Classically Activated Macrophages Protect against Lipopolysaccharide-induced Acute Lung Injury by Expressing Amphiregulin in Mice

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ABSTRACT

Background: Alveolar macrophages (AMs) activated into M1 phenotype are involved in the development of lipopolysaccharide-induced acute lung injury (ALI). However, whether AMs express amphiregulin and what roles amphiregulin plays in lipopolysaccharide-induced ALI remain poorly understood.

Methods: Acute lung injury was induced by intratracheal instillation of lipopolysaccharide in male C57BL/6 mice. Lung injury scores, level of protein, and level of neutrophils in bronchial alveolar lavage fluid of lipopolysaccharide-induced ALI mice were compared with those in mice challenged with recombinant exogenous amphiregulin and antiamphiregulin antibody. Amphiregulin expression in macrophages and neutrophils in bronchial alveolar lavage fluid of lipopolysaccharide-induced ALI mice was determined by using immunofluorescence technique and further detected in M0, M1, and M2 phenotypes of both peritoneal macrophages and AMs. The effect of amphiregulin on apoptosis of MLE12 cells and activation of epithelial growth factor receptor-AKT pathway were, respectively, examined by using flow cytometry and western blotting.

Results: Alveolar macrophages were found to highly express amphiregulin in ALI mice. Amphiregulin neutralization aggravated, whereas recombinant exogenous amphiregulin attenuated lipopolysaccharide-induced ALI in mice (n = 6). In cultured AMs and peritoneal macrophages, amphiregulin was mainly generated by M1, rather than M0 or M2 phenotype (n = 5). Apoptosis ratio of lipopolysaccharide-challenged MLE12 cells was significantly reduced by recombinant exogenous amphiregulin from 16.60 ± 1.82 to 9.47 ± 1.67% (n = 5) but significantly increased from 17.45 ± 1.13 to 21.67 ± 1.10% (n = 5) after stimulation with supernatant of M1-polarized AM media conditioned with amphiregulin-neutrolizing antibody. Western blotting revealed that amphiregulin activated epithelial growth factor receptor and AKT in the lung tissues and MLE12 cells (n = 5).

Conclusions: Different from the common notion that classically activated AMs have just a detrimental effect on the lung tissues, the results of this study showed that classically activated AMs also exerted a protective effect on the lung tissues by producing high-level amphiregulin in lipopolysaccharide-induced ALI. **(Anesthesiology 2016; 124:1086-99)**

ACUTE lung injury (ALI) is a serious respiratory condition with high mortality and is ascribed to the disrupted epithelial and endothelial barrier and infiltration of neutrophils and protein-rich fluid into the alveolar space.[1] Although neutrophils within the lung play an important part in the pathogenesis of ALI,[2,3] mounting evidence indicates that macrophages also contribute to the modulation of inflammatory responses, including the initiation and resolution of these responses.[4] On the basis of the patterns of gene and protein expressions, in combination with the different roles they play in host defense, macrophages fall into two categories, *i.e.*, classically activated (M1) and alternatively activated (M2) macrophages. Macrophages, when stimulated with lipopolysaccharide, can be activated into M1 phenotype, which is characterized by high level of proinflammatory cytokines and reactive oxygen species, thereby result in damage of adjacent tissues.[5] Conversely, M2 macrophages,

What We Already Know about This Topic

- Immune responses are critical to protect hosts from pathogens. An important aspect of this immune response is also to minimize the harmful effects of inflammation on tissue and to promote tissue healing and repair.
- Amphiregulin is a protein that plays important roles in tissue tolerance to infection. Its role in acute lung injury is not clear.
- In an experimental model of acute lung injury, the role of amphiregulin was evaluated.

What This Article Tells Us That Is New

- Amphiregulin was expressed in alveolar macrophages after acute lung injury.
- Exogenous amphiregulin protected, whereas amphiregulin antibodies exacerbated lung injury.
- The results are consistent with the notion up-regulation of amphiregulin in activated alveolar macrophages can exert a protective effect on the lung tissue in a model of acute lung injury.

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induced by interleukin-4 and/or interleukin-13, release high-level arginase-1 (Arg-1), Fizz1, and Ym1, thus promoting inflammation resolution and tissue repair.[6] Previous studies exhibited that alveolar macrophages (AMs) are activated into M1 phenotype at the early phase of ALI,[4,7] and depletion of macrophages from lung tissues[8,9] or inhibition of M1 products, such as nitric oxide and interleukin-1β,[9,10] protect against pulmonary injury in some animal models. These findings suggest that M1 macrophages may be one of the initiators of the acute phase of ALI.

Amphiregulin, a ligand of epithelial growth factor receptor (EGFR), was reported to be up-regulated in a ventilator-associated ALI model.[11] Amphiregulin expression was also found to be increased in damaged lung tissues in patients with chronic obstructive pulmonary disease and asthma.[12–15] Even smoking and urban fine particulate materials can increase amphiregulin expression in the lung cells.[16–18] Interestingly, amphiregulin was shown to promote the proliferation of epithelial cells and modulate the gene expression of proinflammatory cytokines.[18,19] But little is known about the expression pattern and role of amphiregulin in lipopolysaccharide-induced ALI.

Given that lung epithelial damage plays an important role in the pathogenesis of ALI and that lipopolysaccharide can stimulate Kupffer cells (liver macrophages) secreting amphiregulin,[20] we were led to hypothesize that amphiregulin may be produced by AMs when challenged with lipopolysaccharide, thereby improving epithelial barrier function and further attenuating lipopolysaccharide-induced ALI. To test this hypothesis, in this study, we determined the level of amphiregulin in classically activated AMs *in vitro* and in lung tissues of lipopolysaccharide-induced ALI *in vivo* and observed protein exudation, infiltration of inflammatory cells, and other indicators of lung functions, with an attempt to understand the protecting role of amphiregulin in lipopolysaccharide-induced ALI.

Materials and Methods

Animals

Male C57BL/6 mice of 6 to 8 weeks old, weighing 20 to 25 g, were obtained from Wuhan University Laboratory Animal Center and were maintained under specific pathogen free conditions at a room temperature of 23° ± 1°C (50 ± 10% relative humidity) and on a 12:12-h light/dark cycle in the Experimental Animal Center, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. All experimental protocols were carried out in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and were approved by the Committee of Experimental Animals of Huazhong University of Science and Technology.

Experimental Procedures

Mice were tagged and randomly allocated to each group before any treatment. Mice were anesthetized intraperitoneally with sodium pentobarbital (80 mg/kg; Sigma, USA) and then orotracheally intubated. To induce ALI, mice were instilled intratracheally with *Escherichia coli* serotype lipopolysaccharide 055:B5 (2 ml/kg; Sigma), and a bolus of 3 × 0.6 ml air was delivered to ensure that all instilled solutions reached the distal lung. Mice in the control group were treated intratracheally with phosphate-buffered saline (PBS; 2 ml/kg, Thermo Scientific, USA). To investigate the role of endogenous amphiregulin, mice were injected intraperitoneally with amphiregulin-blocking antibody (amphiregulin antibodies; 50 μg per mouse; R&D Systems, USA) 30 min before instillation of 1 mg/kg lipopolysaccharide. To determine the effect of exogenous amphiregulin on lipopolysaccharide-induced ALI, mice were administrated intraperitoneally with recombinant mouse amphiregulin (rmAreg; 5 μg per mouse; R&D Systems), and 30 min later, they were challenged with 3 mg/kg lipopolysaccharide as we previously reported.[21] Mice were killed 6 and 24 h after lipopolysaccharide or PBS challenge. To examine amphiregulin expression in this model, mice were intratracheally instilled with lipopolysaccharide (3 mg/kg) and properly killed at 0, 3, 6, 12, and 24 h.

Histologic Examination of Lung Tissues

The left lungs were fixed in 4% paraformaldehyde and embedded in paraffin. Lung sections (5 μm) were stained with hematoxylin and eosin (HE) and examined under a light microscope. Lung injury was evaluated and scored by an investigator blinded to experimental grouping against a recently released criterion, and the lung damage was assessed on a two-point scale, ranging from 0 to 1.[22]

Bronchial Alveolar Lavage Fluid Analysis

To collect bronchial alveolar lavage fluid (BALF), 1 ml PBS was instilled into mouse lung and slowly withdrawn. The procedure was repeated three times for each mouse. Cells were collected after centrifugation at 400*g* for 10 min and counted on a hemocytometer. For differential cell counting, cells were stained with phycoerythrin-conjugated anti-mouse Gr-1 (eBioscience, USA) for 45 min and flow cytometrically analyzed by using a Fluorescence Activated Cell Sorter machine (BD Biosciences, USA). Neutrophils were calculated as the total cell number in BALF multiplied by the percentage of $Gr\text{-}1^{+}F4/80^{-}$ cells. The supernatants of centrifuged BALF were used to measure the protein concentration by using a Pierce bicinchonininc acid protein assay kit (Thermo Scientific) or stored at −80°C for later cytokine and IgM analysis.

Giemsa Staining of Cells in BALF

To differentiate cells in BALF, cells in BALF were first removed by centrifugation (400*g*, 4°C, 6 min), counted on a hemocytometer, stained with Giemsa fluids after centrifugation at 400*g* for 6 min and smeared onto a glass slide.

To distinguish the attached cells and unattached cells in BALF of mice challenged with lipopolysaccharide, cells in

BALF were collected and cultured in a 24-well plate. Forty minutes later, the unattached cells in the supernatant were smeared onto a glass slide after centrifugation for 6 min at 400*g*. Both attached and unattached cells were stained with Giemsa fluid for morphologic identification (n = 5 for each group).

Quantitative Real-time PCR

Ten milligrams of lung tissues from mice in different groups were harvested, and total RNA was extracted by using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. Five hundred nanograms of total RNA was isolated to generate cDNA in a reverse transcription reaction by using PrimeScript RT Master Mix (TaKaRa, China). Quantitative reverse transcription polymerase chain reaction (PCR) for amphiregulin (specific primers: forward 5′-AATGAGAACTCCGCTGCTAC-3′, reverse 5′-CCCCTGTGGAGAGTTCACTG-3′) and β-actin (forward 5′-TGTCCACCTTCCAGCAGATGT-3′, reverse 5′-AGCTCAGTAACAGTCCGCCTAGA-3′) was conducted using a QuantiFast SYBR Green PCR kit (Qiagen, Germany) on a StepOne Real-time PCR System (Applied Biosystems, USA). Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method, with β-actin serving as an internal control.

Immunofluorescence

After deparaffinization, rehydration, and antigen retrieval, lung sections were blocked with 2% donkey serum for 2 h and then incubated with rabbit monoclonal anti-Iba1 (1:100, Wako, Japan) and goat polyclonal antiamphiregulin (10 μg/ml, R&D Systems) overnight at 4°C. The sections were washed and incubated with Dylight 488-conjugated donkey anti-rabbit (1:100; Abbkine, USA) and Dylight 549-conjugated donkey anti-goat (1:300, Abbkine) antibody for 1 h and then stained with dihydrochloride (DAPI) (1μg/ml) for 10 min for nuclei labeling. Sections were observed under an Olympus fluorescence microscope (Olympus, Germany) using the Cellsense software package (Olympus).

To evaluate which type of cells (neutrophils or macrophages) in the BALF secret amphiregulin, the cells in the supernatant centrifuged on a glass slide and the attached cells were stained with rabbit polyclonal antiamphiregulin (1:50, Santa Cruz Biotechnology, USA) overnight at 4°C and then incubated with Dylight 549-conjugated goat anti-rabbit secondary antibody (1:200, Abbkine) for 1 h, followed by stained with DAPI (1 μg/ml) for 10 min for nuclei labeling.

Liposome-encapsulated Clodronate Treatment

Alveolar macrophage was depleted by using liposome-encapsulated clodronate (Department of Molecular Cell Biology, Free University Medical Center, Amsterdam, the Netherlands).[9,23] Briefly, mice were instilled intratracheally with 100 μl liposome-encapsulated clodronate (5 mg/ml). Mice in control group were administrated PBS-containing liposomes. Forty-eight hours after liposome treatment, mice were stimulated intratracheally with lipopolysaccharide (3 mg/kg) for 6 h. Cell-free BALF was collected for determination of amphiregulin level.

Cell Culture and Stimulation

Alveolar macrophages were obtained as described previously.[24] Briefly, cells from the lavage fluid for each mouse were pooled, centrifuged at 400*g* for 10 min at 4°C, and resuspended in Roswell Park Memorial Institute 1640 medium (Hyclone) containing 10% fetal bovine serum (FBS; Life Technologies, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Hyclone). After incubation at 37°C and in 5% CO_2 overnight, cells were washed with PBS to remove unattached cells. For M1 and M2 polarization, AMs were seeded into 24-well plates (2×10^5 cells/well) and incubated for 24 h with lipopolysaccharide (1 μg/ml) and interleukin-4 (20 ng/ml; PeproTech, USA), respectively. AMs treated with PBS were designated as M0. The supernatants were collected for amphiregulin detection. To obtain the conditioned medium of M0 and M1, AMs were incubated with PBS and lipopolysaccharide (1 μg/ml), respectively, in a 6-well plate (1×10^6 cells/well) for 24 h. The supernatants were collected and designated as M0-CM and M1-CM. M1-CM was pretreated with amphiregulin antibodies (3 μg/ml) or IgG (3 μg/ml) at 37°C for 3 h.

Peritoneal macrophages (PMs) were isolated as described elsewhere.[25] Briefly, the abdominal cavity of the animals was lavaged with 5 ml PBS supplemented with 5% FBS, and then the recovered buffer was centrifuged at 400*g* for 10 min. Cells were resuspended in Roswell Park Memorial Institute 1640 medium (Hyclone) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Hyclone) and were seeded into 24-well plates (2×10^5 cells/well). Cells were incubated at 37°C and in 5% CO_2 overnight and then washed with PBS to remove unattached cells. PMs were treated with PBS or lipopolysaccharide (1 μg/ml) and interleukin-4 (20 ng/ml) separately for 4 or 24 h.

The murine lung epithelial cell line (MLE12 cells) was purchased from American Type Culture Collection (USA). MLE12 cells were cultured in Dulbecco's Modified Eagle's Medium:Nutrient Mixture F-12 (Dulbecco's Modified Eagle's Medium/F12, Hyclone) with 10% FBS at 37°C and in 5% CO_2. For western blotting, MLE12 cells were seeded into 6-well plates (1×10^6 cells/well) and stimulated with rmAreg (50 ng/ml) at indicated time points or with M0-CM and M1-CM containing IgG or amphiregulin antibodies for 10 min.

Enzyme-linked Immunosorbent Assays

Enzyme-linked immunosorbent assays were carried out using commercially available kits to measure concentrations of amphiregulin (R&D systems), IgM (Plymouth Meeting, USA), tumor necrosis factor (TNF)-α, and interleukin-6 (Neobioscience Technology, China) by following the manufacturer's instructions.

Cell Apoptosis Analysis

MLE12 cells were seeded into 12-well plates at 2×10^5 cells/well and allowed to grow to 70% confluence. Programmed cell death was induced by incubation of the cells with lipopolysaccharide (100 μg/ml) for 24 h. To examine the effect of amphiregulin on apoptosis, MLE12 cells were pretreated with indicated dosage of amphiregulin before lipopolysaccharide stimulation. To examine the effect of amphiregulin secreted by M1 AMs, MLE12 cells were preincubated with M1-CM containing IgG or amphiregulin antibodies for 30 min and then stimulated with lipopolysaccharide (100 μg/ml) for 24 h. The challenged MLE12 cells were harvested, stained with annexin-V-fluorescein isothiocyanate and PI (BD Biosciences), and analyzed by the Fluorescence Activated Cell Sorter flow cytometry (BD Biosciences). In this experiment, a nonstained control tube and a single color tube were used to control the gate according to the manufacturer's constructions.

Western Blotting

Total protein from lung tissues or cells was extracted by using a commercially available kit (KGP250; Nanjing Keygen Biotech Co. Ltd., China). Protein concentrations were determined by the bicinchoninic acid protein assay (Thermo Scientific). Equal amount of protein extract was separated on sodium dodecyl sulfate-polyacrylamide gels and then transferred onto a polyvinylidene difluoride membrane (Millipore, USA). The membranes were blocked with 5% nonfat milk in Tris-buffered saline and Tween 20 (0.1% Tween in Tris-buffered saline) for 1 h at room temperature and then incubated overnight at 4°C with rabbit monoclonal anti-EGFR (1:1,000; Abcam, UK), rabbit monoclonal anti-p-EGFR-Tyr 1068 (1:1,000; Cell Signaling Technology, USA), rabbit monoclonal anti-AKT (1:1,000, Cell Signaling Technology), rabbit monoclonal anti-p-AKT-Thr 308 (1:1000, Cell Signaling Technology) or rabbit polyclonal anti-β-actin (1:2,000; Santa Cruz Biotechnology), and then with horse radish peroxidase–conjugated goat anti-rabbit IgG antibody (1:5,000; Proteintech Group, USA). The immunoreactive bands were visualized and photographed by using an EC3 Imaging System (UVP Inc., USA).

Statistical Analysis

Data were expressed as mean ± SD. Gaussian distribution of the data was assessed by using the d'Agostino and Pearson omnibus normality test, and the equalities of variances were examined by using the F test. Student's *t* test was employed for pairwise comparisons. One-way ANOVA followed by Bonferroni *post hoc* comparison test was used for multiple comparisons. The lung injury scores were compared by utilizing nonparametric Mann-Whitney U test. The sample size was based on our previous research work.[21] All statistical analyses were performed by using the GraphPad Prism software package (version 5 for Windows, GraphPad Software. Inc., USA), and statistical significance was accepted at two tailed $P < 0.05$. Four mice died after intratracheal intubation, and the data were lost.

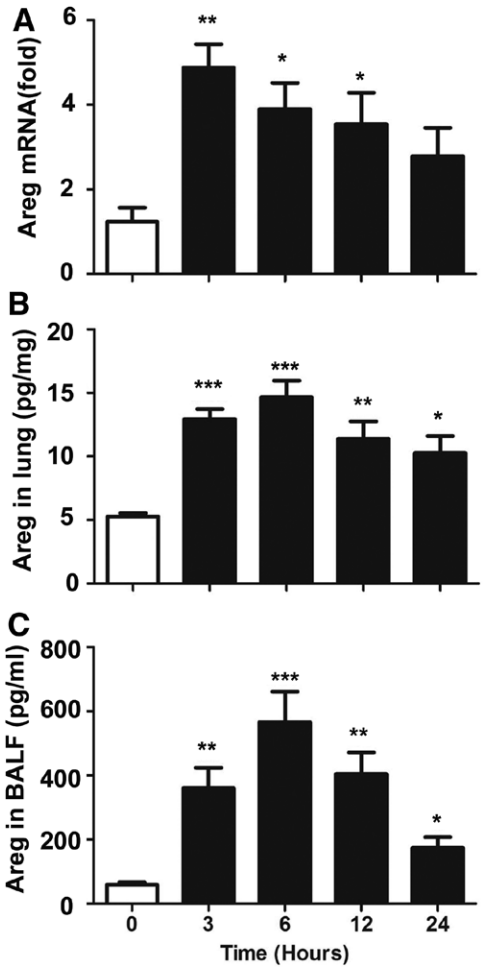

Fig. 1. Amphiregulin (Areg) expression in lung tissues is up-regulated in lipopolysaccharide-induced acute lung injury. Mice were instilled intratracheally with lipopolysaccharide (3 mg/kg) and killed at indicated time points. (*A*) Quantitative reverse transcription polymerase chain reaction data for relative gene expression of Areg in the lung tissues. Gene expression was normalized to β-actin expression levels, and the mean value for mice in 0 h group was set to 1. Data are shown as mean ± SD of five independent samples. Concentrations of Areg in lung tissue homogenate (*B*) and bronchial alveolar lavage fluid (BALF; *C*) were measured by enzyme-linked immunosorbent assay. Data were shown as mean ± SD from five independent samples. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ *versus* 0 h; one-way ANOVA Bonferroni posttest.

Results

Amphiregulin Expression in Lung Tissue in Lipopolysaccharide-induced ALI

In the mouse model of lipopolysaccharide-induced ALI, amphiregulin gene expression (fig. 1A) in lung tissues was significantly induced between 3 and 12 h after lipopolysaccharide challenge. The levels of amphiregulin protein in both lung tissues (fig. 1B) and BALF (fig. 1C) were significantly increased 3 h after intratracheal lipopolysaccharide challenge, reached a peak at 6 h, and declined thereafter, but staying at high level throughout the experiment.

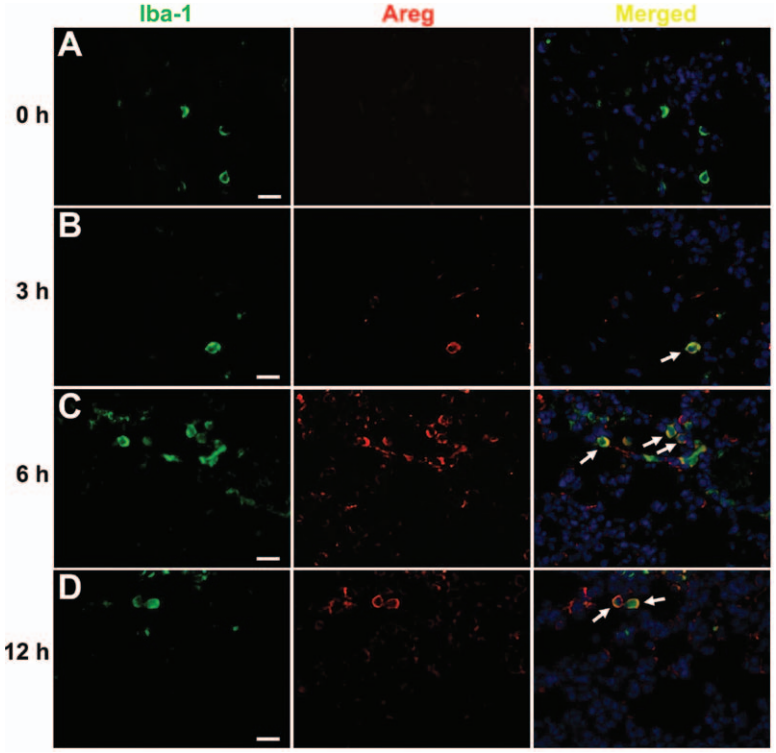

Fig. 2. Amphiregulin (Areg) is mainly expressed by alveolar macrophages in lipopolysaccharide-induced acute lung injury (ALI) in mice. (*A–D*) Double-staining immunofluorescence of Iba-1 and Areg on lung sections obtained from ALI-bearing mice at 0, 3, 6, and 12 h after lipopolysaccharide (3 mg/kg) challenge. *Scale bar* = 20 μm. *White arrows* indicate the Iba-1$^+$/Areg$^+$ cells.

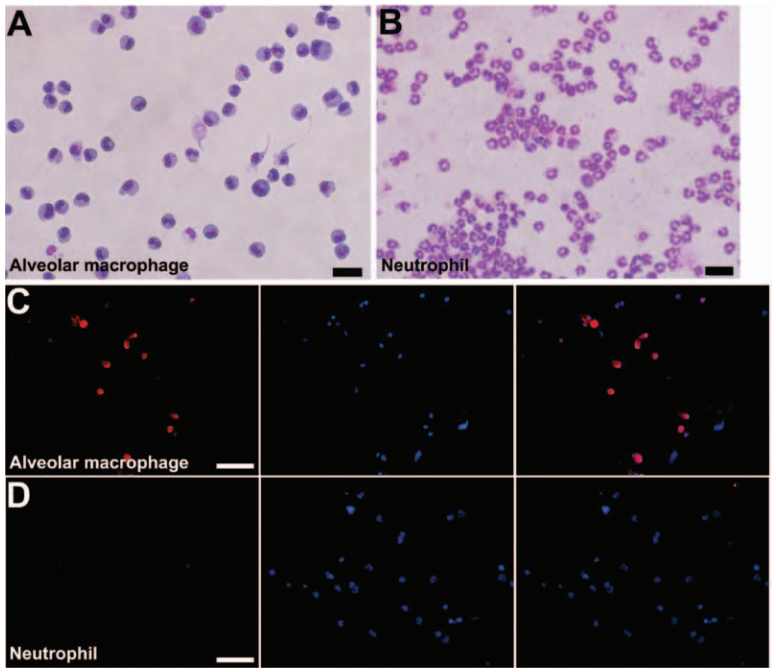

Fig. 3. Amphiregulin expression in alveolar macrophages and neutrophils in bronchial alveolar lavage fluid (BALF) collected from lipopolysaccharide-induced acute lung injury mice. Mice were challenged with lipopolysaccharide (3 mg/kg) for 6 h. BALF was equally divided and placed into 2 wells in a 24-well plate. Forty minutes later, cells were washed with phosphate-buffered saline. The cells in the supernatant were smeared on a glass after centrifugation for 6 min at 400*g*. All the cells, including the cells that attached to the substrate, were separately stained with Giemsa fluids (n = 5 for each group), shown in (*A*) for attached cells and (*B*) for cells in the supernatant. At the same time, cells adherent to the substrate (*C*) or in washed solution (*D*) were double stained with immunofluorescence of amphiregulin and dihydrochloride (n = 5). (*A*, *B*) *Scale bar* = 20 μm and (*C*, *D*) *Scale bar* = 100 μm.

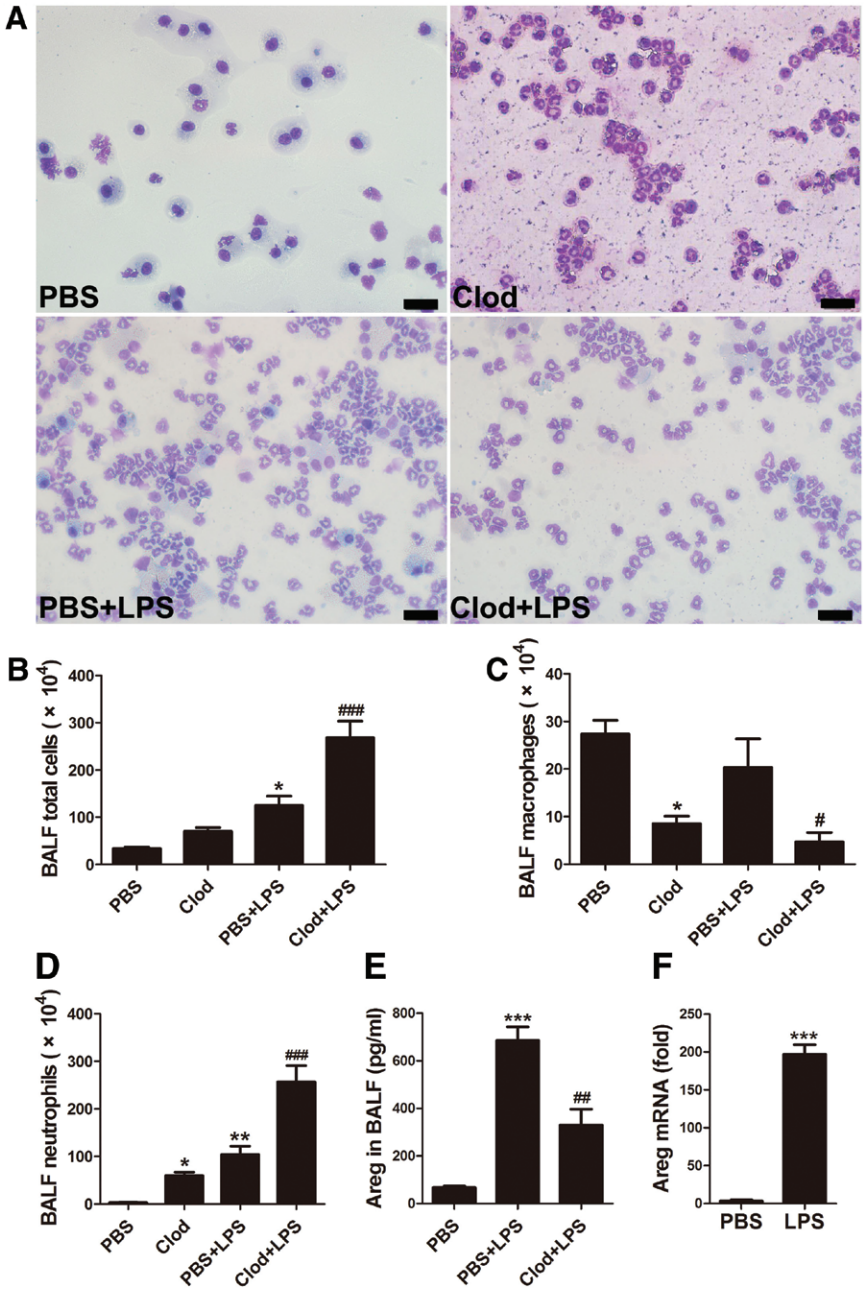

Fig. 4. The effect of liposome-encapsulated clodronate (Clod) on number of cells in bronchial alveolar lavage fluid (BALF) of mice without or with lipopolysaccharide (LPS) challenge for 6 h. Mice were treated intratracheally with phosphate-buffered saline (PBS) liposomes for 48 h, followed by intratracheal treatment for 6 h with PBS (PBS group); treated intratracheally with Clod liposomes for 48 h, followed by intratracheal stimulation for 6 h with PBS (Clod group); treated intratracheally. with PBS liposomes for 48 h, followed by intratracheal stimulation for 6 h with LPS (PBS + LPS group); or treated intratracheally with Clod liposomes for 48 h, followed by intratracheal challenge for 6 h with LPS (Clod + LPS group). The dose of LPS was 3 mg/kg. Giemsa staining of cells in BALF isolated from mice (*A*). *Scale bar* = 20 μm. Total BALF cells were measured using a hematocytometer (*B*), and the numbers of macrophages (*C*) and neutrophils (*D*) were calculated as the total BALF cell number multiply their percentage got from the Giemsa staining. (*E*) *In vivo* depletion of alveolar macrophages (AMs) decreased LPS-induced amphiregulin (Areg) release in mouse lung. Mice were treated intratracheally with Clod or PBS liposomes for 48 h, followed by intratracheal stimulation with LPS (3 mg/kg) for 6 h. Concentrations of Areg in BALF were measured by enzyme-linked immunosorbent assay. Data are shown as mean ± SD (n = 5). Data are shown as mean ± SD. * $P < 0.05$ and ** $P < 0.01$ *versus* PBS; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ *versus* PBS + LPS; one-way ANOVA Bonferroni posttest. (*F*) Quantitative reverse transcription polymerase chain reaction data for Areg expression in AMs isolated from mice before LPS challenge and at 3 h after LPS challenge. Data, normalized to β-actin and relative to expression levels in PBS group, are shown as mean ± SD (n = 6). *** $P < 0.001$ *versus* PBS; Student's *t* test.

Amphiregulin Expression in AMs in Lung Tissues of Mice with ALI

To confirm whether macrophages express amphiregulin when stimulated with lipopolysaccharide, we first detected amphiregulin expression in macrophages by using immunofluorescent staining. *In vivo* results showed that only green immunofluorescence, an indicator of Iba-1 antibody, was observed, whereas no obvious amphiregulin protein expression was found in the control group (fig. 2A, 0 h). In contrast, immunofluorescence indicating amphiregulin protein expression was enhanced conspicuously in Iba^+ cells at 3 h (fig. 2B) and stayed at a high level of 6 (fig. 2C) and 12 h (fig. 2D) after lipopolysaccharide stimulation.

To confirm that amphiregulin was expressed by macrophages rather than neutrophils in lipopolysaccharide-induced ALI, we, by using Giemsa staining, demonstrated that the attached cells in BALF were mainly macrophages (fig. 3A) and that the unattached cells in the supernatant were, for the most part, neutrophils (fig. 3B). Then we stained the attached cells and the unattached cells separately on glass slides with amphiregulin antibody and DAPI. Our result showed that the attached cells (macrophages) secreted amphiregulin (fig. 3C). Conversely, unattached cells (neutrophils) did not express amphiregulin (fig. 3D).

To further confirm that activated AMs produce amphiregulin, we determined amphiregulin expression in the mice depleted of AMs by intratracheal administration of liposome-encapsulated clodronate as reported previously.[9,23] We found that liposome-encapsulated clodronate challenge reduced the number of macrophages and increased the number of neutrophils in BALF irrespective of exposure to lipopolysaccharide (fig. 4, A–D). At the same time, AM depletion significantly lowered amphiregulin protein production in the BALF of lipopolysaccharide-challenged mice when compared with their liposome-PBS–treated counterpart (fig. 4E). But lipopolysaccharide challenge significantly increased the expression of amphiregulin mRNA compared with control group (fig. 4F).

Amphiregulin Expression in Different Phenotypes of AMs and PMs

To evaluate the amphiregulin expression among different phenotypes of macrophages, we then detected amphiregulin expression in both alveolar and PMs in M1 and M2 phenotypes. Macrophages were stimulated with lipopolysaccharide and interleukin-4 for M1 and M2 polarization, respectively. Cells in control group (M0) were treated with PBS. As shown in fig. 5, A and B, amphiregulin was highly expressed at both mRNA and protein levels exclusively in M1-polarized AMs, whereas amphiregulin expression was very low in M0 and M2 phenotype. Similar results were observed in the PMs (fig. 5, C and D).

Effect of Amphiregulin Antibody on Lipopolysaccharide-induced ALI in Mice

To investigate the role of amphiregulin in ALI, mice were given amphiregulin Abs to neutralize amphiregulin 30 min before lipopolysaccharide (1 mg/kg) challenge. IgG was used as an isotype control. HE staining (fig. 6A) showed that treatment with amphiregulin antibodies alone did not cause

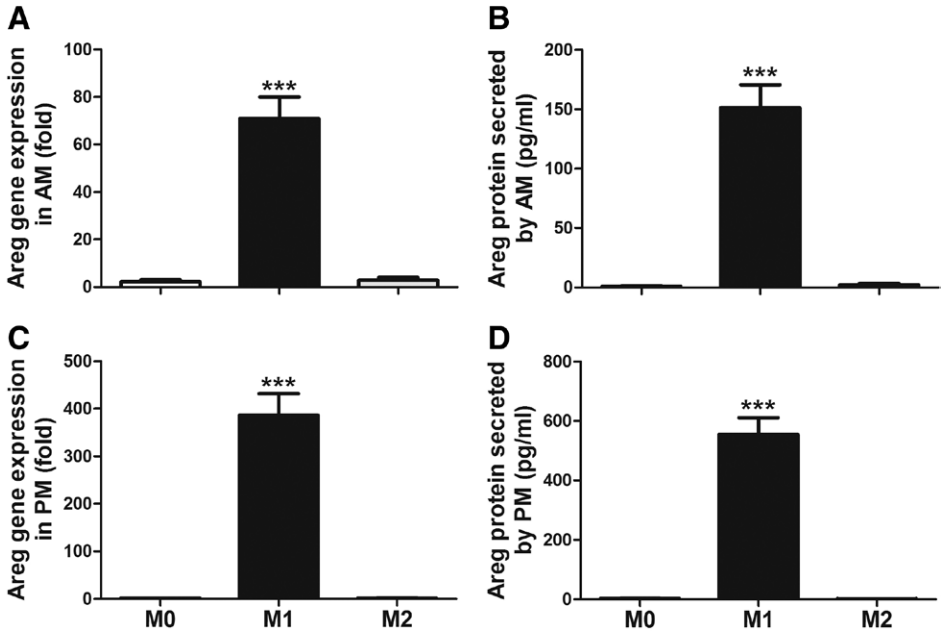

Fig. 5. Amphiregulin (Areg) expression in different phenotypes of both alveolar macrophages (AMs) and peritoneal macrophages (PMs). Mouse AMs and PMs were treated with phosphate-buffered saline (M0), 1 μg/ml of lipopolysaccharide (M1) or 20 ng/ml of interleukin-4 (M2) for 4 or 24 h. Areg mRNA expressions in AMs (*A*) and PMs (*C*) treated for 4 h were detected by quantitative reverse transcription polymerase chain reaction. Areg expression was normalized to β-actin expression levels, and the mean value for M0 was set to 1. Data were shown as mean ± SD of five independent samples. Areg protein concentrations in supernatants of AMs (*B*) and PMs (*D*) treated for 24 h were detected by bicinchoninic acid . Values are mean ± SD of five independent experiments. *** $P < 0.001$ *versus* M0; one-way ANOVA Bonferroni posttest.

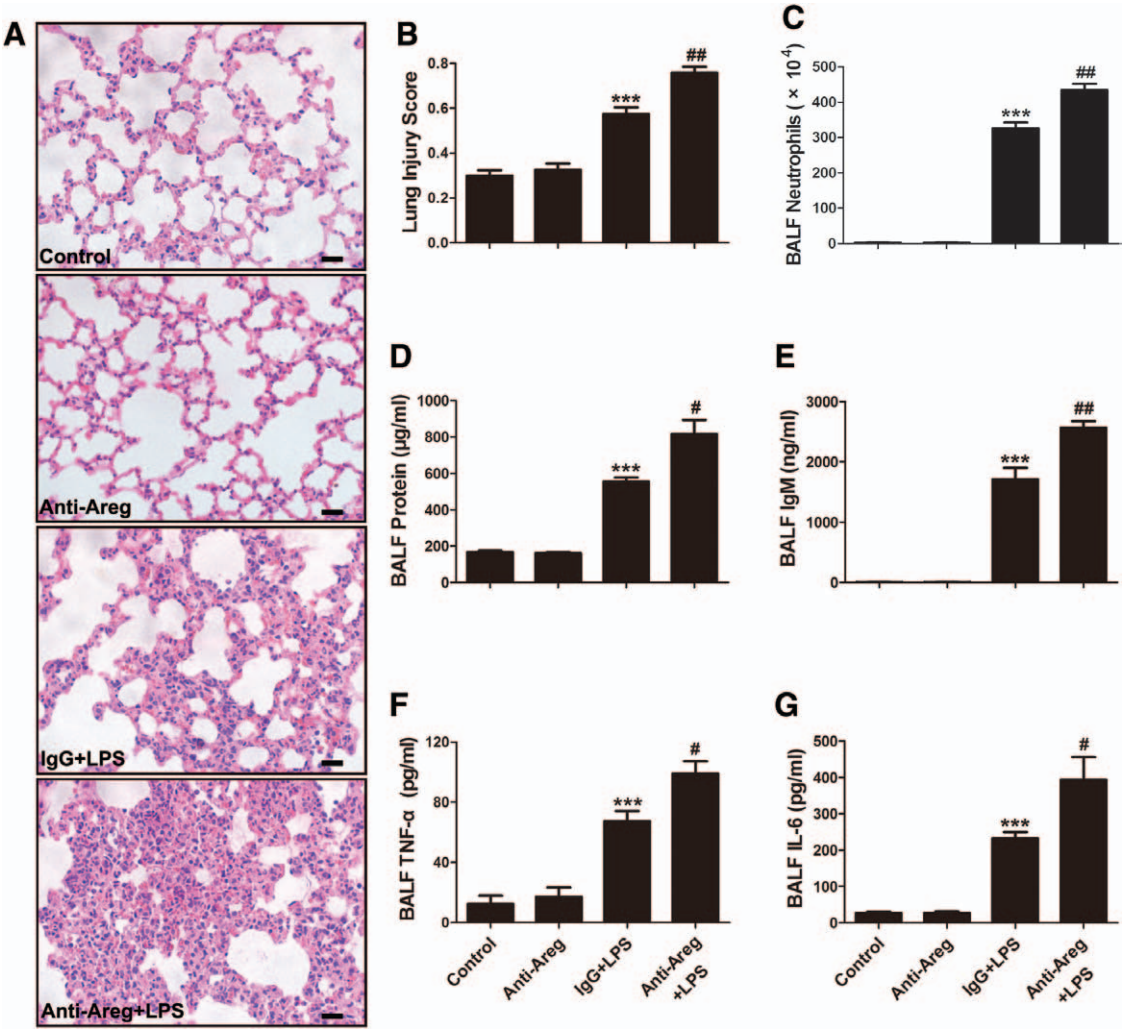

Fig. 6. Amphiregulin (Areg) neutralization aggravates lipopolysaccharide (LPS)-induced acute lung injury in mice. Mice were injected intraperitoneally with IgG (50 μg/mouse) or Areg antibodies (anti-Areg, 50 μg/mouse), then challenged 30 min later with 1 mg/kg LPS for 24 h. (*A*) Hematoxylin and eosin histopathology of left lungs of mice. Each image is representative of histologic images from six independent samples. *Scale bar* = 20 μm. (*B*) Analysis of lung sections by lung injury score. Data are shown as mean ± SD (n = 6). (*C*) The numbers of neutrophils in bronchial alveolar lavage fluid (BALF). (*D*) Total protein concentration in BALF was detected by bicinchoninic acid kits. Levels of IgM (*E*), tumor necrosis factor (TNF)-α (*F*), and interleukin (IL)-6 (*G*) in BALF were measured by enzyme-linked immunosorbent assay. Data are shown as mean ± SD from four independent samples. *** $P < 0.001$ *versus* control; # $P < 0.05$ and ## $P < 0.01$ *versus* IgG + LPS; (*B–G*) one-way ANOVA Bonferroni posttest.

damage to lung tissues in mice. However, when exposed to lipopolysaccharide, mice pretreated with amphiregulin antibodies suffered from severe lung injury compared with their IgG-pretreated counterparts, as evidenced by more severe alveolar destruction, thicker alveolar wall, higher lung injury score (fig. 6B), more neutrophil infiltration (fig. 6C), increased levels of total protein (fig. 6D), IgM (fig. 6E), and proinflammatory factors (TNF-α and interleukin-6, fig. 6, F and G) in BALF compared with the IgG-pretreated mice.

Effect of Exogenous Amphiregulin on Lipopolysaccharide-induced ALI in Mice

Then, we examined the protective effect of exogenous amphiregulin on lipopolysaccharide-induced ALI. We made an ALI model by intratracheal administration of 3 mg/kg of lipopolysaccharide. Briefly, mice were first injected intratracheally with rmAreg or PBS and then challenged with lipopolysaccharide 30 min later. HE staining (fig. 7A) exhibited that treatment with 3 mg/kg lipopolysaccharide resulted in severe lung injuries, and rmAreg pretreatment obviously ameliorated alveolar disarray, interstitial edema, and neutrophil infiltration compared with PBS pretreatment (fig. 7B). In contrast, rmAreg administration significantly lowered the lipopolysaccharide-induced increase of neutrophil number, (fig. 7C), TNF-α (fig. 7D), and interleukin-6 (fig. 7E), total protein (fig. 7F), and IgM (fig. 7G) expressions in BALF.

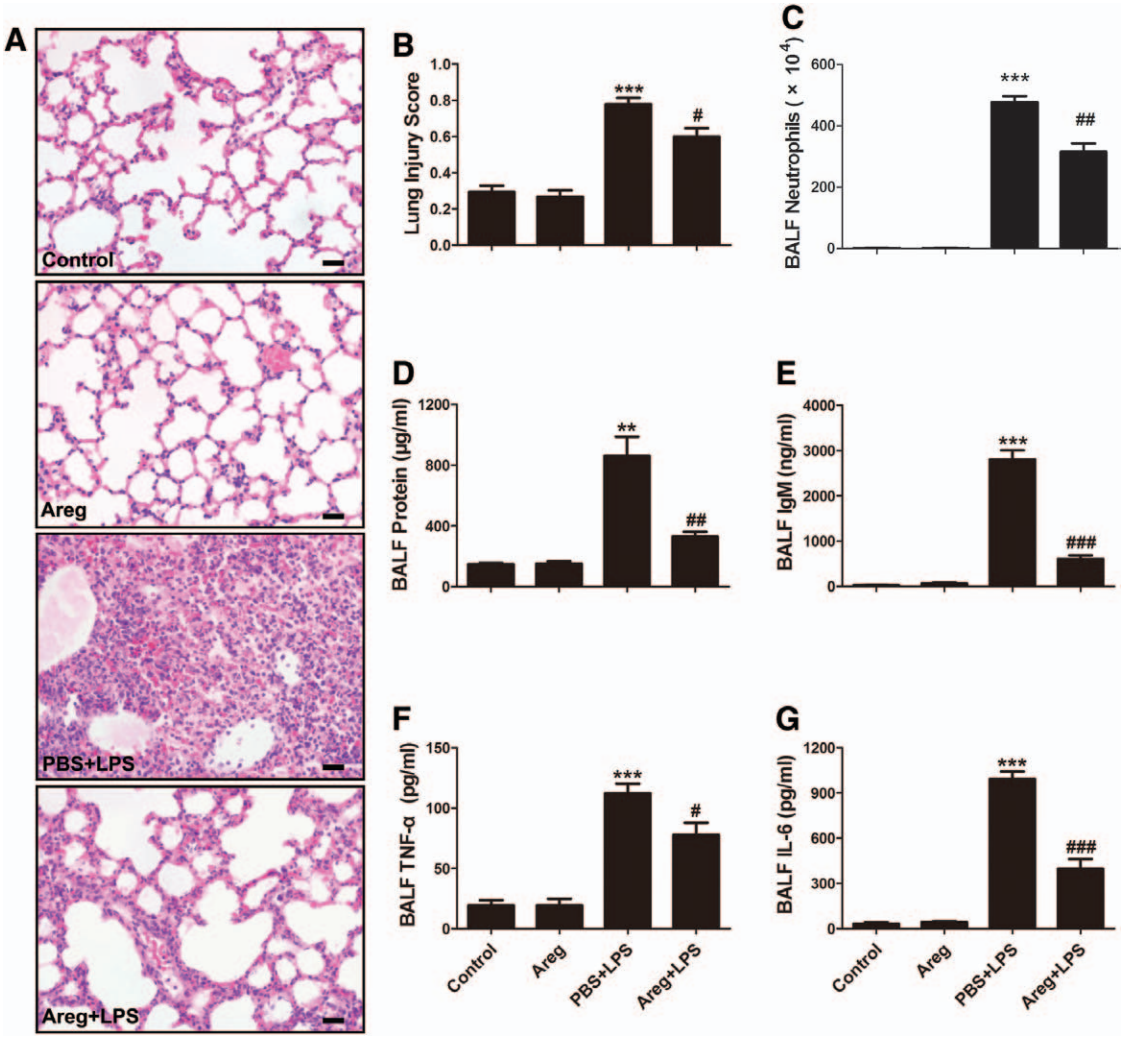

Fig. 7. Recombinant mouse amphiregulin (Areg) attenuated lipopolysaccharide (LPS)-induced acute lung injury in mice. Mice were injected intraperitoneally with phosphate-buffered saline (PBS; 250 μl/mouse) or recombinant mouse Areg (5 μg/mouse) and then stimulated 30 min later with 3 mg/kg of LPS for 24 h. (*A*) Hematoxylin and eosin–stained lung sections (n = 6/group). *Scale bar* = 20 μm. (*B*) Analysis of lung sections by lung injury score. Data are shown as mean ± SD (n = 6). (*C*) The numbers of neutrophils in bronchial alveolar lavage fluid (BALF). (*D*) Total protein concentration in BALF was detected by bicinchoninic acid kits. Concentrations of IgM (*E*), tumor necrosis factor (TNF)-α (*F*), and interleukin (IL)-6 (*G*) in BALF was determined by enzyme-linked immunosorbent assay. Data are shown as mean ± SD from four independent samples. ** $P < 0.01$, *** $P < 0.001$ *versus* control; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ *versus* PBS + LPS; (*B–G*) one-way ANOVA Bonferroni posttest.

Effect of Amphiregulin on Lipopolysaccharide-induced Apoptosis of MLE12 Cells

We then investigated whether amphiregulin has protective effect on pulmonary epithelial cells *in vitro*. MLE12 cells were challenged with lipopolysaccharide to mimic *in vivo* lung epithelial cells in sepsis, and cell survival was determined by flow cytometry. Lactate dehydrogenase assay found that the difference in cell death was no different among all the groups (shown in fig. 1, Supplemental Digital Content 1, http://links.lww.com/ALN/B246). Figure 8A shows that apoptosis ratio of MLE12 cell significantly increased from 5.41 ± 0.60% in control group to 16.60 ± 1.82% after lipopolysaccharide challenge, but this pro-apoptosis effect of lipopolysaccharide was significantly attenuated by administration of rmAreg in a dose-dependent manner with only 9.47 ± 1.67% cells suffering apoptosis in Areg(50) + lipopolysaccharide group, suggesting that amphiregulin can protect MLE12 cells against apoptosis induced by lipopolysaccharide challenge. To further confirm the effect of AM-released amphiregulin on epithelial apoptosis, MLE12 cells were preincubated with conditioned media of PBS-treated AMs (M0-CM), conditioned media of M1-polarized AMs (M1-CM) treated with IgG, and amphiregulin antibodies. Preincubation with M0-CM and IgG-treated M1-CM exerted no effect on lipopolysaccharide-induced apoptosis of MLE12 cells, but amphiregulin antibodies–treated M1-CM significantly exacerbated lipopolysaccharide-induced apoptosis of MLE12 cells, increasing the apoptosis ratio from 17.45 ± 1.13% to 21.67 ± 1.10% (fig. 8B).

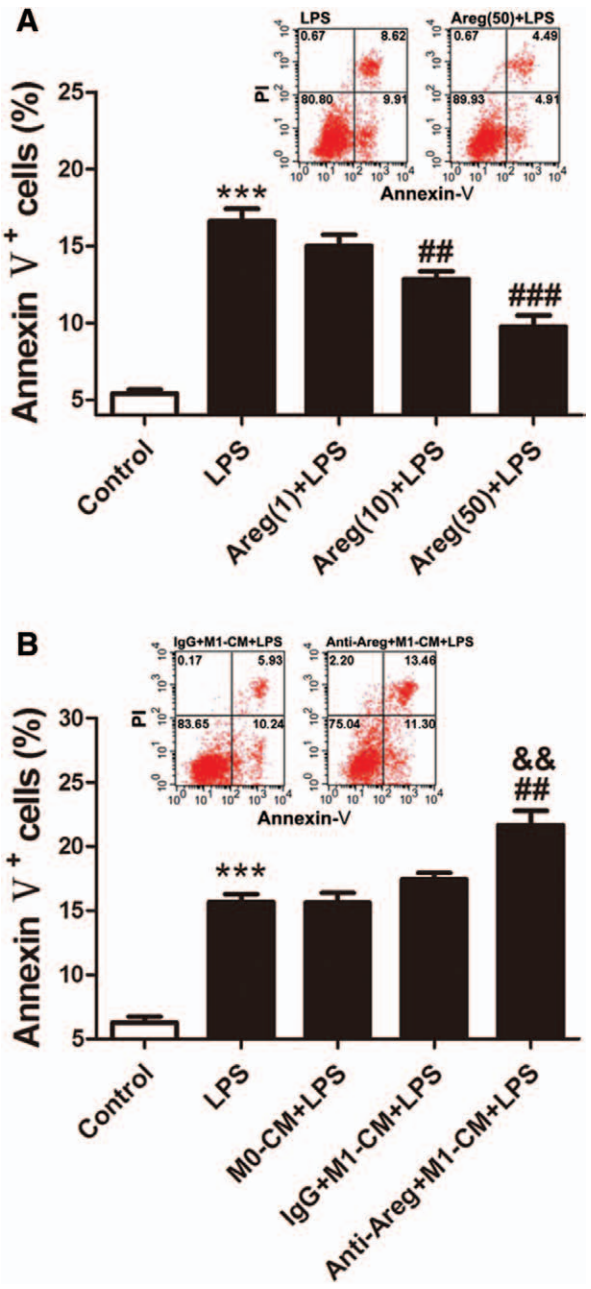

Fig. 8. Effects of amphiregulin (Areg) on lipopolysaccharide (LPS)-induced MLE12 cell apoptosis. (*A*) Areg reduced MLE12 cell apoptosis caused by LPS. MLE12 cells were incubated with LPS (100 μg/ml) for 24 h in the absence or presence of recombinant exogenous Areg (1, 10, or 50 ng/ml). The percentage of total annexin-V$^+$ cells was determined by flow cytometry analysis. Data are shown as mean ± SD of five different experiments. *** $P < 0.001$ *versus* control; ## $P < 0.01$ and ### $P < 0.001$ *versus* LPS; one-way ANOVA Bonferroni posttest. (*B*) Areg secreted by M1-alveolar macrophages (AMs) prevented MLE12 cells from apoptosis. AMs were stimulated with phosphate-buffered saline (PBS) or LPS (1 μg/ml) for 24 h, and the conditioned medium (M0-CM or M1-CM) was collected. M1-CM was treated with IgG (5 μg/ml) or Areg antibodies (Anti-Areg, 5 μg/ml) for 3 h. MLE12 cells were incubated for 30 min with M0-CM or M1-CM containing

Activation of EGFR and AKT by Amphiregulin in Lung Tissues of Mice

Western blotting (fig. 9, A–C) showed that the levels of EGFR and AKT phosphorylation in lung tissues were very low in the control group but obviously increased in lipopolysaccharide-challenged lung. Amphiregulin antibodies alone did not affect phosphorylation of EGFR and AKT, but pretreatment with amphiregulin antibodies significantly inhibited lipopolysaccharide-induced EGFR and AKT activation. Conversely, rmAreg alone activated both EGFR and AKT in mouse lung tissues, and amphiregulin treatment further enhanced lipopolysaccharide-induced EGFR and AKT phosphorylation (fig. 9, D–F).

Activation of EGFR and AKT by Amphiregulin in MLE12 Cells

To understand the role of amphiregulin in EGFR and AKT activation in pulmonary epithelial cells, we stimulated MLE12 cells with rmAreg. It was found that EGFR phosphorylation rapidly reached a peak within 5 min and declined thereafter to almost baseline levels 30 min after the stimulation (fig. 10, A–C). In contrast, AKT was activated 5 min after challenged with rmAreg, but the phosphorylation level remained unchanged over a time period of 30 min. To examine the effects of amphiregulin secreted by AMs on phosphorylation of EGFR and AKT in MLE12 cells, MLE12 cells were exposed to M1-CM treated with IgG or amphiregulin antibodies and M0-CM, respectively (fig. 10, D–F). The M0-CM did not alter the phosphorylation of EGFR or AKT compared with control cells. Conversely, IgG-treated M1-CM significantly activated EGFR and AKT in MLE12 cells, but the activation could be dramatically attenuated when the MLE12 cells were exposed to amphiregulin antibodies-treated M1-CM.

Discussion

Activated AMs are generally believed to exert a damaging effect on lung tissues when they work as a defense against infection. In this study, however, we found that M1 phenotype of AMs protect lung tissues by expressing high level of amphiregulin. Our study showed that amphiregulin reduced the influx of protein-rich fluid into alveolar space, inhibited infiltration of inflammatory cells and expression of inflammatory cytokines, and thereby protected the lung against lipopolysaccharide-induced ALI by strengthening epithelial barriers.

Amphiregulin expression in lung tissues of ALI models is poorly understood. Our data showed that lipopolysaccharide challenge caused a significant increase in amphiregulin

Fig. 8 (*Continued*). IgG or anti-Areg antibodies, followed by stimulation with LPS (100 μg/ml) for 24 h. *Vertical bars* represent mean ± SD of percentage of total annexin-V$^+$ cells detected by flow cytometry analysis (n = 5). *** $P < 0.001$ *versus* control; ## $P < 0.01$ *versus* LPS; && $P < 0.01$ *versus* IgG + M1-CM + LPS; one-way ANOVA Bonferroni posttest. M0-CM = the conditioned medium of PBS-stimulated macrophages; M1-CM = the conditioned medium of LPS-stimulated macrophages.

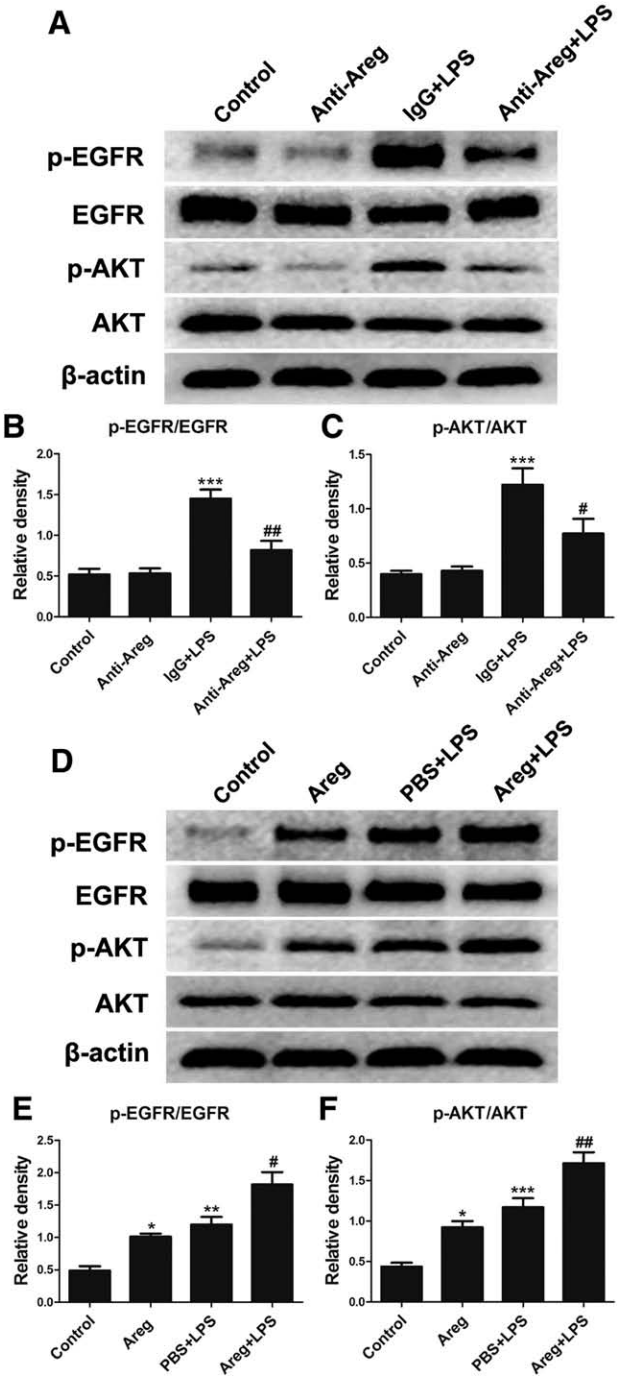

Fig. 9. Effects of amphiregulin (Areg) on lipopolysaccharide (LPS)-induced epithelial growth factor receptor (EGFR) and AKT activation in lung tissues. (*A–C*) Mice received pretreatment with Areg antibodies (anti-Areg, 50 μg per mouse) or IgG (50 μg per mouse) for 30 min, followed by intratracheal challenge with 1 mg/kg of LPS for 6 h. (*A*) Total and phosphorylated EGFR and AKT in lung lysate were measured by western blot. Densitometric analysis of phosphorylated/total EGFR (*B*) and AKT (*C*). Values were mean ± SD of five independent samples. *** $P < 0.001$ *versus* control; # $P < 0.05$ and ## $P < 0.01$ *versus* IgG + LPS; one-way ANOVA Bonferroni posttest. (*D*, *E*) Mice were pretreated with phosphate-buffered saline (PBS) or recombinant exogenous Areg (5 μg per mouse) for 30 min, followed by intratracheal stimulation with 3 mg/kg of LPS for 6 h. (*D*) Western blot of phosphorylated EGFR and AKT in lung lysate. Densitometric analysis of phosphorylated/total EGFR (*E*) and AKT (*F*). Data are shown as mean ± SD representative five independent samples. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ *versus* control; # $P < 0.05$ and ## $P < 0.01$ *versus* PBS + LPS; one-way ANOVA Bonferroni posttest.

expression in lung tissues of ALI mice. At the same time, we found that, although the gene expression increased at early stage after lipopolysaccharide challenge, the amphiregulin protein expression sustained for a long time, suggesting that amphiregulin effects may be prolonged during the recovery stage of inflammatory response to balance the proinflammatory response. This finding was consistent with previous results that amphiregulin expression was increased in lung tissues damaged by mechanical ventilation or bleomycin.[11,26] We also observed the concomitant appearance of amphiregulin and Iba-1 (a macrophage biomarker) fluorescence in lipopolysaccharide-challenged lung tissues and coexistence of fluorescence of both amphiregulin and attached cells (macrophages) in BALF isolated from ALI mice. Further investigation revealed that AM depletion resulted in dramatically reduced amphiregulin concentration in BALF of ALI mice. However, lipopolysaccharide challenge of macrophages significantly increased the expression of amphiregulin. Collectively, these findings suggested that AMs were the main sources of amphiregulin production in lipopolysaccharide-induced ALI. Because AMs are activated to M1 phenotype at early phage of ALI,[4,7] we further evaluated the amphiregulin expression in different phenotypes of AMs and PMs *in vitro*. We found that only M1 macrophages secreted high-level amphiregulin. Both *in vivo* and *in vitro* results suggested that amphiregulin was mainly expressed by classically activated macrophages.

To evaluate the biologic significance of amphiregulin upregulation in lipopolysaccharide-induced ALI, we examined the effects of both exogenous recombinant amphiregulin and amphiregulin antibody on pulmonary pathology (including inflammation and changes in permeability). Our results consistently showed that exogenous recombinant amphiregulin reduced influx of protein-rich fluid into alveolar space, inhibited infiltration of inflammatory cells, down-regulated the expression of inflammatory cytokines, and resultantly protected the lung against lipopolysaccharide-induced ALI. Conversely, amphiregulin antibody abolished the protective effect of endogenous amphiregulin. These results indicated that amphiregulin produced by activated AMs had a protective effect on the lung in the ALI mice.

Previous studies reported that alveolar epithelial cell death in ALI is associated with severity of lung injury, and inhibition of epithelial apoptosis can improve lung function and animal survival in ALI models.[27–29] In view of these findings, we investigated the mechanism underlying the protective effect of amphiregulin by examining the effect of amphiregulin on lipopolysaccharide-induced apoptosis of MLE12 cells (lung

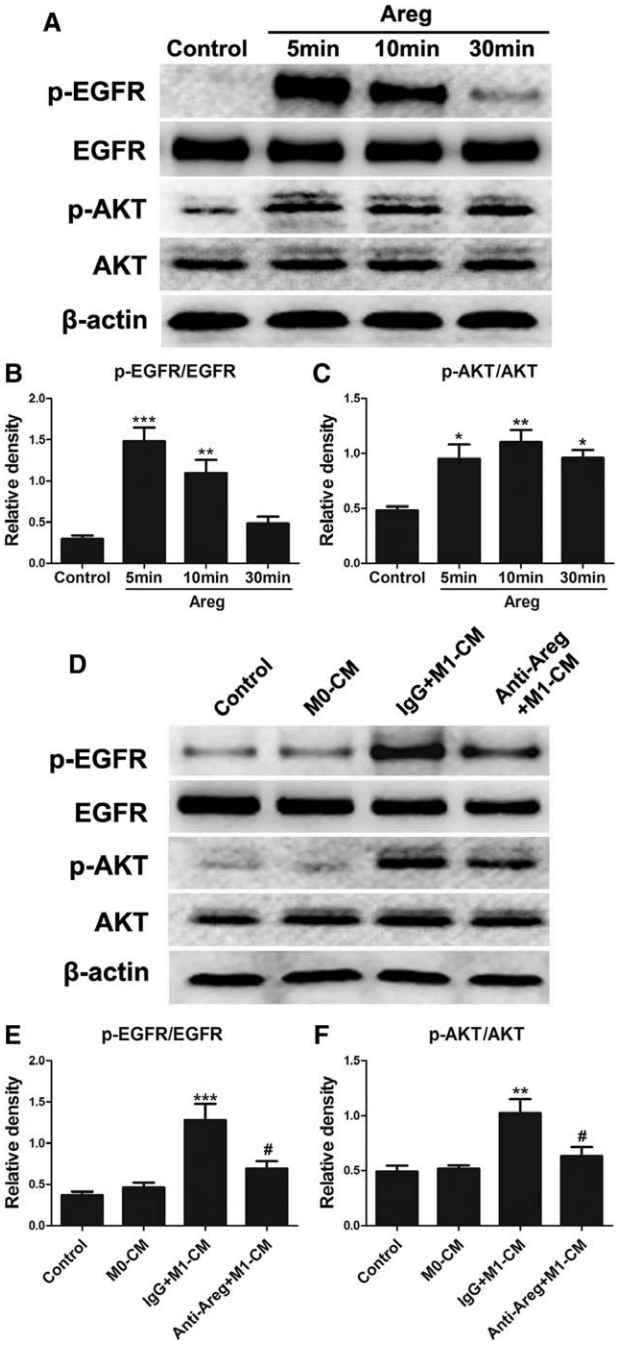

Fig. 10. Effects of amphiregulin (Areg) on epithelial growth factor receptor (EGFR) and AKT phosphorylation in MLE12 cells. Western blots (*A*) and densitometric analysis of phosphorylated/total EGFR (*B*) and AKT (*C*) in MLE12 cells incubated with Areg (50 ng/ml) for indicated times. Data are shown as mean ± SD of five independent samples. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ *versus* control; one-way ANOVA Bonferroni posttest. (*D*, *E*) Alveolar macrophages were stimulated with phosphate-buffered saline (PBS) or lipopolysaccharide (LPS, 1 μg/ml) for 24 h, and supernatants (M0-CM or M1-CM) were collected. M1-CM was treated with Areg antibodies (anti-Areg, 5 μg/ml) or IgG (5 μg/ml) for 3 h. MLE12 cells were incubated in M0-CM or M1-CM containing Areg Abs or IgG for 10 min. Western blots (*D*) and densitometric analysis of phosphorylated/total EGFR (*E*) and AKT (*F*) in MLE12 cell lysate. Values are presented as mean ± SD of five independent experiments. ** $P < 0.01$ and *** $P < 0.001$ *versus* control; # $P < 0.05$ *versus* IgG + M1-CM; one-way ANOVA Bonferroni posttest. M0-CM = the conditioned medium of PBS-stimulated macrophages; M1-CM = the conditioned medium of LPS-stimulated macrophages.

epithelial cells). By mimicking events that epithelia experienced in lipopolysaccharide-induced ALI, we found that amphiregulin inhibited the apoptosis of lipopolysaccharide-treated epithelial cells in a concentration-dependent manner. Interestingly, we observed that the conditioned media of M1-polarized AMs did not directly protect MLE12 cells against lipopolysaccharide-induced apoptosis, and neutralization of amphiregulin in M1-CM by amphiregulin Abs significantly enhanced the proapoptotic effect of lipopolysaccharide on MLE12 cells. The absence of protective effect of M1-CM on apoptosis of MLE12 cells might be explained by the fact that high concentrations of proinflammatory cytokines and molecules in M1-CM might promote the lipopolysaccharide-induced proapoptotic effect of MLE12 cells, thereby abolishing the protective effect of amphiregulin in M1-CM on MLE12 cells. These findings suggested that, without the protection of amphiregulin secreted by M1 macrophages in lipopolysaccharide-induced ALI, lung epithelial cells might otherwise have suffered from more severe damage.

Amphiregulin, by exclusively binding to its receptor EGFR, induces phosphorylation of EGFR. In this study, lipopolysaccharide-induced phosphorylation of EGFR and AKT in lung tissues was increased by exogenous amphiregulin but decreased by amphiregulin neutralization. The enhancing effect of exogenous amphiregulin on the phosphorylation was consistent with our *in vitro* findings that both the recombinant amphiregulin and the amphiregulin protein secreted by M1 AMs directly activated EGFR and AKT in pulmonary epithelial cells. Moreover, the simultaneous phosphorylation of both EGFR and AKT by amphiregulin is in agreement with a previous study demonstrating that phosphorylation of EGFR could further activate the AKT signaling pathway.[30] We also found that, amphiregulin protected the pulmonary epithelial cells against lipopolysaccharide-induced apoptosis, thereby strengthening the epithelial barrier, which was coincident with a previous finding that decreased AKT phosphorylation led to more severe damage in lipopolysaccharide-challenged lung tissues.[31] Westphalen *et al.*[31] proposed that the activated AMs could prevent lung injury by initiating Ca^{2+}-dependent AKT activation in epithelia at the onset of lipopolysaccharide-induced inflammation. Whether amphiregulin activates AKT signal pathway in a Ca^{2+}-dependent manner warrants further investigation. Overall, this study demonstrated that amphiregulin was capable of promoting the phosphorylation of EGFR and AKT in mouse lung epithelial cells under lipopolysaccharide challenge and, as a result, preventing the lung against ALI.

In summary, different from the common concept that classically activated AMs just exert a damaging effect on the lung tissues, this study showed that that classically activated

AMs have a protective effect on the lung tissues by expressing a high-level of amphiregulin, which reduced influx of protein-rich fluid into alveolar space, inhibited infiltration of inflammatory cells, down-regulated the expression of inflammatory cytokines through activation of EGFR-AKT pathway, and resultantly protected lung tissues against lipopolysaccharide-induced ALI by strengthening the epithelial barrier.

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Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Shihai Zhang: Department of Anesthesiology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. zhangshihai@vip.163.com. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. Anesthesiology's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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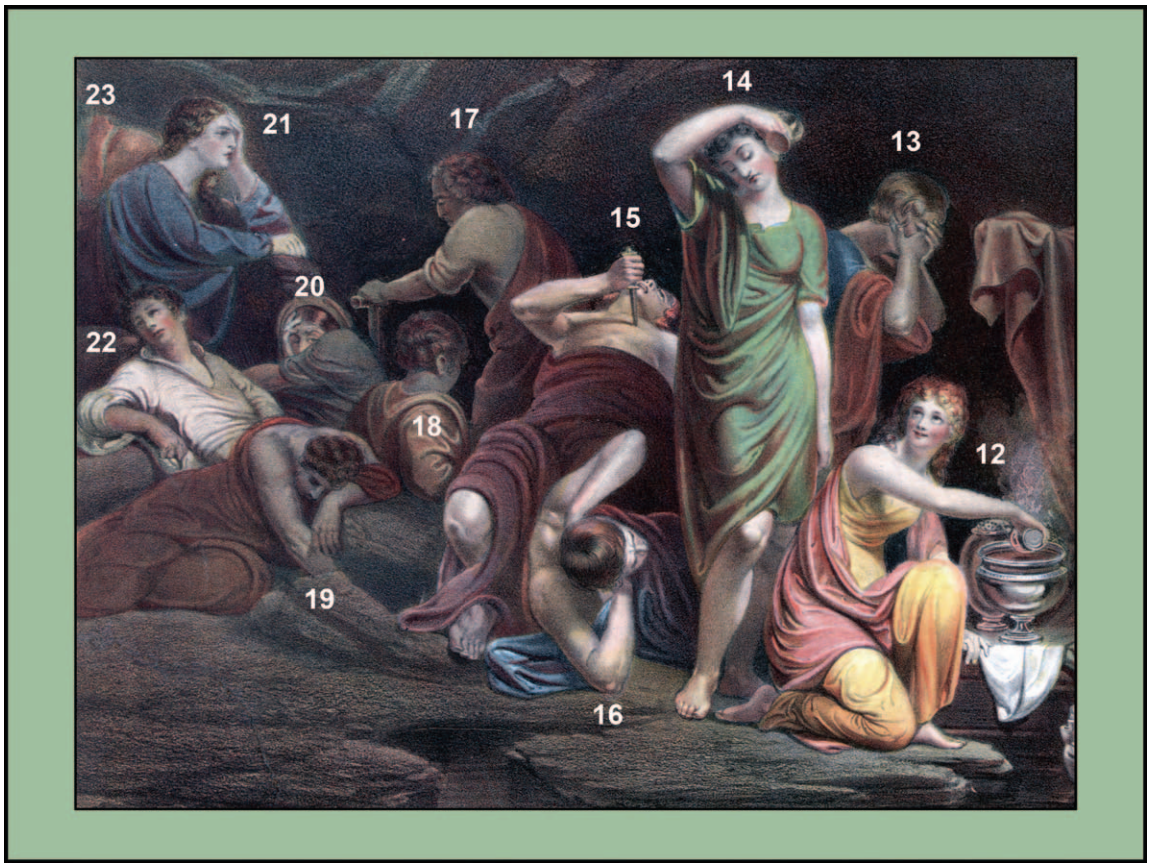
ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

Portrayed by Peale, Would "Apoplexy" Haunt Colton's Near-asphyxial Anesthetics?



By mass-producing chromolithographs of *The Court of Death*, an allegorical painting by Rembrandt Peale (1778–1860), nitrous oxide pioneer Gardner Q. Colton (1814–1898) hoped to decorate 100,000 American homes with this "parlor ornament." On the left third of Colton's print of Peale's painting, a closer view (*above*) reveals sensual Pleasure (12) and Intemperance (14), which Peale associated with Gout (17), Fever (22), and Delirium Tremens (16). Of course, Pleasure and Intemperance were also linked with mental afflictions ranging from Hypochondria (20), Remorse (13), and Despair (23) to Suicide (15). Accumulating products of self-destructive behaviors could congest the lungs, the heart, or the brain and were depicted by Peale as Consumption (21), Dropsy (18), and Apoplexy (19), respectively. Hypoxic brain damage, often manifesting as apoplexy or stroke, would afflict patients, possibly hundreds of them, over the century following Colton's 1863 revival of using unoxygenated nitrous oxide for dental anesthesia. (Copyright © the American Society of Anesthesiologists, Inc.)

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