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ORIGINAL RESEARCH

Cigarette Smoke Triggers IL-33–associated Inflammation in a Model of Late-Stage Chronic Obstructive Pulmonary Disease

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Abstract

Chronic obstructive pulmonary disease (COPD) is a worldwide threat. Cigarette smoke (CS) exposure causes cardiopulmonary disease and COPD and increases the risk for pulmonary tumors. In addition to poor lung function, patients with COPD are susceptible to bouts of dangerous inflammation triggered by pollutants or infection. These severe inflammatory episodes can lead to additional exacerbations, hospitalization, further deterioration of lung function, and reduced survival. Suitable models of the inflammatory conditions associated with CS, which potentiate the downward spiral in patients with COPD, are lacking, and the underlying mechanisms that trigger exacerbations are not well understood. Although initial CS exposure activates a protective role for vascular endothelial growth factor (VEGF) functions in barrier integrity, chronic exposure depletes the pulmonary VEGF guard function in severe COPD. Thus, we hypothesized that mice with compromised VEGF production and challenged with CS would trigger human-like severe inflammatory progression of COPD. In this model, we discovered that CS exposure promotes an amplified IL-33 cytokine response and severe disease progression. Our *VEGF*-knockout model combined with CS recapitulates severe COPD with an influx of IL-33–expressing macrophages and neutrophils. Normally, IL-33 is quickly inactivated by a post-translational disulfide bond formation. Our results reveal that BAL fluid from the CS-exposed, VEGF-deficient cohort promotes a significantly prolonged lifetime of active proinflammatory IL-33. Taken together, our data demonstrate that with the loss of a VEGF-mediated protective barrier, the CS response switches from a localized danger to an uncontrolled long-term and long-range, amplified, IL-33–mediated inflammatory response that ultimately destroys lung function.

Keywords: alarmins; ST2; chronic obstructive pulmonary disease; alveolar epithelial cells; vascular endothelial growth factor

Clinical Relevance

Cigarette smoke pollutant exposure is common and causes lung destruction via poorly understood inflammatory mechanisms that progress to end-stage chronic obstructive pulmonary disease (COPD). Activation of vascular endothelial growth factor (VEGF) is the first response to sudden contact with pollutants. However, chronic exposure results in downregulation of VEGF. The main barrier to battling COPD is the lack of a model recapitulating the VEGF-dependent progression to end-stage inflammatory disease. We present a VEGF-deficient COPD model that overcomes this scientific barrier. This mouse model has a weakened protective lung barrier and displays amplified inflammation upon cigarette smoke exposure. This response is mediated by upregulation and prolonged activation of the proinflammatory cytokine IL-33. Our data reveal a robust model for future studies and implicate prolonged IL-33 signaling as a therapeutic target in COPD.

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Chronic obstructive pulmonary disease (COPD) is a global health burden, projected to be the third leading cause of death by 2030 (1). However, suitable animal models do not exist to study end-stage disease. Most animal exposure models result in only mild inflammation and reflect early stages of disease, which is not readily diagnosed in humans. Although specific targeting of destructive proteases or single-gene knockouts gives insights into potential mechanisms, this approach does not provide clues about the transition from mild lung destruction to emphysema and complex inflammatory pathways that occur in severe COPD. This last phase of the disease is important to understand because acute inflammatory episodes strike in an already weakened pulmonary system and result in a rapid and irreversible decline in lung function in late-stage COPD. Therefore, investigation of the immune response mechanisms triggered by pollutants such as cigarette smoke (CS), which accelerate disease progression, is an area of intense research interest worldwide (2, 3).

The repeated insult by CS acts to repeatedly cause small injuries in the defensive airway barriers, which require continuous repair. As the innate immune system weakens, it leaves the lung more susceptible to an inflammatory episode when it encounters pollutants, whether these pollutants be CS, smoke from burning biomass, industrial toxins, or infectious agents. What we observe in our mouse model is an initial increase in epithelial expressed vascular endothelial growth factor (VEGF) that dies off with continued insult. By exposing mice to CS with and without a VEGF-mediated compromised alveolar epithelium, we are able to model the transition from mild disease to severe end-stage pulmonary inflammation. Inhibiting VEGF expression results in excessive apoptosis concomitant with oxidant generation that contributes to damaging the alveolar cell wall (2, 3). This system is biologically relevant because increased apoptosis and oxidative stress and decreased VEGF levels are all detected in patients with late-stage COPD (4).

Key to protecting and maintaining pulmonary bronchial and alveolar barriers, together with VEGF, is a group of locally acting cytokines, referred to as "alarmins." Among this group of cytokines are some of the S100 proteins, A6 and A8, as well as HMGB1 (high mobility group protein B1) and IL-33. This latter cytokine, IL-33, is linked to several chronic lung diseases, including COPD (5–8). In a healthy lung environment, the alarm signal of IL-33 is localized to the epithelial barrier and self-regulated in part by its short half-life (8). In the CS-exposed lung, IL-33 is released into the extracellular space by macrophages and neutrophils, in addition to epithelial cells. One rapidly acting post-translational mechanism to inactivate released IL-33 is to form an internal disulfide bond (DSB) within the β-trefoil fold with a concomitant conformational change, rendering it incapable of binding the ST2 (IL-1 receptor-like 1) receptor (9). This localized, very rapid reaction can prevent an exaggerated cytokine response and signal the restoration of a quiescent epithelium. Thus, the balance of oxidants and antioxidants, which are altered in the lung milieu with a compromised VEGF-deficient barrier, can dictate the biological activity of IL-33. We predicted that if DSB inactivation of IL-33 is suppressed, the epithelial barrier can no longer be quickly restored upon CS exposure to a resting state, because an exaggerated inflammatory cell influx and persistent cytokine signaling will result in disease.

We hypothesize that VEGF, which functions as both a proproliferative and antiapoptotic/antioxidant factor, protects the respiratory tract from injurious pollutants and accelerated aging (2, 3). When the barrier is compromised, CS exposure can act as a danger signal, much like a virus, to trigger an IL-33–mediated inflammatory response. In addition, we hypothesize that the CS environment minimizes the normal rapid DSB-mediated downregulation of IL-33 (9). To test these hypotheses, we first used *VEGF* promoter-luciferase reporter mice to determine if *VEGF* transcriptional activation was an early response to CS challenge. In a separate experiment, mice with normal or inhibited pulmonary VEGF expression were exposed to chronic CS and tested for changes in lung function (static lung mechanics and leukocyte profile). In addition, IL-33 cellular localization and the rate of post-translational conversion to an inactive, disulfide-bonded form in a CS-exposed lung environment were evaluated. Taken together, our results suggest that the multiple insults of CS and a weakened innate immune system provide the "perfect storm" to override the inactivation mechanisms that protect against persistent, potentially lethal inflammation.

Methods

Ethical Approval

This study was approved by the University of California, San Diego, Animal Care and Use Committee. Three to four mice per cage were housed in a vivarium maintained on a 12-hour/12-hour day–night cycle and were provided standard chow (Harlan Teklad 8604; Envigo) and water *ad libitum*.

CS Exposure

Mice were exposed to "nose-only" smoke from five University of Kentucky Reference Cigarettes without filters (3R4F; University of Kentucky Tobacco and Health Research Program) over 30–40 minutes delivered for 10 seconds of each minute at 3 L/min using the InExpose system from SCIREQ. Mice were exposed twice per day for 5 days per week for 4 months.

In Vivo Detection of VEGF Transcriptional Activity

Adult *VEGF* promoter-luciferase reporter mice (2 mo of age) (10) were exposed to daily periods of CS. Mice were optically scanned with the IVIS *in vivo* imaging system (Caliper Life Sciences, Inc.). *See* the data supplement for experiment details.

Lung-targeted *VEGF* Gene Deletion

Adult VEGF*LoxP* mice (aged 5–8 mo) were instilled with 10^4 viral genomes (VG)/mouse with either AAV2-Cre-GFP (>1.0E + 12 viral genomes (VG)/ml, catalog no. SL100814; SignaGen Laboratories) or AAV-LacZ as previously described by Tang and colleagues (3). One month later, half the mice in each group were exposed to daily periods of "nose-only" CS, which was continued for 4 months.

Static Lung Mechanics

Mice were anesthetized with ketamine/xylazine, and a cannula (20 gauge) was inserted into the trachea. Airway pressures were measured as the lungs

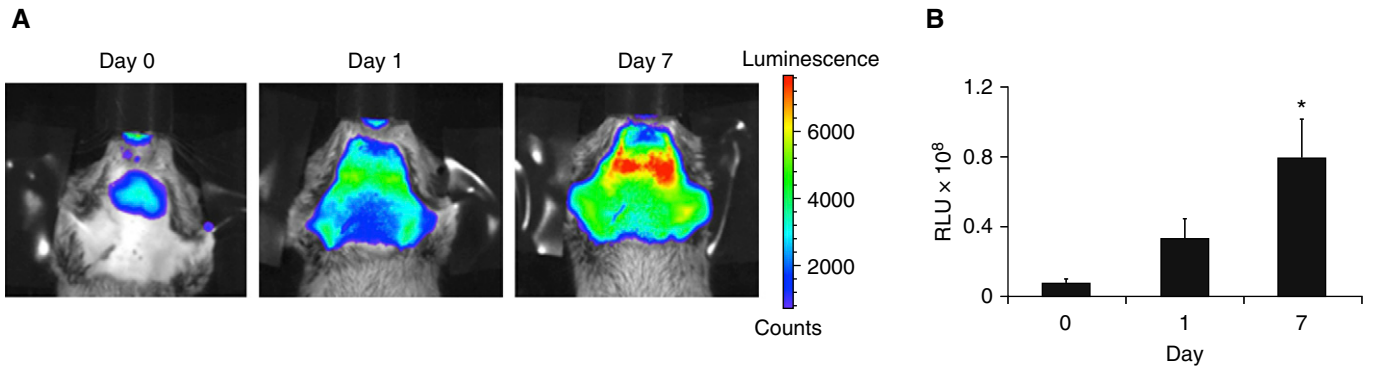

Figure 1. *In vivo* detection of *VEGF* (vascular endothelial growth factor) transcriptional activity in *VEGF* promoter-luciferase reporter mice exposed to cigarette smoke. (*A*) Optical images. (*B*) Relative light units (RLU) measured in the thoracic area. Data are expressed as the mean ± SE ($n = 7$). *Day 7 values are significantly different from Day 0 values ($P < 0.05$).

were inflated in 0.1-ml increments to an airway pressure of 20 cm H_2O and then deflated.

Lung Histology

BAL cells were collected with three 0.8-ml washes with sterile saline, cytospun onto glass slides, and detected with May-Grünwald Giemsa stain. The pulmonary vasculature was perfused with PBS at 15 cm H_2O, and the right lung was instilled and fixed at airway pressure of 25 cm H_2O with Z-Fix (Anatech Ltd.). Paraffin-embedded sections (5 μm) were stained with hematoxylin and eosin to evaluate the structural integrity of the airspaces and the presence of inflammatory cells. IL-33, ST2, F4/80, and Gr-1 were detected by immunofluorescence and viewed by confocal microscopy. *See* the data supplement for details.

BAL Fluid and IL-33 Incubations

IL-3 levels were detected in BAL fluid (BALF) by ELISA (catalog no. DY3626; R&D Systems). BALF from experimental mouse groups (20 μl) was incubated with purified IL-33 (20 μg) at 37°C for 0, 4, or 8 hours. The incubation mixture was separated by electrophoresis on 15% SDS-PAGE gels without reducing agent and stained with Coomassie Blue dye. IL-33 was expressed in a Novagen pET-24 vector (EMD Millipore) and amplified in BL21 cells (EMD Millipore). The protein product was purified by osmotic shock cell lysis followed by anion exchange chromatography as previously described (11).

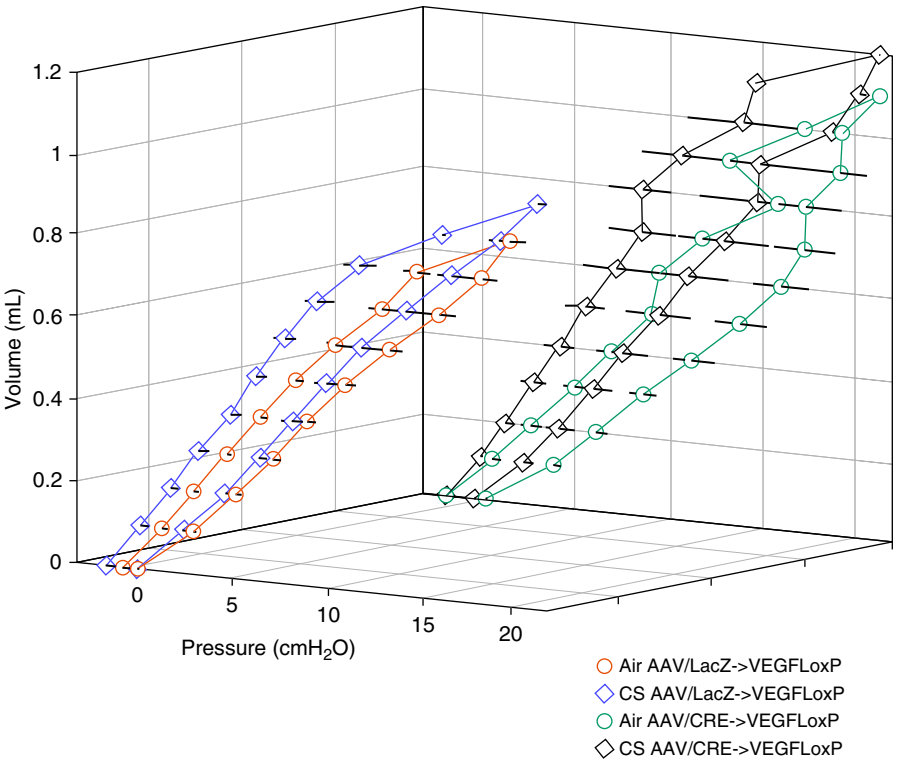

Figure 2. Static lung mechanics of control and VEGF-deficient mice exposed to air (AIR) or cigarette smoke (CS). The pressure–volume relationship was measured in mouse lungs *in situ*. (*A*) AAV-LacZ–instilled VEGF*LoxP* mice exposed to AIR or CS. (*B*) AAV-Cre–instilled VEGF*LoxP* mice exposed to AIR or CS. Data are expressed as mean ± SE (AIR-AAV/LacZ→VEGF*LoxP*, $n = 11$; CS-AAV/LacZ→VEGF*LoxP*, $n = 7$; AIR-AAV/Cre→VEGF*LoxP*, $n = 6$; CS-AAV/Cre→VEGF*LoxP*, $n = 7$). AAV/Cre = adeno-associated cre recombinase virus; AAV/LacZ = adeno-associated β-galactosidase virus.

Results

In Vivo Pulmonary *VEGF* Transcriptional Activity after Daily CS Exposure

With daily exposure to CS, there is an increase in *VEGF* promoter-luciferase reporter activity in the lungs (Figure 1). Luciferase activity was detected in the lungs without CS exposure (Day 0; 5.22e6 relative light units [RLU]). Repeated measurements on Day 1 and Day 7 were 3.40e7 RLU and 7.97e7 RLU, respectively ($P < 0.05$). Luciferase activity was localized in the

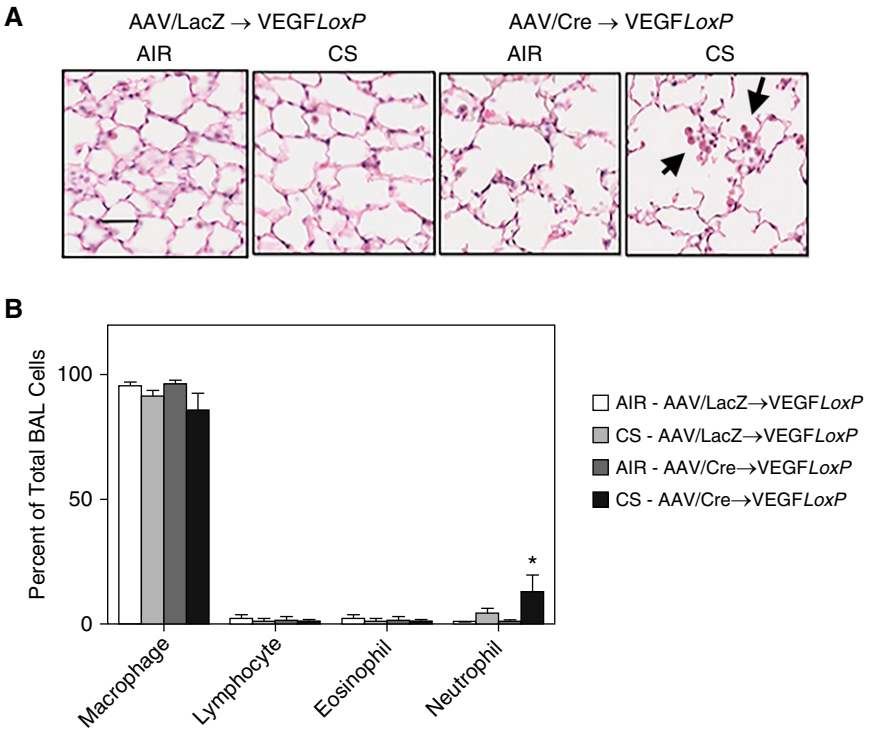

Figure 3. Analysis of inflammatory cells. (*A*) Representative hematoxylin and eosin–stained lung sections from each mouse group. Scale bar = 40 μm. Arrows indicate inflammatory cells. (*B*) Differential cell analysis of May-Grünwald Giemsa staining of BAL cells. *Significant difference between neutrophil proportions of CS-AAV/Cre→VEGF*LoxP* and AIR-AAV/LacZ→VEGF*LoxP* mice ($P < 0.05$).

upper thoracic region of the lungs (Figure E1 in the data supplement).

VEGF-Deficient Lungs Exposed to CS Exhibited Failure to Efficiently Collapse

Static lung mechanics were evaluated by measuring the pressure–volume relationship (Figure 2). Mice that were exposed to CS with *VEGF* pulmonary gene deletion (AAV/Cre→VEGF*LoxP*) had lungs characterized by increased compliance/decreased elastic recoil. At any given airway pressure up to 20 cm H_2O, the CS-exposed AAV/Cre→VEGF*LoxP* lungs exhibited the greatest expansion (lung volume) upon positive pressure but failed to efficiently collapse upon deflation. CS exposure resulted in a leftward shift of the curves in both VEGF*LoxP* mice instilled with AAV/LacZ (control virus) and those instilled with AAV/Cre (*VEGF* gene deletion). *VEGF* gene deletion in both groups resulted in increased hysteresis. Of particular note, the greatest increase in compliance was observed in the CS-exposed mice with *VEGF* gene deletion. At an inflation volume of 0.9 ml, the average airway pressure for the virus control mouse group was as follows: air-exposed (AIR)-AAV/LacZ→VEGF*LoxP*, 19.95 ± 0.87 cm H_2O; CS-AAV/LacZ→VEGF*LoxP*, 19.14 ± 1.45 cm H_2O. For the *VEGF*-knockout group, the average airway pressure was as follows: AIR-AAV/Cre→VEGF*LoxP*, 16.25 ± 0.19 cm H_2O; CS-AAV/Cre→VEGF*LoxP*, 14.84 ± 2.23 cm H_2O.

IL-33–expressing Inflammatory Cells Are Present in CS-exposed VEGF-Deficient Mice

BALF collected from CS-AAV/Cre→VEGF*LoxP* mice had increased levels of neutrophils as compared with the other mouse groups (Figure 3B). In CS-AAV/Cre→VEGF*LoxP* lungs, IL-33 was detected along the circumference of macrophages as well as in neutrophils in a punctate pattern. For the CS-AAV/LacZ→VEGF*LoxP* group, 43% of the macrophages and 76% of the neutrophils were IL-33 positive. For the CS-AAV/Cre→VEGF*LoxP* group, 72% of the macrophages and 80% of the neutrophils were IL-33 positive. At a higher magnification, IL-33 could be observed in microvesicles that ranged in size from 200 to 300 nm (Figure 4 and Video E1). Macrophages expressed both IL-33 and its receptor, ST2 (Figure 4).

A CS Lung Environment Slows Downregulation of Mature IL-33

Active IL-33 is downregulated by conversion to an inactive, disulfide-bonded form to prevent the onset of a prolonged inflammatory state (9). Detection of IL-33 measured by ELISA, which detects predominantly the inactive, disulfide-bonded form, showed an overall increase in *VEGF* gene deletion (AAV/Cre groups, $P = 0.005$). CS exposure showed a nonsignificant trend toward decreased IL-33 levels in the BALF ($P = 0.09$) (Figure 5A). *Post hoc* tests revealed a difference between AIR-AAV/Cre→VEGF*LoxP* and CS-AAV/Cre→VEGF*LoxP* groups ($P < 0.05$). BALF from the separate mouse groups was also incubated with purified mature IL-33. Initially, one prominent band at approximately 19 kD is observed under nonreducing conditions (Figure 5B). After a 4-hour incubation at 37°C, there is an overall disappearance of the active, mature, approximately 19 kD IL-33 band and the appearance of a lower DSB band in both of the AAV/LacZ-instilled groups. In the AIR-AAV/Cre→VEGF*LoxP* samples, the proportion of active/reduced IL-33 to DSB IL-33 was approximately 45% to 55%, respectively. In the CS-AAV/Cre→VEGF*LoxP* group, the proportions observed were 66% active to 34% DSB. After an 8-hour incubation time, there was an overall disappearance of both active and DSB IL-33 bands in the AAV/LacZ groups. Also, in the AIR-AAV/Cre→VEGF*LoxP* group, only the DSB IL-33 band was detectable. In the CS-AAV/Cre→VEGF*LoxP* group, the active IL-33 band remained highly populated, with the beginnings of the emergence of the disulfide-bonded form.

Discussion

We made several important observations in this study. First, we showed that airway-expressed VEGF is necessary to defend the lungs from CS-induced

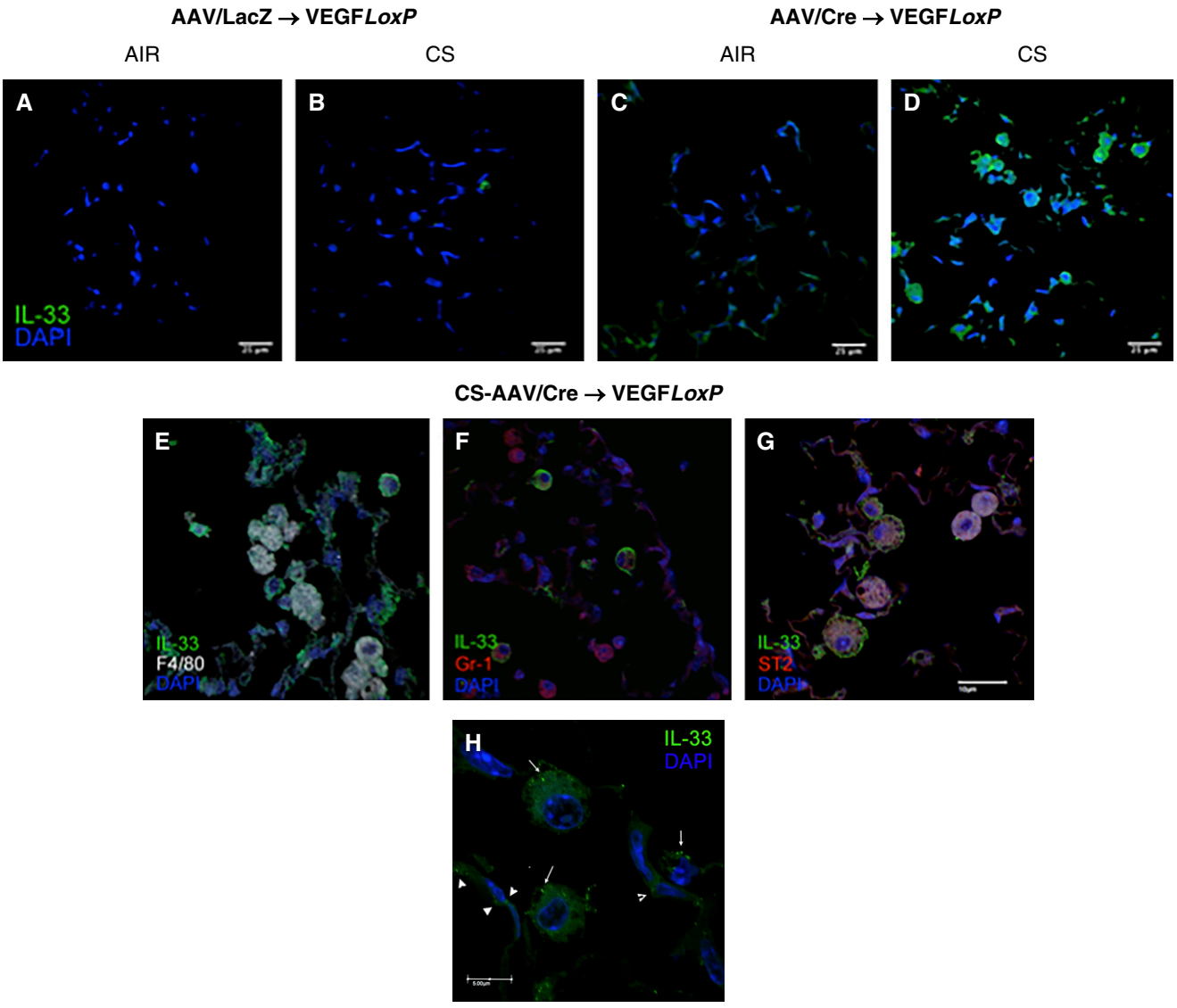

Figure 4. IL-33 colocalization with macrophages, neutrophils, and ST2. Immunohistochemistry was performed on lung sections across all mouse groups and viewed by confocal microscopy. (*A–D*) All mouse groups. Scale bars = 25 μm. (*E–G*) CS-AAV/Cre→VEGF*LoxP*. Scale bars = 10 μm. (*H*) IL-33–containing microvesicles detected at a higher magnification. Arrows indicate microvesicles in macrophage. Arrowheads indicate microvesicles associated with alveolar wall cells.

inflammation. In wild-type mice, 4 months of daily exposure to mainstream CS results in minimal changes in lung mechanics, despite the beginnings of the influx of inflammatory cells. Deletion of the *VEGF* gene from airway lining cells resulted in an increased compliance or decreased elastic recoil and hysteresis characteristic of the beginnings of an emphysema-like lung phenotype. However, a robust inflammatory response characteristic of severe disease was not present. A second environmental hit such as CS exposure combined with *VEGF* gene deletion further compromised lung mechanics and resulted in the presence of numerous macrophages and neutrophils, chronic inflammation, and the onset of a severe COPD phenotype.

VEGF Expression in Defense of CS Insult

Although it has been known for several years that VEGF is involved in COPD, this comes mainly from the observation that biopsies from smokers with severe emphysema have reduced VEGF content and a loss of alveolar wall structures (12, 13). Studies have suggested that CS functions at the receptor level by disrupting VEGF165-VEGFR2 complex–receptor interactions, and this has been proposed as a mechanism leading to emphysema in a rodent model and smokers with emphysema (4, 14). This is supported by the early studies from both our laboratory and that of Kasahara and colleagues (3, 12), in which VEGF receptor signaling was blocked by targeting *VEGF* gene deletion to the airway cells or inhibition of the VEGF receptor tyrosine kinase with SU5416. As a result, excessive apoptosis in both endothelial

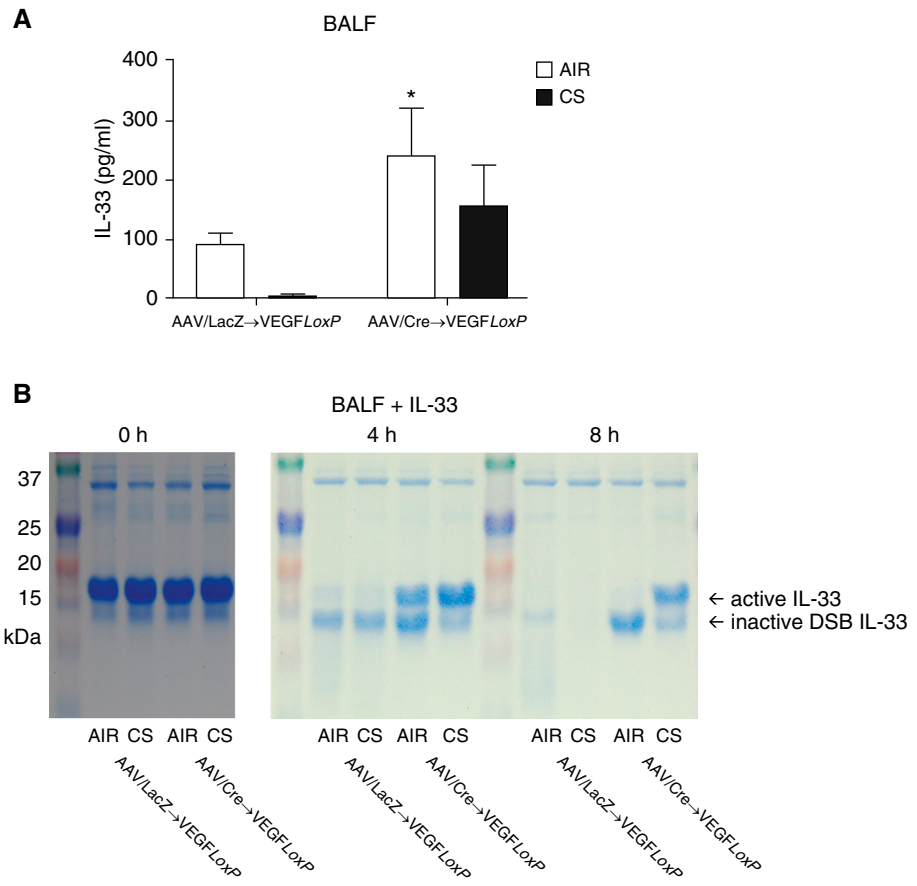

Figure 5. BAL fluid (BALF) collected from CS-AAV/Cre→VEGF*LoxP* mice delays the conversion of active IL-33 into its inactive, disulfide-bonded (DSB) form. (*A*) IL-33 measured in the BALF. *Significant difference between AIR-AAV/Cre→VEGF*LoxP* and CS-AAV/Cre→VEGF*LoxP* groups ($P < 0.05$). (*B*) Purified IL-33 incubated with BALF from mouse groups at 37°C, electrophoresed under nonreducing conditions, and stained with Coomassie Blue.

and alveolar type II cells was suggested to contribute to an emphysema-like lung structure.

The data presented in the present study suggest that activation of *VEGF* transcription in the lungs occurs after just 1 day of mice being exposed to two 30-minute sessions of CS followed by exposure to a healthy environment. In addition, *VEGF* transcription continued to increase up to at least 1 week after the initial CS exposures. CS can injure the epithelium and initiate apoptosis (15). This early *VEGF* transcriptional response may function to counteract CS insult by maintaining an intact, defensive barrier against harmful pollutants (16). However, eventually, repeated exposure will either lead to the loss of VEGF-expressing cells or inhibit signaling, and, consequently, VEGF-regulated maintenance of the barrier will fail. This is what is observed in end-stage lung disease, and it is this damaged epithelium in which CS triggers out-of-control inflammatory responses.

CS Is a Danger Signal in VEGF-Deficient Lungs

Persistent inhibition of VEGF expression leads to excessive lung cell apoptosis (3) and downregulation of surfactant protein D that decreases macrophage clearance of apoptotic cells and pathogens (13, 17). Thus, in lungs with a VEGF-compromised epithelial layer, a secondary defense mechanism is primed for the activation of the alarmins upon challenge with insult from environmental toxins. Alarmins, including S100 proteins, HMGB1, and IL-1β, and the newly discovered IL-33 are abundantly expressed by lung epithelial cell types. The early or "first responder" function of alarmins is to restore an intact barrier through cell proliferation (18). The detailed mechanism for IL-33 release from epithelial cells or pulmonary macrophages is unknown, but IL-33 was detected in microvesicles that formed a punctate halo around macrophages in the VEGF-deficient, CS-challenged lungs. CS has been reported to stimulate the secretion of microvesicles from endothelial, epithelial, and inflammatory cells in patients with COPD (19–21). After microvesicle secretion into the extracellular milieu, several post-translational mechanisms can act to downregulate the action of IL-33 and return cells to a quiescent state. This includes competitive binding to a soluble form of the IL-33 receptor (22, 23), cleavage by the apoptotic enzymes caspases 3 and 7 (24), and the formation of two DSBs that convert IL-33 to a misfolded, inactive form (9). Thus, lung cells undergoing active apoptosis may rapidly inactivate the IL-33 that is released into the BALF. However, many inflammatory cells could be detected in the lungs with the "double hit" of excessive apoptosis and CS insult, and these cells expressed the ST2 receptor for IL-33. Furthermore, when BALF collected from mice with lung-targeted *VEGF* gene deletion and CS exposure was incubated with pure IL-33, there was a delay in the conversion from the mature, active form of IL-33 (~19 kD) to the inactive, disulfide-bonded form. These experiments suggest that there are mediators in this lung environment that keep IL-33 in its reduced, active form. A similar pulmonary post-translational mechanism was reported by Cohen and colleagues (9) in mice challenged with *Alternaria* extract and patients with moderate to severe asthma. In the study by Kearley and colleagues (6), acute CS exposure was the insult that weakened the epithelial barrier, and the second "hit" was an acute challenge with influenza A virus. This second challenge allowed the release of IL-33 that signaled a T-helper cell type 1–mediated inflammatory response. Thus, two triggers are required to overwhelm the lung defense system and result in inflammation: *1*) CS then a respiratory pathogen or *2*) VEGF-dependent apoptosis then CS. Whether it is the hypoxic environment promoted by CS or mediators released from inflammatory cells, the components involved in regulating post-translational processing of IL-33 oxidation remain unclear. However, this VEGF-deficient CS lung environment

initiates a mechanism to prolong the life cycle of an IL-33–mediated cytokine response in ST2-expressing inflammatory cell populations.

Inflammation Further Decreases Lung Function

In the present study, we observed a modest shift in compliance when the mice were exposed to CS alone. This is not an unexpected result, because enlarged airspace size is only observed after 6–10 months of CS exposure in mice (25). The loss of alveolar wall structures due to CS-induced apoptosis or protease activity may just begin to be taking place at the 4-month time point reported in our present study (4, 15, 26–28). As we previously reported, a loss of airway-expressed VEGF results in decreased lung elastic recoil, greater hysteresis, and enlarged airspaces. The addition of CS exposure to *VEGF*-deficient lungs, however, shifted the pressure–volume loops much farther to left, and this observation indicates that compliance was further increased.

Overall, our study suggests that long-term cigarette smoking is an initial insult that facilitates accelerated aging with progressive lung destruction. An initial insult resulting in alveolar wall destruction primes the lungs for an inflammatory response. Once damaged, lungs are at risk for severe damage. Thus, when additional insults are encountered, such as pollutants (29), respiratory pathogens (29, 30), or aging (31), the airway epithelial alarmin system may be activated to trigger an exaggerated inflammatory response. Delayed downregulation of the conversion from an active, reduced, highly lung-expressed IL-33 protein to an inactive, disulfide-bonded form suggests that the damaged lung environment can prolong IL-33 signaling. This in turn can lead to an amplified cytokine response in cells expressing the IL-33 receptor ST2, as well as to major lung damage. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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