Cutting Edge: The Absence of C3 Demonstrates a Role for Complement in Th2 Effector Functions in a Murine Model of Pulmonary Allergy

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Asthma is a chronic disease of the lung characterized by airflow obstruction, airway hyperresponsiveness (AHR), and airway inflammation. Although asthma is multifactorial in origin, atopy, the genetic predisposition for the development of an IgE-mediated response to common aero-allergens, is the strongest identifiable predisposing factor for the development of asthma (1). The inflammatory process is thought to arise from inappropriate responses to commonly inhaled allergens (2, 3) with subsequent exposure to allergens initiating a secondary humoral response. Following an allergen provocation, the allergic reaction can be divided into both early (within minutes) bronchospastic responses and late (hours after exposure) inflammatory responses. The early response is associated with mast cell degranulation and is characterized by increases in mucosal edema, airway smooth muscle tone, and airway narrowing (4). The late-phase response begins 3–6 h after allergen challenge, and the airway narrowing is associated with migration of neutrophils, eosinophils, and lymphocytes from the blood into lung parenchyma and airway epithelium (4–6).

This classification of asthma has led to the concept that the immediate response after allergen challenge is mediated by mast cells, whereas eosinophils are the predominant effector cells in the late asthmatic reaction. Recent research efforts have also demonstrated that CD4+ T cells which produce a Th2 pattern of cytokines play a pivotal role in the pathogenesis of this disease (7). Through the release of cytokines such as IL-4, IL-13, and IL-5, these cells are thought to contribute to bronchial hyperreactivity and mucus hypersecretion as well as orchestrate the recruitment and activation of mast cells and eosinophils. Studies have demonstrated that blocking of IL-4 reduces AHR in the lung (8) and that RAG−/− mice, which lack Th2 cells, fail to develop AHR, mucus hypersecretion, and eosinophilia during the course of asthma (9). IL-4 also plays a prominent role in allergy by initiating B lymphocyte differentiation and production of IgE Abs (10, 11).

Traditionally, classical atopic reactions and asthma were considered simple type I reactions, and, as such, the complement system was not considered important in their pathogenesis (12). This bias has persisted even though the complement anaphylatoxin peptides exhibit properties resembling IgE-mediated anaphylaxis, such as contracting smooth muscle, activating mast cells, and increasing vascular permeability (reviewed in Ref. 13). However, recent evidence has surfaced forcing a reevaluation of the importance of complement in asthma. Specifically, elevated levels of complement anaphylatoxin peptides have been observed in the lungs of asthmatic patients (14–16), and the C5 gene and the C5αR receptor genetic region have been identified as putative asthma susceptibility loci (1, 17). Furthermore, models of pulmonary allergy have demonstrated that OVA-challenged C5a receptor (C5aR)-deficient mice exhibit decreased AHR upon methacholine challenge (16), guinea pigs deficient for the C5αR display reduced immediate bronchoconstriction upon challenge with OVA (18), and guinea pigs exposed to cobra venom factor have reduced lung eosinophilia when challenged with the occupational allergen trimetallic anhydride (19, 20). Finally, C5αR and C5αR expression...

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has been demonstrated on bronchial smooth muscle cells, implicating these receptors as mediators of bronchoconstriction (21). Although significant, these studies have not provided information that address the overall requisite role of the complement system in the pathogenesis of asthma using a model of pulmonary allergy in the complete absence of C3, nor have they described alterations in the regulation of IL-4 or IgE, which are major characteristics of asthma. Accordingly, to elucidate the role of complement in asthma, we have studied a murine model of pulmonary allergy using an allergen derived from the fungus Aspergillus fumigatus and chicken OVA in mice deficient in the third component of the complement system (21). Data from these mice reveal diminished AHR, eosinophilia, and IL-4 production in the lung as well as reduced Ag-specific IgE and IgG1 responses and clearly demonstrate that these major symptomatic hallmarks of asthma are significantly attenuated in the absence of C3, thereby indicating the major contribution of complement in the pathogenesis of asthma.

Materials and Methods

Mice
The C3-deficient (C3−/−) mice used have been described previously and produce no serum C3 (22). These mice lack the ability to generate the anaphylatoxins C3a and the opsonin C3b and have no serum complement lytic activity (22). The C3−/− mice were backcrossed eight generations onto the C57BL/6 background, and their wild-type littermates (C3+/+) were used as controls. This work was conducted in accordance with institutional and National Institutes of Health guidelines and care.

Sensitization and challenge protocol
The Ag preparation is a mixture of A. fumigatus culture filtrate prepared free of living organisms as described (23) and OVA (Sigma, St. Louis, MO). A. fumigatus was used in these studies because it is an allergen known to cause human asthma and it induces consistent AHR in C57BL/6 mice whereas OVA alone does not (8). OVA was added to detect Ag-specific immunoglobulin levels after AHR measurements to ascertain immunization, whereas OVA alone does not (8). OVA was added to detect Ag-specific immunoglobulin levels after AHR measurements to ascertain immunization, whereas OVA alone does not (8). OVA was added to detect Ag-specific immunoglobulin levels after AHR measurements to ascertain immunization, whereas OVA alone does not (8). OVA was added to detect Ag-specific immunoglobulin levels after AHR measurements to ascertain immunization, whereas OVA alone does not (8).

Bronchoalveolar lavage (BAL) measurements
Airway inflammation was assessed by BAL. Lungs were lavaged three times with 1 ml of PBS. Approximately 2.4 ml of fluid was routinely recovered, and total cell numbers were determined using a hemacytometer. Differential cell counts were obtained from BAL cells spun onto slides with a cytocentrifuge (Shandon Lipshaw, Pittsburgh, PA) and treated with Wright-Giemsa stain (Sigma). A total of 500 cells were classified based on characteristic morphology revealed after staining. Absolute numbers of specific cell types (neutrophils, lymphocytes, macrophages, and eosinophils) were calculated from the recovered BAL volume, total cell count, and percent abundance of specific cells.

ELISPOT assays
Single-cell suspensions from whole lung cells were distributed in duplicate aliquots (104 cells of RPMI 1640 with 5% FBS and antibiotics) into 96-well microtiter plates (Immulon IV; Dynatech Laboratories, Chantilly, VA) that had been precoated with either mAb 11B11 against IL-4 or mAb AN-18 against IFN-γ. Serial 2-fold dilutions were prepared, and the plates were incubated undisturbed for 8 h at 37°C. Wells were washed with PBS to remove cells and incubated with biotinylated secondary Abs against IL-4 (BVD6-24G2) or IFN-γ (XMG1.2). After 1 h, wells were washed and incubated for 1 h with 100 μl of streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS with 0.05% Tween 20 and 5% FBS. Color was developed with 5-bromo-4-chloro-3-indoyl phosphate in 0.1 M 2-amino-2-methyl-1-propanol buffer (Sigma) suspended in 0.6% agarose. After solidification of the agar, individual blue spots were counted by inverted microscopy (8).

Total IgE and Ag-specific immunoglobulin measurements
Serum IgE titers were determined by two-site sandwich ELISA using an ELISA kit (BD PharMingen, San Diego, CA) as per the manufacturer’s specifications. For OVA-specific Ab analyses, wells of microtiter plates were precoated with 40 μl OVA (20 μg/well) in PBS and blocked with 5% BSA in PBS with 0.05% Tween 20. Serum diluted 1/500 was then added and incubated for 1 h at 37°C. Plates were washed, and biotinylated isotype specific Abs (anti-IgE, IgG1, and IgG2a; Southern Biotechnology Associates, Birmingham, AL) were added for an additional hour of incubation. Plates were washed again and streptavidin-conjugated alkaline phosphatase was added for 30 min at room temperature. After a final wash, plates were developed with 0.5 M nitrophenyl phosphate in 0.1 M 2-amino-2-methyl-1-propanol buffer (Sigma). Data are expressed as optical density readings at 405 nm.

Statistical analysis
Statistical analysis was performed using the Prism software package (GraphPad, San Diego, CA). Statistical significance of all results were assessed using the two-tailed, unpaired Student’s t test.
Results

Effect of C3 deficiency on AHR after Ag challenge

Mice deficient in C3 lack a functional complement system and are devoid of numerous activation fragments that mediate potent biological activities during immune and inflammatory responses (22). Given the wide ranging effects of the complement system and their potential contribution to the pathogenesis of asthma, we assessed the role of the complement system in vivo. In a mouse model of pulmonary allergy, C3<sup>−/−</sup> and C3<sup>+/+</sup> mice were challenged with an allergen derived from the fungus <i>A. fumigatus</i> and OVA. Consistent with the model, wild-type mice developed an asthma-associated response characterized by an increase in AHR as revealed by enhanced sensitivity to ACh challenge (8) (Fig. 1). In contrast, allergen-challenged C3<sup>−/−</sup> mice showed an attenuation in AHR sensitivity and revealed a 50% reduction in AHR compared with the wild-type littermates. Furthermore, the AHR in the C3<sup>−/−</sup> mice approached that observed in the PBS C3<sup>+/+</sup> (Fig. 1) and C3<sup>−/−</sup> (data not shown) controls, indicating that complement plays a significant role in regulating AHR in this model.

Effect of C3 deficiency on inflammatory cell recruitment in Ag-challenged lungs

An additional facet of asthma is the pulmonary inflammation that results from the presence of white blood cells, specifically eosinophils, recruited into the airways (8). Eosinophils are chemotactic to the C3a and C5a anaphylatoxins (25), and lack of production of these anaphylatoxins in the C3-deficient mice could affect eosinophil recruitment into the lung during asthma. To determine whether complement contributes to eosinophil recruitment, lungs from challenged mice were lavaged after the AHR measurements, and the BAL fluid was examined for influx of white blood cells (Fig. 2). Quantitation of white blood cells from the BAL revealed a 42% reduction in eosinophil recruitment in C3<sup>−/−</sup> mice relative to wild-type controls, supporting the view that complement activation plays an important role in regulating eosinophil recruitment into the lung during asthma.

Effect of C3 deficiency on IL-4 and IFN-γ production in Ag-challenged lungs

In contrast to the reduced eosinophil levels in the BAL, C3-deficient mice had 1.7- and 2.9-fold higher levels of macrophages and neutrophils, respectively, compared with their wild-type littermates (Fig. 2). Previous reports have described that blocking of IL-5 or the Th2 response in T cell-deficient RAG<sup>−/−</sup> mice not only reduces eosinophilia but also results in a concomitant increase of lung neutrophils and macrophages in this model (8, 9). Considering these observations and that T cells in the lung are polarized toward a Th2 response during asthma (1), we performed additional studies to understand the role of complement with respect to T cell effector function. To assess T cell activation in C3<sup>−/−</sup> mice, IL-4 and IFN- γ production in the lung was quantitated by ELISPOT and evaluated as a marker of Th2 vs Th1 responses after allergen challenge (Fig. 3). No statistical differences in the number of IFN-γ-producing cells were detected in the lungs of C3-deficient and wild-type animals suggesting no effect on Th1 cells. However, cells expressing IL-4 were reduced 69% in C3<sup>−/−</sup> mice compared with the C3<sup>+/+</sup> controls. The reduced numbers of IL-4-producing cells indicate that complement activation is involved in IL-4 regulation in the lung and, thus, may promote a Th2 response during asthma.

Effect of C3 deficiency on Ag-specific immunoglobulin levels

Increased production of IgE Abs during the course of asthma is another characteristic of the disease. Studies have demonstrated that C3 activation fragments can directly influence B cell responses through CD21 (26) and that IL-4 can also stimulate B lymphocyte production of IgE Abs (27). To determine whether IgE levels were suppressed in the C3-deficient mice in the pulmonary allergy model, total serum IgE was measured after allergen challenge. IgE levels in C3-deficient mice were significantly reduced by 49% compared with the wild-type mice (C3<sup>+/+</sup>, 1.95 ± 0.21 μg/ml, n = 5; C3<sup>−/−</sup>, 0.98 ± 0.31 μg/ml, n = 5; p < 0.05). Furthermore, Ag-specific IgE, IgG1, and IgG2a isotypes were examined in C3<sup>−/−</sup> mice (Fig. 4). C3 knockout and wild-type mice had similar levels of IgG2a, but Ag-specific IgE and IgG1 levels were decreased 70 and 80%, respectively, in the C3<sup>−/−</sup> animals. These results are consistent with the premise that IgE and IgG1 production, which is controlled by IL-4 (27), should be reduced as a result of decreased IL-4 production in the C3-deficient mice. Moreover, expression of IgG2a, which is regulated by IFN-γ (27), would be similar between C3-deficient and wild-type animals because no differences were observed with IFN-γ production. Although C3 could still contribute to Ab synthesis through the binding of C3d to CD21 expressed on B cells (26), these results suggest that the decreased IgE levels result from reduced IL-4 production in the C3-deficient mice.

FIGURE 2. Effect of C3 deficiency on inflammatory cell recruitment in Ag-challenged lungs. Quantitation of cells in the BAL fluid from C3<sup>−/−</sup> (■) and C3<sup>+/+</sup> littermates (□) was assessed 24 h after the last Ag challenge. Mac, macrophages; Neu, neutrophils; Eos, eosinophils; Lym, lymphocytes. Results are plotted as means ± SEM (C3<sup>−/−</sup>, n = 7; C3<sup>+/+</sup>, n = 7), and significant differences between wild-type and knockout mice are indicated as *p < 0.05 and **p < 0.01 as determined by Student’s t-test.

FIGURE 3. Effect of C3 deficiency on IL-4 and IFN-γ production in Ag-challenged lungs. IL-4 and IFN-γ-producing cells in the lungs from C3<sup>−/−</sup> (■) and C3<sup>+/+</sup> littermates (□) were quantitated 24 h after the last Ag challenge. Results are plotted as means ± SEM (C3<sup>−/−</sup>, n = 5; C3<sup>+/+</sup>, n = 5), and significant differences between wild-type and knockout mice are indicated as *p < 0.01 as determined by Student’s t-test. For the IFN-γ ELISPOT, only 10.0 ± 10 cells/ml IFN-γ-producing cells were observed in C3<sup>−/−</sup> mice, and no IFN-γ-producing cells were detected in C3<sup>+/+</sup> littermates.
Discussion

The importance of this study resides not only in the determination that complement contributes significantly to the pathogenesis of asthma in a pulmonary allergy model but also in the novel discovery that this branch of the innate immune system impacts production of IL-4, a Th2 cytokine crucial to the development of AHR and IgE responses in asthma. The complement system has recently received attention in studies of this disease, as animals deficient in the C3aR have decreases in AHR and bronchoconstriction when challenged with allergen (16, 18). However, these studies did not describe alterations in the regulation of IL-4 or IgE, which are major characteristics of asthma. The data reported here provide important mechanistic insight and implicate the complement system as a regulator of Th2 development in asthma. The failure in eosinophil recruitment and IL-4 and IgE production, as well as the attenuation of AHR, in both C3-deficient and T cell-deficient mice (9) support this view. These results lay the foundation for additional studies directed at delineating the proteins of the complement system that contribute to airway obstruction in the lung as well as regulate Th2 responses. Initial studies have documented that C5a modulates production of IL-12 (28), a cytokine which suppresses Th2 responses, and implicates the parent protein C5 in the pathogenesis of asthma through IL-12 regulation (29). The gene encoding C5 has also been identified as a susceptibility locus for allergen-induced allergic airway responses in a murine model of asthma (29). Furthermore, although other white blood cells make up a smaller fraction of IL-4-producing cells in asthma compared with Th2 lymphocytes (30), C5a has been shown to regulate IL-4 expression by basophils (31), which may contribute to Th2 development in the initial stages of asthma. Additional complement receptors such as the C3ar (32) and Crry (33) have been shown to activate T lymphocytes in vitro but, as yet, do not have a known role in regulating T cell responses in vivo. Given the important contribution of Th2 responses and IL-4 production in asthma, further study of complement-mediated regulation of T lymphocytes may shed light in the causative events leading to AHR and airway inflammation in this disease.

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References


