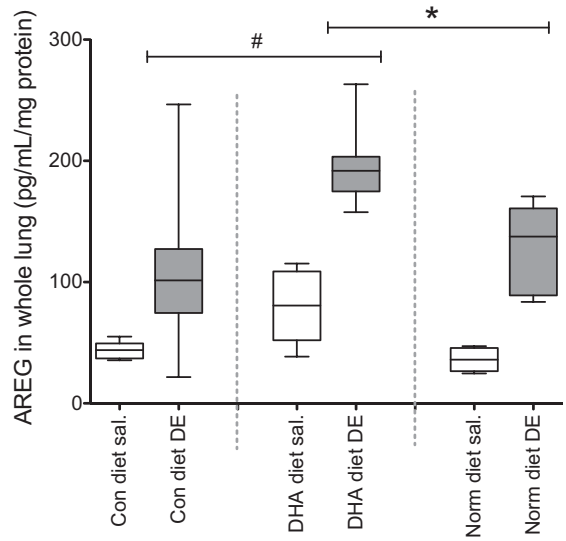

Fig. 9. Effects of docosahexaenoic acid (DHA) dietary supplementation on dust extract (DE)-induced amphiregulin (AREG) in mouse lung. Mice (minimum of 5 mice per condition) were fed a diet supplemented with DHA or with a control diet containing equivalent high-oleic safflower oil (Con), or normal, unmodified rodent chow (Norm) for 4 wk before being exposed to a single dose of 12.5% DE intranasally. At 5 h post-exposure, lung tissues were collected, and AREG was measured in whole lung homogenates. *$P < 0.05$; #$P < 0.01$ for indicated comparisons.

example, is required for full autocrine and paracrine signaling activity (5). Once released, AREG binds to EGFR on surrounding cells to induce pro-proliferative and repair signaling processes while also inducing positive feedback signaling/upregulation of its own transcription (5). Interestingly, both TACE and EGFR are activated in BEC in response to DE (Fig. 11) (14, 59, 60). The activity of TACE is necessary for the release of TNFα and activation of protein kinase C signaling that leads to the release of several inflammatory cytokines induced by DE, including IL-6 and IL-8, from BEC (59, 60). In addition, EGFR is activated within 15 min of BEC stimulation with DE, leading to downstream activation of ERK; inhibition of this pathway also decreases IL-6 and IL-8 release from BEC following DE treatment (14). Despite the appreciation for the role of TACE and EGFR in contributing to the proinflamma-

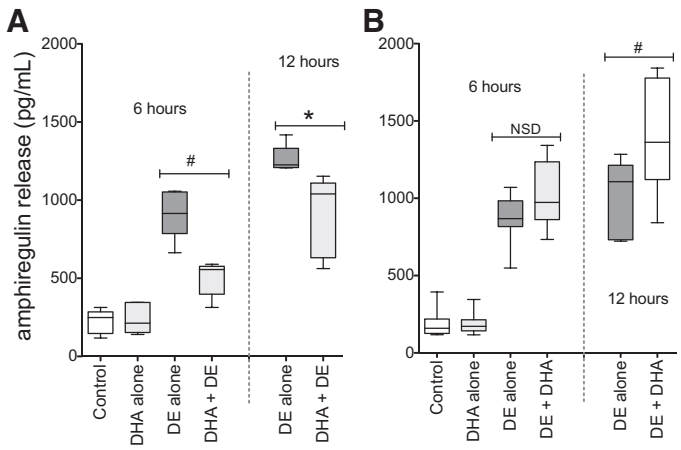

Fig. 8. Timing of polyunsaturated fatty acid (PUFA) treatment affects the inhibitory capacity of docosahexaenoic acid (DHA) in dust extract (DE)-induced amphiregulin (AREG) release. *A*: BEAS-2B cells were given DHA either 18 h before or 2 h after 5% DE challenge, and supernates were assayed for AREG 6 or 12 h thereafter. Pretreatment with DHA significantly reduced DE-induced AREG at both 6 and 12 h. *B*: when given after DE exposure, DHA prompted a significant increase in AREG at 12 h. In parallel experiments using primary bronchial epithelial cells, results were very similar (not shown). Boxes represent median and min/max AREG release for 3 independent experiments (a minimum of 12 technical replicates for each condition). *$P < 0.05$; #$P < 0.01$ vs. DE alone at corresponding time points. NS, no significant difference.

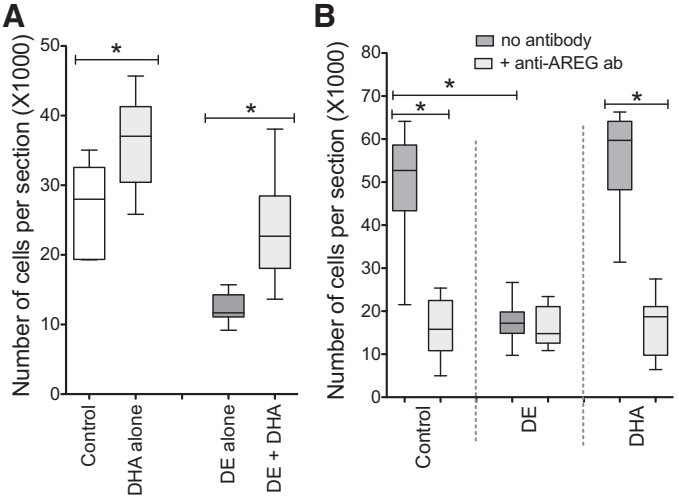

Fig. 10. Augmentation of lung scaffold recellularization by docosahexaenoic acid (DHA) is amphiregulin (AREG) dependent. *A*: primary bronchial epithelial cells were cultured on scaffold matrices in the continuous presence of 5% dust extract (DE) ± 1 μM docosahexaenoic acid (DHA) for 7 days, and MTT uptake was quantified. DHA treatment enhanced cell colonization, and reversed the DE-stimulated decrement. *B*: when scaffold cultures were coincubated with an AREG-neutralizing antibody, both the constitutive recellularization and the stimulatory effect of DHA were abolished, but the effect of DE was unchanged. Boxplots represent the median and min/max for 18 scaffolds per condition. *$P < 0.05$ for indicated comparisons.

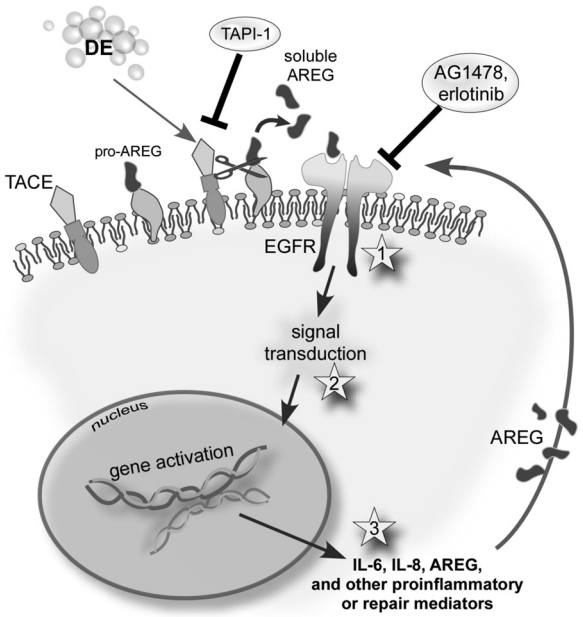

Fig. 11. Schematic summary of amphiregulin (AREG)/epidermal growth factor receptor (EGFR)-mediated actions on dust extract (DE)-exposed bronchial epithelial cells, and proposed docosahexaenoic acid (DHA) interaction points. DE exposure induces TNFα converting enzyme (TACE)-mediated shedding of AREG at the membrane surface, leading to autocrine and paracrine signaling through EGFR binding. As reported in the literature, DHA may modulate DE- and AREG-stimulated effects by altering lipid raft dynamics that regulate EGFR signaling [1] (45), by modifying EGFR-mediated signaling activities through GPR120 and/or peroxisome proliferator-activated receptor (PPAR) binding [2] (10, 15, 25, 29), and by enzyme-mediated production of proresolving lipid effector molecules that enhance repair processes and reduce inflammation [3] (35, 41, 42, 56).

tory response of BEC to DE, there is little known regarding the role of these proteins in repair following this inflammatory stimulus. Interestingly, we have recently identified that AREG levels are increased in lung homogenates during recovery from repetitive DE exposure in vivo (53), implicating this pathway in repair or recovery from DE-induced airway inflammation. Indeed, our current data indicate that, while TACE and EGFR are important components of the inflammatory response of BEC to DE, these proteins are similarly vital to the repair process as well. We found that DE stimulation of BEC leads to a temporal release of AREG in vitro and that stimulation with a single intranasal challenge of DE in mice also leads to a significant temporal increase in lavage AREG levels. AREG release by BEC is dependent on the activities of both TACE and EGFR, likely owing to the combined plasma membrane release via TACE, as well as continued transcriptional upregulation of AREG through EGFR-mediated signaling pathways. These findings suggest that the actions of AREG in BEC during DE responses are largely due to its role as a soluble EGFR ligand. Similarly, when EGFR is inhibited in our in vivo model of acute DE exposure, soluble AREG in whole lung is likewise decreased (Fig. 6*C*).

The regulated production of AREG by BEC in response to DE is of functional significance. Utilizing a decellularized human lung scaffolding model of lung epithelial repair, we identified functional consequences in repair capacity of altered AREG signaling in BEC. We (44) previously identified that DE induces a BEC migration/wound repair defect in cultured monolayers of BEC. Using the matrix scaffolding model of wound repair, we identified a similar deficit in repair capacity when primary BEC were treated with DE, whereby DE-treated cells exhibited reduced capacity to reepithelialize the lung scaffolds. Interestingly, treatment with AREG alone during seeding and reepithelialization led to enhanced recellularization of primary BEC on the lung scaffolds, and providing exogenous AREG in DE-treated cultures rescued the DE-induced repair deficit. Conversely, use of an AREG-neutralizing antibody abrogated these positive effects and enhanced the DE-induced recellularization deficit (Fig. 4*B*), whereas inhibition of TACE and EGFR had similarly negative effects on the ability of BEC to recellularize the lung scaffolds (Fig. 6*D*). Notably, in the context of exogenous AREG treatment, the direct EGFR inhibitor AG1478 was a more potent inhibitor of AREG-mediated recellularization than was the TACE inhibitor, potentially because the availability of exogenous AREG diluted the comparatively modest contribution of TACE-mediated AREG shedding, although TACE inhibition still likely had a negative impact on AREG-mediated positive regulation of its own transcript and protein expression. However, in the context of DE treatment without exogenous AREG, TACE inhibition had a more pronounced effect on BEC recellularization, implicating the important role of TACE in initiating the AREG-mediated signaling cascade via initial pro-AREG cleavage from the plasma membrane. In addition, TAPI-1 treatment alone (in the absence of DE or exogenous AREG) significantly decreased recellularization. We believe this is likely because TACE is responsible for the cleavage of a large number of mediators in various different pathways (40), some of which appear to also be involved in the recellularization process. Together, these data highlight the functional significance of TACE and EGFR activities in not only the proinflammatory response of BEC to DE but in BEC repair activities as well.

Furthermore, these data support the use of the human decellularized lung scaffolding model of wound repair for use in inflammation-related investigations. This technique for modeling airway epithelial wound repair has been similarly used in investigations assessing the regenerative capacities of the lung, including assessment of lung epithelial progenitor populations, mesenchymal cell growth, and modifications to lung extracellular matrix during disease states and its contribution to cell growth and function (7, 16, 51). Our studies further support the utility of this model in investigating the effects of inhaled toxicants on lung cellular functions. Future studies are warranted using this model to assess not only total recellularization capacities of BEC within the context of DE but to specifically address changes in both cellular attachment as well as cellular growth changes associated with DE challenge. We have previously found that DE treatment causes changes in cell adhesion molecule expression, growth and migration (26, 27, 44, 58), supporting these future investigations. Studies specifically addressing attachment and growth of cells in the large-airway extracellular matrix (ECM; e.g., Fig. 2*F*) compared with alveolar ECM could potentially identify differences in repair capacity within these different areas of the lung. These studies could provide valuable information regarding how different areas of the lung are impacted by DE exposure, warranting further investigation.

In addition to our findings for the role of AREG in regulating epithelial lung repair processes following agri-

cultural dust exposures, we have identified a role for the omega-3 PUFA DHA in modulating this pro-repair pathway. Omega-3 fatty acids are recognized for their protective effects against numerous inflammatory diseases, including cardiovascular and numerous lung diseases (17, 25, 55). During an inflammatory process, PUFA play several key roles in controlling inflammatory physiology. In the initiation of inflammation, PUFA are cleaved from the plasma membrane (the primary site of storage/function of PUFA in a cell) and acted upon by cyclooxygenase, lipoxygenase, or cytochrome *P*-450 enzymes to produce eicosanoid lipid mediators, including leukotrienes, thromboxanes, and prostaglandins, the majority of these inflammatory mediators being derived from the omega-6 fatty acid arachidonic acid (32, 35, 55). Additionally, several anti-inflammatory and proresolving mediators are produced from PUFA, including omega-3 fatty acid-derived resolvins, protectins, and maresins, and arachidonic acid-derived lipoxins (35, 41, 42, 56). In this way, PUFA contribute to both the initiation and resolution phases of inflammatory processes. In addition, free PUFA can bind to G protein-coupled receptors on the surface of cell membranes (e.g., free fatty acid receptor 4; FFAR4/GPR120), or bind to nuclear receptors (e.g., peroxisome proliferator-activated receptors; PPAR) to modulate cell signaling pathways and inflammatory responses (10, 15, 25, 29). DHA in particular, due to its highly unsaturated structure, can alter membrane fluidity, influencing membrane lipid raft dynamics and allowing for increased mobility in the membrane (45). This function leads to surface receptor signaling changes and thereby influences downstream cell signaling events. Indeed, PUFA supplementation has been shown to influence the signaling activities of EGFR through several activities. Signaling by PUFA through the FFAR4/GPR120/GPR120R4 receptor for medium and long-chain fatty acids, including DHA, leads to transactivation of the EGFR (24, 57), while limiting inflammatory signaling via inhibition of TAK1 phosphorylation (19, 30). Meanwhile, incorporation of DHA into the plasma membrane also alters EGFR signaling through modulation of EGFR-rich lipid rafts and alteration of downstream EGFR signaling events with cofactors (37, 47).

Within this context, our findings provide further evidence linking DHA to EGFR signaling regulation and attribute this pathway to wound repair processes in BEC. In our in vitro investigations, we have found that when DHA is given as a pretreatment to DE exposure, AREG release is decreased (Fig. 8*A*). This is consistent with our previously reported findings of reduced overall BEC inflammatory responsiveness to DE in DHA-pretreated cells (27), that perhaps also includes a reduction in DE-stimulated AREG release. Interestingly, when DHA is given one hour after DE stimulation of BEC (after an inflammatory response has been initiated), we find that DHA-treated cells release significantly more AREG in the presence of DE than cells stimulated with DE alone (Fig. 8*B*). These data compellingly suggest that cellular utilization of the free fatty acid DHA differs during inflammatory stimulation compared with noninflammatory/normative conditions. We hypothesize that the initiation of an inflammatory response preferentially directs the use of DHA toward stimulation of pro-repair pathways, based on increased AREG production that we found under these conditions. Concordant with this hypothesis, utilizing a lung scaffolding model of repair, we further observed that DHA treatment alone could enhance BEC recellularization of the scaffolds while partially reversing the DE-induced recellularization deficit. Interestingly, these recellularization-enhancing effects of DHA on BEC were AREG dependent, as cotreating the BEC with an AREG-neutralizing antibody along with DHA completely abrogated the DHA-mediated recellularization enhancement on the scaffolds (Fig. 10*B*). Consistent with the proposed role of DHA in promoting pro-repair pathways during DE-induced inflammation, AREG protein levels were significantly increased in lung tissues of mice fed a high-DHA diet for 4 wk before a single DE challenge. These observations provide compelling evidence to suggest a role for DHA in influencing AREG-mediated EGFR signaling in inflammatory and wound repair processes.

Taken together, these findings identify an important role for AREG in mediating repair processes in BEC, including enhancing repair capacities of BEC in the context of agricultural dust-induced inflammation and injury. Furthermore, these findings indicate that the omega-3 fatty acid DHA plays a regulatory role in controlling AREG production and wound repair processes in BEC during DE-induced inflammatory processes. These results confirm the value of future investigations addressing the mechanistic relationship between DHA intake, AREG production, and EGFR signaling in the context of lung inflammation and injury. By better understanding the inflammation and wound repair physiology associated with agricultural dust-induced lung inflammation, new preventive or therapeutic modalities may be devised to reduced the negative inflammatory consequences of these exposures.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

T.M.N., K.L.B., and D.J.R. conceived and designed research; T.M.N., A.J.H., K.L.B., and D.M.K. performed experiments; T.M.N., A.J.H., K.L.B., M.L.T., and C.S.W. analyzed data; T.M.N., K.L.B., M.L.T., and D.J.R. interpreted results of experiments; T.M.N., A.J.H., and D.M.K. prepared figures; T.M.N., A.J.H., D.M.K., and D.J.R. drafted manuscript; T.M.N., A.J.H., K.L.B., D.M.K., M.L.T., C.S.W., and D.J.R. edited and revised manuscript; T.M.N., A.J.H., K.L.B., D.M.K., M.L.T., C.S.W., and D.J.R. approved final version of manuscript.

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