Complement Factor 3 Mediates Particulate Matter–Induced Airway Hyperresponsiveness

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Epidemiologic studies have suggested that exposure to airborne particulate matter (PM) can exacerbate allergic airway responses; however, the mechanism(s) are not well understood. We and others have recently shown that development of airway hyperresponsiveness (AHR) may be a complement-mediated process. In the present study, we examined the role of complement factor 3 (C3) in the development of PM-induced AHR and airway inflammation by comparing responses between C3-deficient ($C3^{-/-}$) and wild-type mice. Mice were exposed to 0.5 mg of ambient particulate collected in urban Baltimore. Forty-eight hours later, airway responsiveness to intravenous acetylcholine was assessed and bronchoalveolar lavage was conducted. PM exposure of wild-type mice resulted in significant increases in AHR, whereas it did not significantly increase airway reactivity in $C3^{-/-}$ mice. Interestingly, PM induced similar inflammatory responses in both wild-type and $C3^{-/-}$ mice. Immunohistochemical staining demonstrated marked C3 deposition in the airway epithelium and connective tissue of wild-type mice after PM exposure. These results suggest that exposure to PM may induce AHR through activation of complement factor 3 in the airways.

The morbidity and mortality of asthma has increased dramatically over the last few decades. Although the factors that contribute to this increase are not well understood, epidemiologic studies have suggested that increased exposure to air pollutants such as ozone and particulate matter (PM) are clearly associated with increased hospitalizations, increased asthma medication usage, and decrements in pulmonary function (1, 2). Animal studies have supported a role for PM exposure in development of asthma-like parameters, in that exposure of animals to PM surrogates such as residual oil fly ash (ROFA) and diesel PM induce significant airway inflammation concomitant with increases in airway responsiveness (3–6). To date, the exact mechanisms by which PM exposure can induce acute bronchoconstriction and airway hyperresponsiveness (AHR) are unknown.

Upon activation of the complement system, cleavage of the third and fifth components of the complement system (C3 and C5, respectively) generates the peptides C3a and C5a, both of which are potent anaphylatoxins. C3a and C5a can trigger contraction of smooth muscle, increase the permeability of small blood vessels, and regulate vasodilation (reviewed in Ref. 7). In addition, complement factors are potent chemoattractants for a variety of inflammatory cells including neutrophils, eosinophils, and macrophage/monocytes. Recent studies suggest that these molecules of the innate immune response are produced by airway epithelial cells and macrophages at the airway surface (8, 9). Receptors for both of these anaphylatoxins (C5aR and C3aR) have been shown to be constitutively expressed on bronchial and alveolar epithelial cells, as well as on vascular endothelial and smooth muscle cells (10). Recent reports suggest that allergen exposure upregulates C3aR expression (11) on bronchial smooth muscle cells. Furthermore, recent studies in C3aR- and C3-deficient mice support a role for C3 in antigen-induced AHR (11, 12). However, the role of complement components in non–antibody-mediated inflammatory responses has not been explored.

We have previously shown that PM collected in urban Baltimore induces AHR concomitant with significant elevations in bronchoalveolar lavage (BAL) cellularity (13). In the present study, we examined the role of C3 in AHR and inflammation induced by ambient Baltimore PM by comparing airway reactivity and inflammatory responses in wild-type and $C3^{-/-}$ mice. We demonstrate that particulate-induced AHR is C3-dependent, whereas the accompanying inflammatory response is independent of C3 activation.

Materials and Methods

Animals

Five- to six-week-old, specific pathogen free, male C57BL/6–129S3/SvImJ mice (wild-type) (Jackson Laboratories, Bar Harbor, ME) and C3-deficient ($C3^{-/-}$) mice (Jackson Laboratories) were housed in laminar flow hoods in an environmentally controlled animal facility for the duration of the experiment. The studies reported here conformed to the principles for laboratory animal research outlined by the Animal Welfare Act and the Department of Health, Education, and Welfare (National Institutes of Health) guidelines for the experimental use of animals.

PM

Ambient PM was collected from a sixth floor window in urban Baltimore city using a high-volume cyclone collector with a theoretical cut-point of 0.85 μm aerodynamic diameter when operated at a flow rate of 0.6 m^3/min (14). The cyclone was intermittently operated over a period of months at a flow rate of 0.6 m^3/min. Collected PM was pooled and refrigerated until use.

The particle size distribution of ambient PM was determined using phase contrast light microscopy at a magnification of 400×. Particles were counted and sized according to the method of Hinds (15). The count median diameter (CMD) of the particulate was 1.78 μm with a geometric standard deviation (GSD) of 2.21.

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Abbreviations: acetylcholine, ACh; airway hyperresponsiveness, AHR; bronchoalveolar lavage, BAL; complement factor 3, C3; phosphate-buffered saline, PBS; particulate matter, PM.

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Particulate Exposure

To ensure equal exposure, PM was delivered via aspiration challenge as previously described by Wills-Karp and coworkers (16). Briefly, anesthetized mice (45 mg/kg ketamine and 8 mg/kg xylazine) were suspended on a 60° incline board. With the tongue gently extended, a 50-μl aliquot of PM suspended in phosphate-buffered saline (PBS) (10 mg/ml) placed in the back of the oral cavity is aspirated by the animal. Using this technique, ~ 80% of 0.5 mg PM delivered is deposited in the lungs of each mouse (17).

Airway Responsiveness Measurements

Airway responsiveness to intravenous acetylcholine (ACh) was assessed 48 h after PM exposure as previously described (18). Briefly, mice were anesthetized (80–90 mg/kg sodium pentobarbital), cannulated, and ventilated at 120 breaths/min with a tidal volume of 0.2 ml. The mice were then paralyzed with decamethonium bromide (25 mg/kg). ACh (50 μg/kg) was injected into the inferior vena cava and changes in airway pressure were recorded. Airway responsiveness is expressed as the airway pressure–time index (APTI), which is the time-integrated change in airway pressure from baseline.

BAL

Immediately after AHR measurements, mice were exsanguinated and the lungs were lavaged three times with a single 1.0-ml aliquot of cold Hanks' balanced salt solution (Biofluids, Rockville, MD). Recovered lavage fluid (70–80%) was centrifuged (300 × *g* for 8 min) and the cell pellet was resuspended in 1.0 ml of 10% fetal bovine serum in PBS. Total cells were counted with a hemacytometer. Slides were prepared by cytocentrifugation (Cytospin 3; Shandon Instruments, Pittsburgh, PA), and stained with Diff-Quik (Dade Behring, Düdingen, Switzerland). BAL cell differential counts were determined using morphologic criteria under a light microscope with evaluation of ⩾ 500 cells/slide.

Measurement of Total Protein in BAL Fluid

Total protein content in BAL fluid was measured using a BCA Protein Assay (Pierce, Rockford, IL) according to manufacturer's recommendations. Optical density (OD) readings of samples were converted to μg/ml using values obtained from a standard curve generated with serial dilutions of bovine serum albumin (20–2,000 μg/ml)

Histologic Examination of Lung Sections

Lungs were excised and immersed in 10% formalin for at least 24 h. The left lobe was removed and washed with 70% ethanol, dehydrated, and embedded in glycol methacrylate. Sections 3 μm thick were cut and stained with hematoxylin and eosin (H&E), or periodic acid Schiff (PAS) reagent. Three sections from each of two unlavaged lungs per treatment group were examined for evidence of inflammation, injury, and increases in mucus-containing cells.

Detection of C3 in Frozen Lung Sections

A separate group of $C3^{-/-}$ and wild-type mice (n = 4/group) were given 0.5 mg of ambient PM or PBS via aspiration challenge as described above. Forty-eight hours after challenge, anesthetized mice (45 mg/kg ketamine and 8 mg/kg xylazine) were cannulated and ventilated at 120 breaths/min with a tidal volume of 0.2 ml. The lungs were perfused with PBS containing 0.1% lidocaine and heparin (10^4 IU/liter) through the right ventricle at a rate of 1 ml/min for 20 min. Lungs were excised and fixed in 4% paraformaldehyde at 4°C overnight, then placed in 0.5 M sucrose (4°C overnight). Lung samples were frozen in OCT and stored at −20°C until sectioning. Sections 10 μm thick were cut and dried on slides for 30 min. Sections were blocked and permeabilized for 2 h in PBS containing 2% normal goat serum, 0.5% Triton, and 10 μg/ml anti-CD16/CD32 (FcγRIII/II) antibody (Pharmingen, San Diego, CA). FITC-conjugated goat anti-C3 antibody or FITC-conjugated nonimmune goat IgG (ICN/Cappel, Costa Mesa, CA) was added at 5 μg/ml and incubated at 4°C overnight with gentle agitation. Slides were washed three times with PBS before observations were made using fluorescence microscopy. C3 ab or isotype control ab staining was examined in three sections of the left lobe of the lung of each mouse in a treatment group (n = 4 mice/group).

Data Analysis

APTI, BAL cell and total protein data are expressed as the mean ± SEM for each group. One-way ANOVA was used to determine differences between groups with *post hoc* comparisons by the method of Fisher. Significance was assumed at $P < 0.05$.

Results

Airway Reactivity

To assess whether C3 plays a role in AHR induced by PM exposure, we compared airway responsiveness to acetylcholine in wild-type and $C3^{-/-}$ mice. Ambient PM exposure induced a significant increase in AHR compared with PBS controls in wild-type mice (Figure 1). In contrast, PM exposure did not induce an increase in airway responsiveness in $C3^{-/-}$ mice as compared with their respective PBS controls. Interestingly, airway responsiveness to ACh in $C3^{-/-}$ PBS exposed mice was lower than in wild-type control mice, although the difference was not significant.

Airway Inflammation

In contrast to the differences in AHR seen between PM-exposed wild-type and $C3^{-/-}$ mice, the cellular profile was remarkably similar in the two groups of mice. Both wild-type and $C3^{-/-}$ mice exhibited a significant increase in the total number of cells recovered in BAL fluid after PM challenge compared with their respective PBS controls

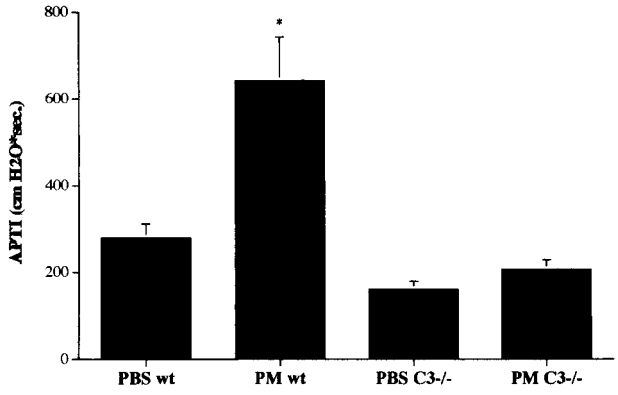

Figure 1. Airway responsiveness to i.v. acetylcholine in C57BL/6-129S3/SvImJ (wildtype) and C3-deficient ($C3^{-/-}$) mice (n = 10/group). Airway responsiveness significantly increased in wild-type, but not in $C3^{-/-}$ mice after PM challenge compared the PBS control groups. Airway responsiveness is expressed as the time-integrated change in airway pressure over baseline pressure (APTI). Values are mean ± SEM. *Indicates a significant increase over PBS control ($P < 0.05$).

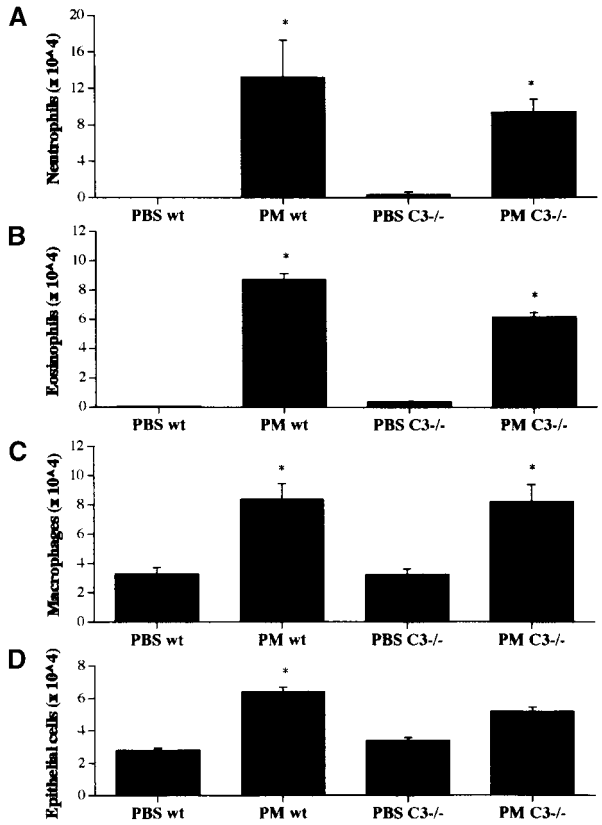

Figure 2. (*A*) Neutrophils, (*B*) eosinophils, (*C*) macrophages, and (*D*) epithelial cells in BAL fluid from C57BL/6-129S3/SvImJ (wildtype) and C3-deficient ($C3^{-/-}$) mice (n = 8/group). All cell types were dramatically elevated after PM exposure in both wildtype and $C3^{-/-}$ mice compared to their respective PBS controls. Values are mean ± SEM. *Indicates a significant increase over PBS control ($P < 0.05$).

(wild-type: 36.7 versus 6.2×10^4 and $C3^{-/-}$: 29.1 versus 7.4×10^4). Increases in neutrophil and eosinophil numbers (Figures 2A and 2B) primarily accounted for the increase in BAL cellularity. PM induced similar increases in macrophage numbers in wild-type and $C3^{-/-}$ mice; however, significant increases in epithelial cells were only noted in the wild-type group (Figures 2C and 2D).

Total Protein in BAL Fluid

Total protein was assessed in lavage fluids as a marker of lung permeability. Total protein levels were significantly increased in BAL fluid of both wild-type and $C3^{-/-}$ mice after PM challenge compared with their respective PBS controls (Figure 3). However, $C3^{-/-}$ mice had significantly higher levels of BAL protein than did wild-type mice exposed to PM. Interestingly, protein levels were elevated even in the PBS-treated $C3^{-/-}$ mice compared with PBS-treated wild-type mice.

Histology

To assess the dependence of PM-induced cellular infiltration on C3, we examined lung architecture in sections from wild-type and $C3^{-/-}$ mice after PM exposure. Both wild-type and $C3^{-/-}$ mice exposed to PBS exhibited normal lung

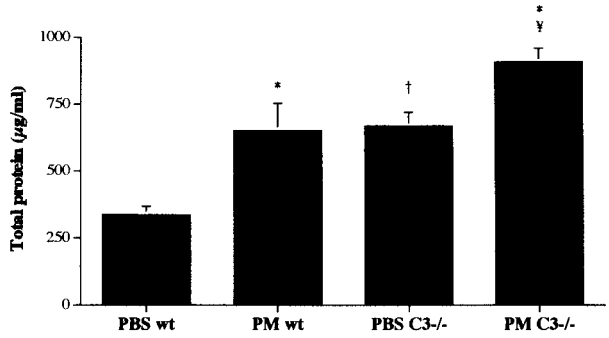

Figure 3. Total protein levels in BAL fluid from C57BL/6-129S3/SvImJ (wildtype) and C3-deficient ($C3^{-/-}$) mice (n = 8/group). PM exposure induced a significant increase in protein levels in both wildtype and $C3^{-/-}$ mice; however, C3-deficient mice had significantly higher baseline levels of protein than did wildtype mice. Values are mean ± SEM. *Indicates a significant increase over PBS control for strain. †Indicates a significant increase over wildtype PBS control. ¥Indicates a significant increase over PM-exposed wildtype ($P < 0.05$).

architecture (Figures 4A and 4C, respectively). Consistent with our previous findings, PM exposure induced primarily granulocytic inflammatory foci in areas of PM deposition in both wild-type and $C3^{-/-}$ mice (Figures 4B and 4D, respectively). Both the degree and distribution of granulocytic inflammation observed in $C3^{-/-}$ mice is similar to that seen in wild-type mice exposed to PM. Taken together, the histologic findings and the BAL data suggest that PM-induced inflammation is not dependent on C3 expression.

Immunohistochemistry

To confirm C3 deposition in the lung in response to PM exposure, we immunohistochemically stained frozen lung sections with a fluorescently labeled antibody to murine C3. Wild-type mice exposed to ambient PM exhibit marked C3 deposition along the epithelium and connective tissue of large airways, as well as vascular endothelium (Figure 5A). In contrast, these areas did not stain with Ig isotype control antibody (Figure 5B). As expected, no C3 was detected in C3-deficient mice in these areas; however, there is nonspecific staining of macrophages which accumulate in areas of PM deposition in both wild-type (Figures 6A and 6B) and $C3^{-/-}$ mice (Figures 6C and 6D).

Discussion

In the present study we demonstrate that ambient PM-induced AHR is dependent on C3. Interestingly, the recruitment and influx of inflammatory cells into the airways were not dependent on C3, as C3-deficient mice mounted similar inflammatory responses to particulate exposure as the wild-type mice. Immunohistochemical staining suggests that indeed PM exposure results in increased C3 deposition in the airway epithelium, connective tissue, and vascular endothelium of the airways.

Consistent with our previous findings, we show that PM collected in urban Baltimore induces significant increases in airway responsiveness to cholinergic stimulation. Interestingly, we demonstrate that PM-induced AHR is depen-

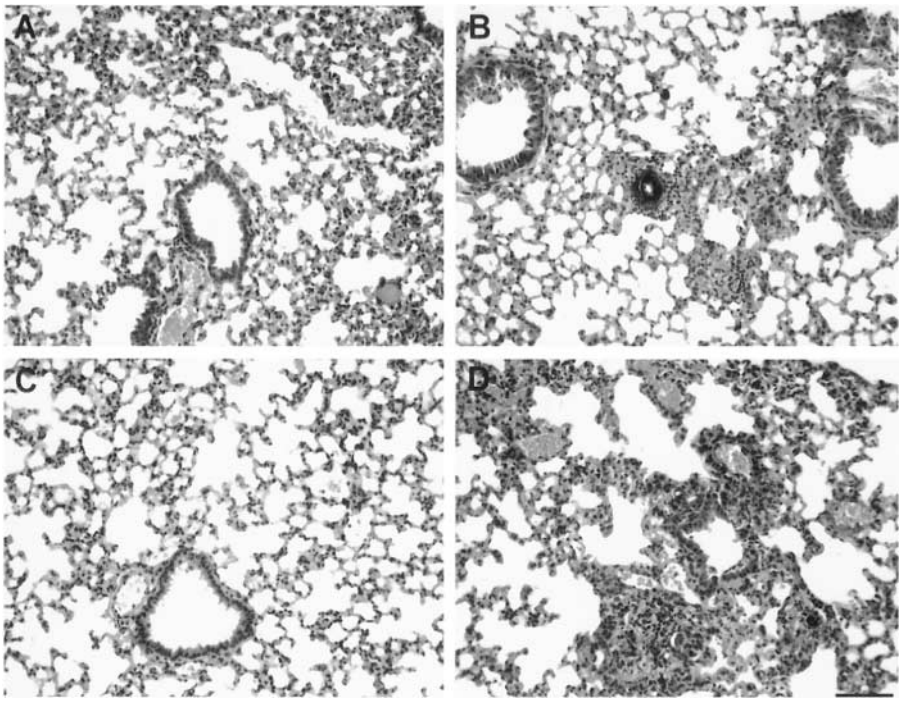

Figure 4. Photomicrographs of histological lung sections from C57BL/6-129S3/SvImJ (wildtype) and C3-deficient ($C3^{-/-}$ mice. PBS control sections appear normal in both wildtype and $C3^{-/-}$ mice (*A* and *C*, respectively), whereas PM-exposed sections exhibit intense granulocytic inflammatory foci in areas of PM deposition (*B* and *D*). H&E stain. *Bar* = 50 μm.

dent on C3, as C3-deficient mice do not develop significant AHR after PM exposure. These findings are consistent with previous studies in guinea pig and mouse models of allergy and asthma (11, 12, 19). Specifically, guinea pigs deficient for the C3aR have impaired immediate bronchoconstriction upon challenge with allergen (19). Similarly, neither C3aR- (11) nor C3 (12)-deficient mice develop AHR in response to allergen challenge. Furthermore, we have previously demonstrated that a defect in the complement component 5 gene is associated with susceptibility to allergen-induced AHR in A/J mice (20). Support for a role for complement in human disease is provided by the demonstration that the levels of the anaphylatoxins (C3a and C5a) are elevated in the lungs of patients with asthma (21, 22), and that segmental allergen challenge induces increases in BAL levels of C3a and C5a (23). Additionally, the chromosomal regions containing the C5 and the C5aR genes have been identified as putative asthma susceptibility loci (24, 25). Taken together, these findings suggest that complement activation is a common pathway in development of AHR in response to a variety of stimuli.

PM exposure induces significant changes in vascular permeability as assessed by protein content in the BAL fluids. Although C3a has previously been thought to induce vascular leakage, depletion of the C3 gene paradoxically appears result in increased vascular permeability, as control $C3^{-/-}$ mice had higher protein content in their lavage fluids than PBS-exposed wild-type mice. This protein level was further elevated after PM exposure. Although the mechanism is unknown at this time, these results suggest that endogenous expression of C3 provides protection against vascular leakage under normal conditions and after exposure to particulates. Further studies are clearly needed to elucidate the role of C3 in maintenance of vascular integrity.

As assessed by immunohistochemical staining, PM exposure leads to C3 deposition along the epithelial surface and connective tissue of large airways after exposure to PM. The exact source of C3 in this model is not known, but it may originate from the bloodstream as a result of leakage of complement components into the airways via the bloodstream, as significant vascular leakage (i.e., increased protein levels) was observed after PM exposure. Alternatively, recent studies suggest that complement components are produced locally in the lung. Studies have shown that there are several sources of complement in the lung, including alveolar macrophages (9) and airway epithelial cells (26). Although epithelial cells stain positive for C3, we do not know whether these cells are the actual producers of C3 in our model or if C3 is produced by other cells and just binds to the epithelium. However, it has been shown that airway epithelial cells produce C3 *in vitro* (8, 26), thus it is likely that the epithelium is the major source of C3. Additionally, C3 was detected in the smooth muscle and connective tissue surrounding the large airways. Again, it is not known whether smooth muscle cells also contribute to PM-induced C3 deposition in the lung or if C3 from another source is binding to smooth muscle cells and/or other components of connective tissue.

Although the mechanisms by which C3 mediates AHR are not known, C3a has many actions that may either directly or indirectly contribute to the development of AHR. First, both C3a and C5a are known to induce airway smooth muscle contraction in guinea pigs and humans both *in vivo* and *in vitro* (*see* review in Ref. 7). This is consistent with the existence of receptors for these mediators on airway smooth muscle (10). Further support for a direct effect of C3 on airway smooth muscle in the current study is the fact that PBS-exposed C3-deficient mice exhibit lower airway reactivity than do PBS-exposed wild-type

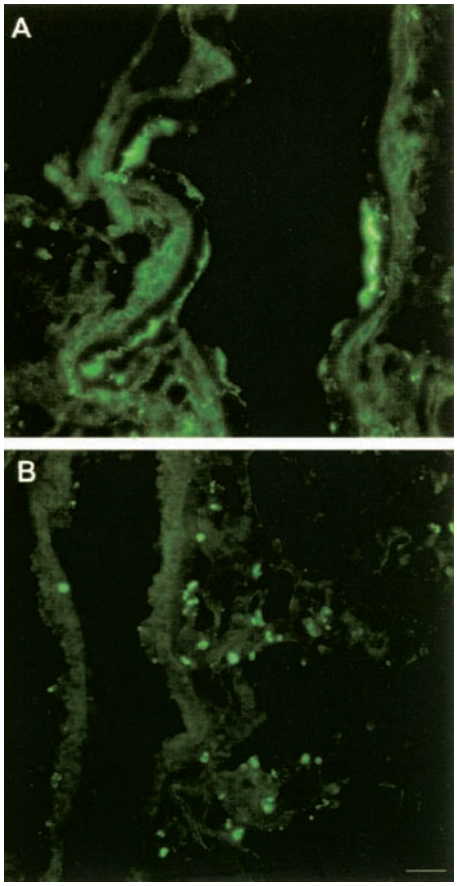

Figure 5. Photomicrographs of frozen lung sections from C57BL/6-129S3/SvImJ (wildtype), mice stained with (*A*) anti-C3 antibody or (*B*) Ig isotype control antibody after challenge with ambient PM. PM exposure induced C3 deposition along the epithelium and connective tissue of large airways. *Bar* = 50 μm.

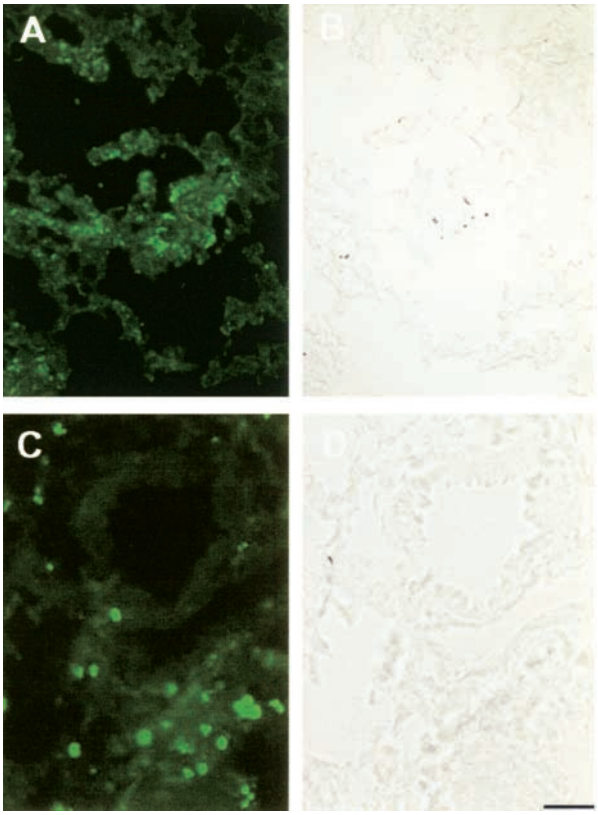

Figure 6. Photomicrographs of frozen lung sections from (*A* and *B*) C57BL/6-129S3/SvImJ (wildtype) and (*C* and *D*) C3-deficient ($C3^{-/-}$) mice stained with anti-C3 antibody or unstained after PM challenge. Macrophages that accumulate in areas of PM deposition stain non-specifically. *Bar* = 50 μm.

mice, suggesting that C3 may play a role in regulation of baseline airway tone. Alternatively, C3 may induce AHR via indirect effects on other cells in the lung which express receptors for these anaphylatoxins such as eosinophils, basophils, mast cells, monocytes, neutrophils, activated lymphocytes, and several nonmyeloid cell populations. For example, C3a and C5a have been shown to cause histamine and mediator release from mast cells (27). In addition, they have been shown to be chemoattractants for effector cells such as eosinophils and neutrophils (7). In the present study, PM-induced AHR does not appear to be dependent on the chemotactic activity of C3, as both the type and magnitude of the inflammatory response to PM exposure were equivalent between $C3^{-/-}$ and wild-type mice. A similar lack of effect on allergen-induced airway inflammation was observed in C3aR-deficient mice (11). Taken together, these studies seem to dispel the notion that anaphylatoxins play a major role in the chemotaxis of inflammatory cells in the lung.

Although the exact component(s) of PM that induce local production of C3 are unclear, other studies support our findings that PM exposure can activate the complement cascade (28–30). Several studies have shown that particulate air pollution can induce increases in the concentration of complement activation products in sera of individuals exposed to high levels of PM (28–30). Additionally, both cigarette smoke and diesel exhaust have been shown to activate complement via the alternative pathway (31–33). Interestingly, reactive oxygen species have also been shown to activate complement, highlighting the possibility that PM may activate complement via induction of reactive oxygen species (34, 35). It is tempting to speculate that AHR induced by other oxidizing substances such as ozone may also be mediated through complement activation. Koren and coworkers found that C3a was increased in BAL fluid from humans exposed to ozone (36). Interestingly, ozone-induced AHR and airway inflammation have been shown to be regulated by different genes, suggesting that complement activation may underlie the development of ozone-induced AHR as well (37).

In summary, our studies demonstrating the requirement of C3 for PM-induced AHR provide a plausible mechanism by which environmental pollutants may induce AHR. Furthermore, our results suggest a common mechanism by which different stimuli such as allergens and pollutants may elicit similar changes in airway reactivity. Further studies are underway to define the exact mechanisms by which PM induces the production of complement in the airways.

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