Asthma progression to airway remodeling and bone marrow eosinophil responses in genetically distinct strains of mice

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Background: Patient factors that cause long-term airway remodeling are largely unidentified. This suggests that genetic differences may determine which asthmatic patients develop airway remodeling. A murine model with repeated allergen exposure leading to peribronchial fibrosis in complement factor 5 (C5)–deficient A/J mice has been used to study asthma progression. No studies have addressed the systemic effects of allergen sensitization or chronic allergen exposure on bone marrow eosinophilopoiesis in this mouse strain.

Objective: To investigate bone marrow eosinophil responses during acute sensitization and chronic allergen exposure using genetically distinct mouse strains differing in persistent airway reactivity and remodeling.

Methods: The C5-sufficient BALB/c and C5-deficient A/J mice were repetitively exposed to intranasal ovalbumin for 12 weeks. Subsequently, the mice were evaluated for airway eosinophilia, mucus-containing goblet cells, and peribronchial fibrosis. Both strains of mice were also acutely sensitized to ovalbumin. Bone marrow eosinophil progenitor cells and mature eosinophils were enumerated.

Results: BALB/c and A/J mice have similar bone marrow responses after acute allergen exposure, with elevations in bone marrow eosinophil progenitor cell and eosinophil numbers. After chronic allergen exposure, only C5-deficient A/J mice that developed peribronchial fibrosis exhibited bone marrow eosinophilia. BALB/c mice lacked peribronchial fibrosis and extinguished accelerated eosinophil production after long-term allergen challenge.

Conclusions: Chronic airway remodeling after repeated allergen exposure in genetically different mice correlated with differences in long-term bone marrow eosinophilopoiesis. Preventing asthma from progressing to chronic airway remodeling with fibrosis may involve identifying genetically determined influences on bone marrow responses to chronic allergen exposure.

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INTRODUCTION

Allergic asthma is a progressively debilitating disease that is initially characterized by periodic reversible airway obstruction and pulmonary accumulation of inflammatory cells in response to allergen exposure. In many patients, repeated exposure to allergen leads to persistent eosinophilic infiltration of the lungs and irreversible airway damage, including epithelial cell thickening, goblet cell metaplasia, peribronchial fibrosis, and smooth muscle hypertrophy.[1] These irreversible changes can result in permanent loss of airway function.[2] The clinical presentation of factors such as atopy, asthma duration, and disease severity does not seem to correlate with the degree of airway remodeling present.[3,4] The biological mechanisms that underlie the persistence of airway disease and progression to chronic irreversible airway remodeling in some patients but not in others are poorly understood.[5] The presence of these dissimilar phenotypes in asthmatic patients suggests that genetic differences may exist in these groups.

The A/J mouse is a well-established model of persistent airway reactivity. Shinagawa and Kojima[6] reported that A/J mice differ in response from C57BL/6, C3H/HeJ, and BALB/c mice in response to chronic allergen exposure. BALB/c mice, for example, were mild responders to allergens and initially developed classic pulmonary findings of eosinophilia, mucus production, increased secretion of inflammatory cytokines, and airway hyperreactivity. However, after 12-week repeated intranasal exposure to allergen, BALB/c mice no longer exhibited airway hyperreactivity, and they lacked chronic airway remodeling. After the same allergen exposure regimen, A/J mice, in contrast, developed pathologic findings consistent with chronic airway remodeling: significant bronchoalveolar lavage eosinophilia, mucus cell hyperplasia, smooth muscle layer thickening, airway collagen deposition, and heightened airway hyperreactivity.

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Recently, Karp et al[7] used microarray analysis combined with quantitative trait locus analysis to identify complement factor 5 (C5) as an asthma susceptibility locus for airway hyperresponsiveness in A/J mice. A/J mice are C5 deficient due to a 2–base pair (bp) deletion in the C5 gene on murine chromosome 2 that renders them lacking in functional C5 protein and, therefore, also lacking in the C5 cleavage product, C5a anaphylatoxin.[8] An additional locus for "allergen-induced bronchial hyperresponsiveness" in the A/J mouse is also located on chromosome 2 but at a location distinct from C5.[9]

Other clues to asthma etiology are emerging from studies of bone marrow changes in asthma. Recent studies demonstrate that allergen exposure systemically affects bone marrow function. Bone marrow eosinophilopoiesis is selectively altered in asthmatic patients[10] and in animal models of asthma after allergen challenge, resulting in bone marrow, blood, and airway eosinophilia.[11–14] This is most clearly demonstrated by studies[14,15] in which allergen challenge in humans and animals resulted in expansion of the eosinophil progenitor cell (CFU-eo) pool and mature eosinophils in bone marrow. One study[16] demonstrated that accelerated eosinophilopoiesis that occurs after initial intranasal sensitizing exposures to ovalbumin is, in part, T-cell independent and precedes the development of specific IgE antibody. In animals sensitized to allergen, the resulting eosinophilia is regulated by extrinsic factors, including T-cell–derived interleukin 5 (IL-5). However, the role of bone marrow in regulating eosinophil production after chronic allergen exposure has not been studied in mice or humans.

Herein, we question whether acute sensitization or long-term exposure to intranasal ovalbumin would result in different bone marrow responses in C5-deficient (with airway remodeling) and BALB/c mice after long-term allergen exposure. These data demonstrate that both strains of mice have similar bone marrow responses to acute airway allergen exposure associated with asthma induction. However, output of mature eosinophils from the bone marrow is dramatically different in these 2 strains of mice after repeated intranasal allergen exposure. C5a-deficient mice that exhibit airway remodeling in chronic asthma also had altered bone marrow eosinophil production compared with BALB/c mice, which rapidly extinguish the eosinophil response and do not develop pathologic evidence of airway remodeling. These results suggest that genetic differences can affect airway remodeling responses and long-term bone marrow eosinophil production in chronic asthma.

METHODS

Mice

Female, 4- to 6-week-old, genetically different (C5a-sufficient) BALB/c or C5a-deficient (A/J) mice were obtained from Jackson Laboratories (Bar Harbor, Maine). The mice were housed in autoclaved microisolator cages (Lab Products, Maywood, New Jersey). Autoclaved food and acidified water (pH, 2.8) were provided ad libitum. A 12-hour light-dark cycle was provided. All the procedures were approved by the West Virginia University Animal Care and Use Committee, which follows the Guide for the Care and Use of Laboratory Animals.

Chronic Allergen-Induced Asthma Model

For mice undergoing allergen sensitization for chronic asthma, ovalbumin, 2 mg/mL (Sigma-Aldrich Corp, St Louis, Missouri), allergen exposure was performed intranasally 3 times weekly after mice were anesthetized with 2 mg of ketamine, 20 mg/mL (A. Webster, Sterling, Massachusetts) delivered intraperitoneally. Ovalbumin was delivered intranasally 3 times weekly in 25 μL of endotoxin-free 0.9% saline (Baxter Pharmaceutical Solutions, Round Lake, Illinois) for 12 weeks.[6] No initial or ongoing exposure to intraperitoneal ovalbumin or intraperitoneal adjuvants, such as alum, were used in this model. Control groups consisted of mice exposed intranasally to 25 μL of endotoxin-free saline only and instilled at the same time that ovalbumin-exposed mice were treated. Groups consisted of 3 to 5 mice. End point measurements included lung abnormalities and morphometric findings. Bone marrow total white blood cell counts, bone marrow eosinophil counts, and CFU-eo numbers also were measured 24 and 72 hours after the last ovalbumin exposure.

Initial Allergen Sensitization Model

Briefly, optimized doses of ovalbumin–aluminum potassium sulfate were provided by means of intraperitoneal injection: 100 mg/kg suspended in a saturated solution of aluminum potassium sulfate (alum; Sigma-Aldrich Corp) in sterile distilled water on days 0 and 10 as previously reported.[16] In addition, mice were sedated with 2 mg of ketamine on day 10, and the first intranasal dose of 100 μg of ovalbumin was instilled into the lungs by the intranasal route. In this model, the sensitization period ends after day 19 when intranasal challenge doses are given on days 20 and 21, and acute asthma responses are studied thereafter.[11] Control groups consisted of mice exposed to intraperitoneal or intranasal endotoxin-free saline only matched to the same time that ovalbumin-exposed mice were treated. All the groups contained 3 to 5 mice. End point measurements included bone marrow total white blood cell count, bone marrow eosinophil count, and CFU-eo numbers on day 13 of the sensitization period (3 days after the first intranasal ovalbumin exposure).

Lung Abnormalities and Morphometric Findings

Lung samples obtained 1 day after the last ovalbumin exposure were embedded in paraffin and were stained with hematoxylin-eosin, an eosinophil stain using a modified Hansel staining technique for eosinophils with substitution of Mayer hematoxylin for methylene blue, Alcian blue/periodic acid–Schiff (PAS), and trichrome. Histologic evaluation was conducted by a board-certified veterinary pathologist (A.F.H.) blinded to the exposure status of the study mice. Histopathologic diagnoses were converted to semiquantitative scores for nonparametric statistical analysis. Severity was converted to numeric scores, as previously described elsewhere.[17]

Morphometric analysis of subepithelial airway fibrosis was quantified by measuring collagen in sections of left lung lobe stained with Masson trichrome using commercial morphometry software (Metamorph; Universal Imaging Corp, Downingtown, Pennsylvania). All bronchioles and associated vessels in the left lung lobe were photographed using a 10× objective. Fibrosis was quantified by measuring the area of trichrome blue collagen contained in the basement membrane and tunica adventitia surrounding the airways and associated vessels. The vasculature was included in the sample area to avoid arbitrary separation of bronchiolar and vascular tunica adventitia in the inflamed lung. The fractional area that contained fibrous connective tissue was the area of fibrous connective tissue divided by the total area obtained in the basement membrane and tunica adventitia surrounding the airways and associated vessels. The percentage of fibrous connective tissue was the fractional area times 100%. In addition, 5 random alveoli samples obtained using a 20× objective were used to measure alveolar fibrosis.

Goblet cell metaplasia was measured in 10× photographs of all bronchioles in Alcian blue/PAS–stained sections of the left lung lobe. The area of mucus in the bronchiolar epithelium and the length of basement membrane beneath that bronchiolar epithelium were measured using commercial morphometry software (Metamorph).

Bone Marrow

Mice were euthanized by means of carbon dioxide asphyxiation. Bone marrow was obtained by flushing femora with α MEM (Gibco, Gaithersburg, Maryland) supplemented with 1% fetal calf serum (Summitt Biotechnology, Fort Collins, Colorado) using a syringe fitted with a 23-gauge needle. Total white blood cell count was obtained using a hemocytometer, and 10^5 bone marrow cells were cytocentrifuged. May-Grünwald-Giemsa stain (Sigma-Aldrich Corp) was used to identify bone marrow eosinophils. CFU-eo cultures were established, counted, and verified as CFU-eo as previously described.[16]

Statistical Analysis

Data analysis was performed using a 1-way analysis of variance with the Tukey posttest. In some studies, an unpaired *t* test was used. All statistical analyses were performed using a software program (GraphPad InStat; GraphPad Software, San Diego, California).

RESULTS

Repeated Airway Allergen Exposure and the Differential Development of Peribronchial Fibrosis

Development of airway remodeling in response to chronic allergen exposure has been reported to differ in BALB/c and A/J mice.[6] We sought to confirm this difference using histopathologic sections from 6-week-old BALB/c and C5a-deficient A/J mice exposed to intranasal ovalbumin 3 times weekly for 12 weeks. Only C5a-deficient mice developed significant peribronchial fibrosis using this protocol ($P < .01$). No significant increase in peribronchial collagen deposition was noted in ovalbumin-exposed BALB/c mice after long-term allergen exposure.

The area of peribronchiolar fibrosis was measured in sections of left lung from C5a-deficient mice. Only A/J mice with 12 weeks' exposure to intranasal ovalbumin had detectable peribronchiolar fibrosis (Fig 1A and B). Morphometric analysis revealed a 3-fold increase in the fractional area of peribronchiolar fibrous connective tissue in A/J mice exposed to ovalbumin compared with mice exposed to the saline diluent ($P < .03$) (Fig 1C). Alveolar fibrous connective tissue in BALB/c mice was unaffected by ovalbumin exposure ($P > .05$, data not shown). These data support the previously observed[6] difference in development of peribronchial fibrous tissue after repeated intranasal ovalbumin installation in BALB/c and C5a-deficient mice.

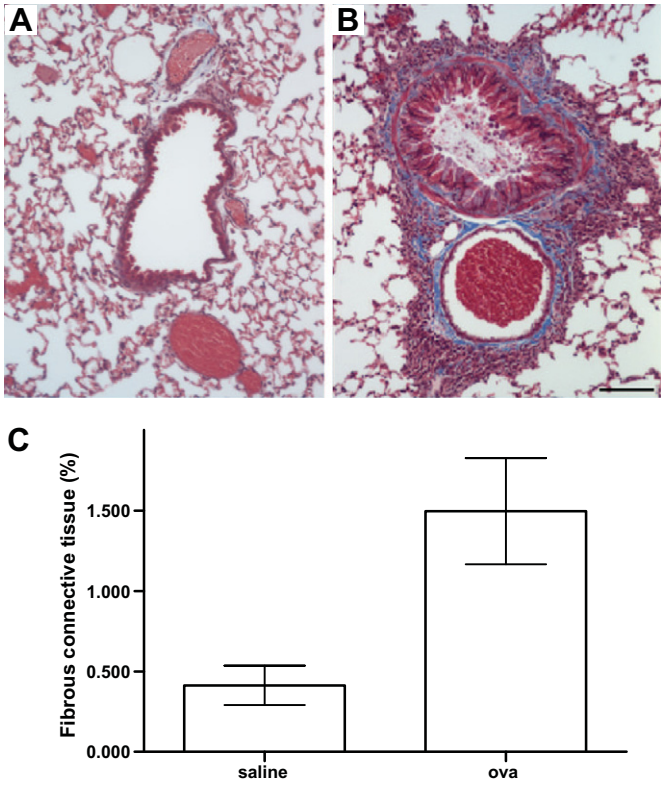

Figure 1. Comparison of airway collagen deposition between saline-treated control and ovalbumin-exposed A/J mice. Ovalbumin-exposed mice developed significant bronchiole-associated fibrosis. A, No evidence of alveolar fibrosis is noted in control mice. B, Fibrous connective tissue is blue and represents normal stromal elements. Bar represents 100 μm. C, Morphometric analysis of airway collagen deposition in complement factor 5a–deficient mice exposed to intranasal ovalbumin (ova) for 12 weeks. Significant increases in the percentage of area containing fibrous connective tissue were evaluated using an unpaired *t* test ($P < .03$). Data are given as mean (SEM).

Repeated Airway Allergen Exposure and Reversible Pathologic Findings of Asthma

In Alcian blue/PAS–stained sections of the left lung lobe, C5a-deficient A/J and BALB/c mice exposed to ovalbumin for 12 weeks had equivalent significant increases in mucus production compared with same strains of mice exposed to the saline diluent ($P \leq .001$, data not shown). Morphometric measurement of mucus-containing epithelial cells in ovalbumin-exposed A/J mice had demonstrable mucus metaplasia compared with mice exposed to saline ($P < .001$) (Fig 2A).

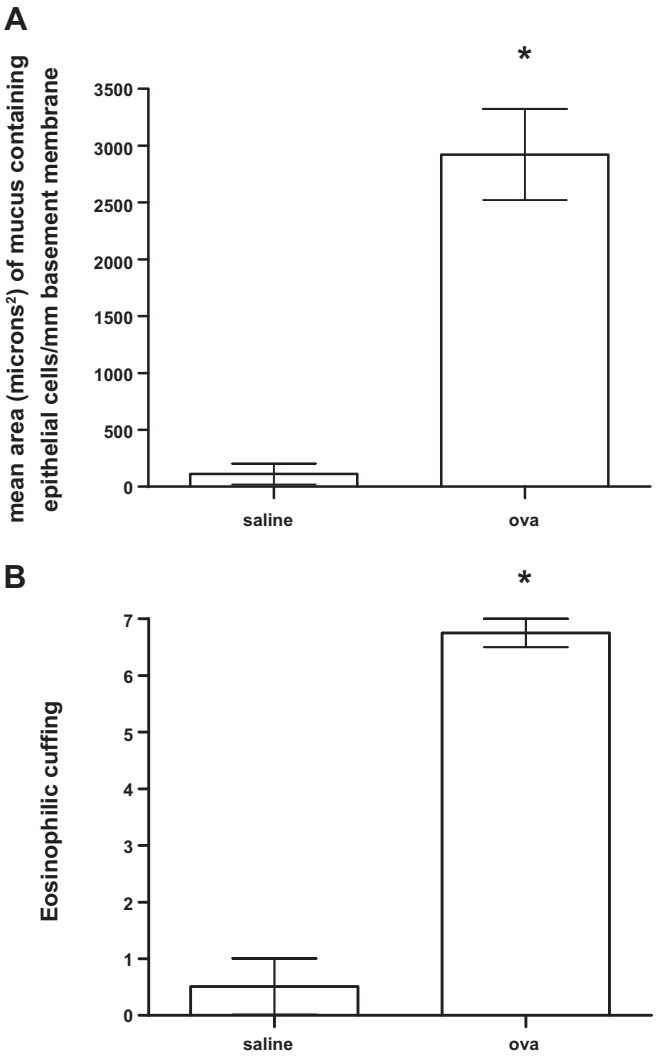

Figure 2. A, Comparison of mucus-containing epithelial cells between saline-treated control and ovalbumin-challenged (ova) A/J mice. A significant increase in the mean area of mucus-containing epithelial cells per millimeter of basement membrane was noted using an unpaired *t* test (*$P <$ .001). B, Histologic evaluation of eosinophilic peribronchial cuffing in saline-treated control vs ovalbumin-challenged A/J mice for 12 weeks. A significant increase in eosinophils in peribronchial areas was noted using an unpaired *t* test (*$P <$ .005). Error bars represent SEM.

Significant eosinophilic peribronchial cuffing was observed in ovalbumin-exposed A/J mice compared with saline-treated control mice ($P \leq .001$) (Fig 2B). Some eosinophilic peribronchial cuffing was detectable in ovalbumin-exposed BALB/c mice; however, this level of eosinophilic infiltration was less than that in C5a-deficient mice treated with the same allergen exposure regimen (data not shown).

Acute Bone Marrow Response to Allergen Sensitization

Initial exposure to allergen in BALB/c mice (allergen sensitization) resulted in a predictable temporal sequence of changes in CFU-eos and eosinophils in the bone marrow of BALB/c mice.[16] On day 3 after the first intranasal allergen exposure, the number of CFU-eos in bone marrow is significantly depressed, and the number of mature eosinophils in that tissue is dramatically increased. The CFU-eos rebound to super-normal levels in bone marrow by day 6 after the second allergen exposure. In the present study, no difference in baseline numbers of bone morrow CFU-eos or eosinophils was noted between A/J and BALB/c mice ($P > .05$) (Fig 3). We also compared the acute bone marrow response of BALB/c and A/J mice to intranasal allergen deposition. Mice were exposed to intraperitoneal and intranasal ovalbumin on days 0 and 10, with CFU-eos enumerated on day 13. BALB/c and A/J mice had significantly depressed CFU-eo numbers on day 3 after the last allergen exposure ($P < .01$) (Fig 3A). Eosinophil numbers were significantly elevated in both mouse strains on day 13 of the sensitization period ($P < .001$) (Fig 3B).

Repeated Airway Allergen Exposure and CFU-eo Numbers in Bone Marrow

To determine whether BALB/c and C5a-deficient mice differed in their bone marrow response to chronically inhaled allergen, age-matched cohorts of mice were exposed to ovalbumin 3 times weekly for 12 weeks, and bone marrow was harvested 24 and 72 hours after the last intranasal exposure. Long-term pulmonary allergen exposure of BALB/c and C5a-deficient mice during the 12-week protocol did not result in altered numbers of total nucleated bone marrow cells in any of the experiments presented ($P > .05$, data not shown). One day after the last ovalbumin dose, bone marrow CFU-eo numbers were elevated in A/J mice compared with their saline-treated controls ($P < .05$) (Fig 4); however, by 72 hours, postexposure numbers of CFU-eo had returned to baseline and were not different from saline-treated control mice ($P > .05$, data not shown).

Repeated Airway Exposure to Ovalbumin and Bone Marrow Eosinophilia

After acute exposure to intranasal ovalbumin, C5a-deficient and BALB/c mice had dramatically elevated numbers of bone marrow eosinophils, which peaked 72 hours after allergen exposure (Fig 5). Bone marrow eosinophilia was again determined 24 and 72 hours after 12 weeks of repeated allergen exposure. At 12 weeks, the number of bone marrow eosinophils in C5a-deficient mice was elevated 24 hours ($P < .05$)

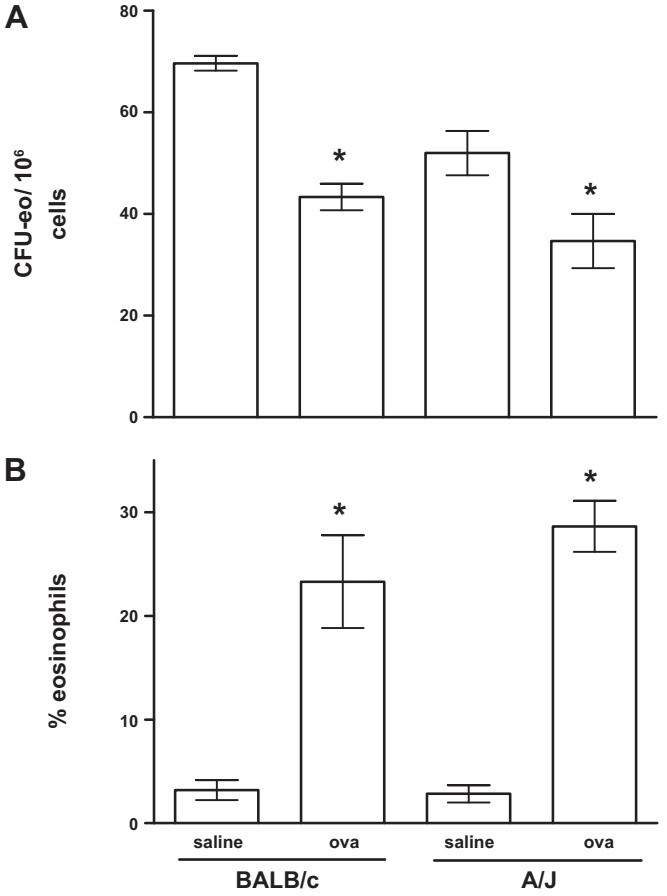

Figure 3. Effect of allergen sensitization on BALB/c and complement factor 5a–deficient bone marrow eosinophil progenitor cell (CFU-eo) (*$P \leq$ .01) (A) and eosinophil (*$P \leq$ .001) (B) numbers. Significant alterations in bone marrow of ovalbumin-exposed (ova) vs control mice were determined using 1-way analysis of variance with the Tukey posttest. Data are given as mean (SEM) and are representative of 2 identical experiments.

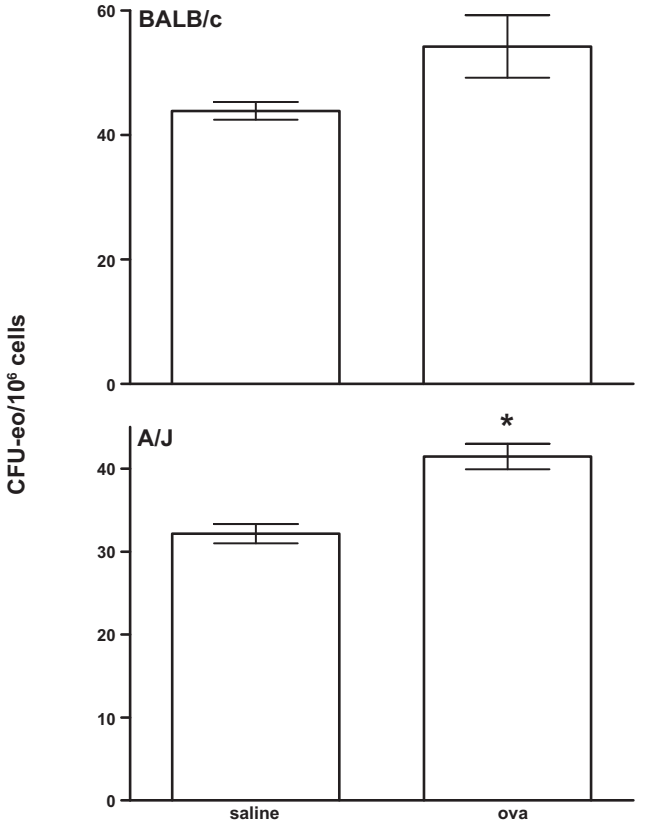

Figure 4. Effect of chronic pulmonary allergen exposure on bone marrow eosinophil progenitor cell (CFU-eo) numbers. Significant differences were noted in bone marrow CFU-eo in complement factor 5a–deficient mice 1 day after the last ovalbumin (ova) exposure (*$P < .05$, 1-way analysis of variance with the Tukey posttest). Data are given as mean (SEM) and are representative of 3 identical experiments.

and 72 hours ($P < .05$) after the final intranasal ovalbumin exposure.

DISCUSSION

In the present study, we evaluated whether bone marrow responses to acute or chronic allergen exposure differed in C5a-deficient and BALB/c mice, which differ in development of chronic peribronchial fibrosis in response to repeated allergen challenge. After acute sensitizing exposures to the allergen ovalbumin, C5a-deficient and BALB/c mice had equivalent reductions in CFU-eo numbers and expansion of mature eosinophils consistent with previous studies in BALB/c mice (Fig 4). As expected, repeated allergen exposure in C5a-deficient mice led to irreversible deposition of peribronchial fibrotic tissue and airway remodeling. The same treatment in BALB/c mice failed to cause peribronchial fibrosis despite persistence of asthma as demonstrated by the ongoing presence of pulmonary eosinophils and airway mucus production. We demonstrate, for the first time, that genetically different strains of mice have differences in ongoing mature bone marrow eosinophil production after chronic allergen challenge. This ongoing accelerated eosinophil production in C5a-deficient mice, which progresses to airway remodeling, may have clinical implications in determining which asthmatic humans have asthma disease persistence vs development of irreversible airway dysfunction.

Eosinophils are bone marrow–derived cells with relatively short life spans that are continuously replenished to maintain the circulating pool of functional cells.[13] After acute airway exposure to allergen in humans and mice with asthma, there is a well-documented wave of increased eosinophil production from bone marrow progenitor cells, followed by circulating and pulmonary eosinophilia.[12–14,16] Regulation of bone marrow eosinophil development remains relatively poorly understood in healthy and asthmatic individuals. Previous studies[18] determined that normal homeostatic eosinophilopoiesis primarily is regulated by a cascade of cytokines derived from resident stromal elements in bone marrow. Maintenance

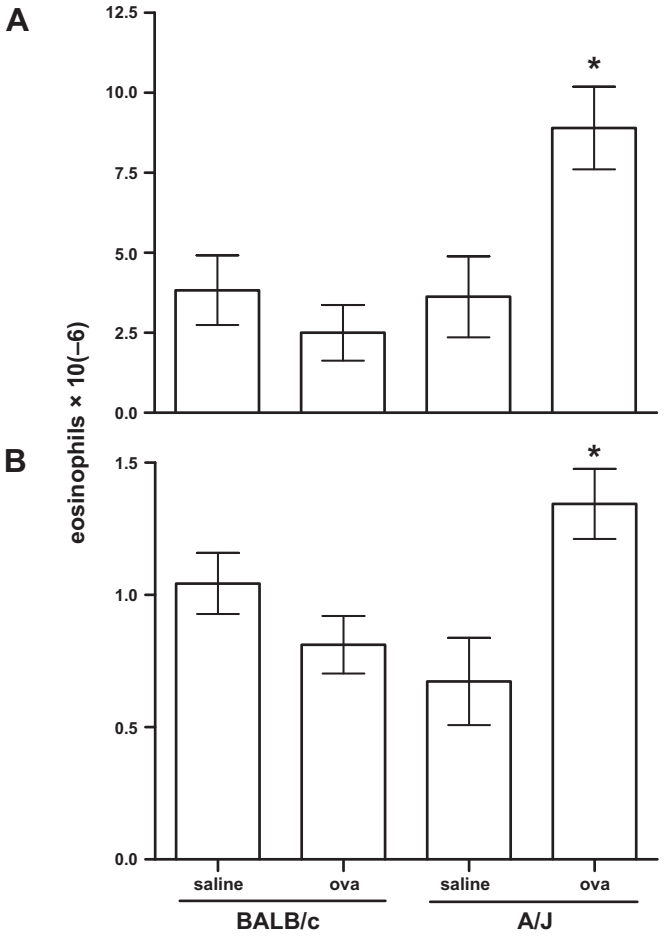

Figure 5. Effect of chronic pulmonary allergen exposure on bone marrow eosinophils in BALB/c and complement factor 5a (C5a)–deficient mice. A, Bone marrow eosinophilia is demonstrated in ovalbumin-exposed (ova) C5a-deficient mice compared with saline-treated control C5a-deficient mice (*$P < .05$, 1-way analysis of variance with the Tukey posttest) 24 hours after the last ovalbumin exposure. B, Continued bone marrow eosinophilia is demonstrated in ovalbumin-exposed A/J mice compared with ovalbumin-exposed BALB/c mice 72 hours after the last ovalbumin challenge (*$P < .05$, 1-way analysis of variance with the Tukey posttest). Data are given as mean (SEM) and are representative of 2 identical experiments.

of steady eosinophil numbers in bone marrow and circulation seems to be controlled primarily by local levels of IL-5 in bone marrow. Production of IL-5 can be demonstrated in bone marrow stromal cells, and normal numbers of eosinophils are produced in athymic nude mice, obviating the need for T lymphocytes in steady-state eosinophilopoiesis.[16,18] However, eosinophilia that follows acute exposure to allergen cannot be supported by bone marrow stromal elements. The sharp rise in eosinophil numbers in marrow that follows intranasal allergen installation requires higher concentrations of IL-5, produced by activated T lymphocytes.[19]

The present study revealed that no inherent difference exists in baseline bone marrow eosinophil numbers between C5a-deficient and BALB/c mice. In addition, after acute sensitizing exposures to the allergen ovalbumin, both C5a-deficient mouse strains had an equivalent reduction in CFU-eo and expansion of mature eosinophils consistent with previous studies in BALB/c mice (Fig 4). This demonstrates that C5a does not affect homeostatic control of bone marrow eosinophil numbers and that there are no substantive differences in these genetically different mice in eosinophil response at allergy-induced asthma initiation. Previous studies[16] documented that T lymphocytes and IL-5 are required for full expression of bone marrow eosinophilia in response to inhaled allergen. For these reasons, we postulate that the difference in the durability of the bone marrow response in BALB/c and C5a-deficient mice is due primarily to differences in T_H2 cell function rather than to intrinsic alteration of hematopoietic regulatory elements.

The C5a-deficient A/J mice have a 2-bp deletion in a 5′ exon of the C5 gene that results in failure to express functional C5.[7] In particular, C5a acts as an anaphylatoxin, stimulating production of many inflammatory cytokines, including IL-12, and suppressing airway hyperreactivity.[7] IL-12 is known to be a potent suppressor of T_H2 responses, and overexpression of IL-12 alone has proved sufficient to inhibit allergic asthma disease progression.[9] Deficiency of C5a in A/J mice resulted in maintenance of the bone marrow response to allergen, whereas C5a-sufficient BALB/c mice extinguished that response across time. This observation suggests that repeated elevation of C5a after multiple episodes of pulmonary inflammation may function by maintaining high concentrations of IL-12, which would, across time, lead to a predominance of T_H1 responses and loss of T-cell production of IL-5 expression in response to allergen exposure. We hypothesize that this skewing of the T-cell response in C5a-sufficient individuals could account for the ability to inhibit asthma progression to include airway remodeling and is a future line of investigation.

Why are these data of interest to clinicians? Clinical evidence of airway remodeling in humans as demonstrated by persistent pulmonary function deficits have been identified in many pediatric patients with atopic asthma.[20–23] However, the development of irreversible airway obstruction does not occur in every allergic asthmatic patient. Some physicians have even questioned the validity of the concept of airway remodeling based on the failure of many asthmatic patients to develop irreversible airway obstruction despite clear evidence of allergies and significant exposures to allergens.[24] Why this process does not occur in every patient is poorly understood.[3] Allergic asthma is known to be genetically linked,[25] and single nucleotide polymorphism–based genotyping has shown a link between C5 genotype and airway hyperreactivity.[26] On the basis of observations in the present study and those of other investigators discussed herein, it is tempting to speculate that chronic allergic asthma may be correlated directly with C5 expression and the ability of bone marrow to maintain responsiveness to inflammatory signals after repeated exposure to offending allergens. Together, these stud-

ies suggest that genetically determined C5a expression (or other unknown genetic determinants) during inflammatory responses may ultimately down-regulate exaggerated bone marrow eosinophil production in asthma and, thus, may protect against chronic tissue damage caused by eosinophil invasion and activation in affected pulmonary sites. The present study suggests that genetic factors could be involved in determining who, among humans, will continue to have accelerated bone marrow eosinophil production and develop airway remodeling with irreversible airway obstruction.

These studies expand our understanding of bone marrow participation in allergic asthma progression. It is now becoming clear in murine and human studies that airway remodeling in response to allergen exposure is linked to eosinophilic inflammation.[27,28] The studies detailed herein suggest a mechanistic link between control of allergen-induced bone marrow eosinophil production and the capacity to generate C5a in this model. They also suggest that clinical intervention strategies that stimulate expression of C5 may blunt bone marrow eosinophilia in patients with allergic asthma and prevent airway remodeling and permanent loss of lung function.

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