

Repeated Aerosol Allergen Exposure Suppresses Inflammation in B-Cell-Deficient Mice with Established Allergic Asthma

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Key Words

Allergy · Asthma · Mouse models · Inhalation tolerance · Immunosuppression

Abstract

Background: Repeated allergen administration is a well-established therapeutic strategy for desensitizing patients with allergic disease. Similarly, repeated inhalation of antigen by mice with established allergen-induced asthma suppresses allergic inflammation. The mechanisms underlying antigen-dependent suppression of allergic immune responses remain unknown. In previous studies, we found that repeated aerosol antigen challenges in sensitized mice reduced eosinophils while increasing plasma cells and antibody in the lungs. We sought to test whether plasma cells and antibody played a role in suppression of allergic disease. **Methods:** We primed wild-type and B-cell-deficient (μ MT) mice with 25 μ g ovalbumin (OVA) precipitated in alum on days 0 and 5, nebulized weekly with 1% OVA, 1 h, twice daily, for up to 6 weeks, and assessed lung inflammation, mucus hypersecretion, and IgE/IgG1. **Results:** Kinetic studies revealed that initial aerosol exposure induced high numbers of eosinophils, lymphocytes, and macro-

phages within lung infiltrates and increased mucus production in wild-type mice. After 3–4 weeks of antigen exposure, eosinophils diminished while lymphocytes, plasma cells, and macrophages and mucus hypersecretion increased. However, by 6 weeks, lung inflammation and mucus hypersecretion were dramatically reduced. In contrast, repeated aerosol challenges maintained OVA-specific IgG1 and IgE production. Repeated aerosol antigen challenges in μ MT mice resulted in reduced lung inflammation and mucus hypersecretion and the development of smooth muscle hypertrophy of the pulmonary microvasculature. **Conclusions:** B cells and antibody do not appear to play a role in antigen-dependent suppression of allergic responses in mice.

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Introduction

Allergic asthma is defined by allergen-dependent intermittent reversible airway obstruction, elevated serum IgE, and lung inflammation. These features can be generated in antigen-primed mice by exposing them to nebulized antigen, which initially leads to the onset of acute allergic asthma characterized by eosinophilic lung infil-

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1018–2438/04/1331–0040\$21.00/0

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trates, elevated serum IgE, mucus hypersecretion, and increased methacholine-induced airway hyperresponsiveness (AHR) [1]. However, when mice with established disease are repeatedly exposed to aerosol antigens, they eventually suppress their immune responses leading to the reduction of eosinophilic inflammation and in some experimental models suppression of serum IgE [2–5]. In allergic patients, repeated administration of allergen reduces allergic symptoms and is the basis of desensitization immunotherapy. The mechanism underlying antigen-dependent suppression of allergic disease has yet to be elucidated.

Repeated inhalation of nebulized protein antigens by naive mice and rats results in a transient rise in IgE followed by tolerance upon subsequent immunization with antigen precipitated in adjuvant [6–11]. These studies differ from those in which the mice are primed and have established allergic asthma. However, the mechanism involved in both types of inhalation tolerance may be related. Inhalation tolerance induced in naive animals was attributed to CD4-T-cell-dependent, IFN γ -producing, antigen-specific CD8 γ δ + T cells [8–11]. However, this was disputed when inhalation tolerance could be induced in mice depleted of CD8 T cells, and in β 2m-, IFN γ - and TCR-deficient mice [12]. Additional mechanisms have been proposed based on data demonstrating that respiratory exposure to antigen in naive mice leads to deletion and inactivation of antigen-specific CD4 T cells [8, 12, 13] and that B cells are critically involved in the process [14].

When mice are weekly nebulized with aerosol antigen following acute onset of disease, there is a dose-dependent increase in serum and airway IgG1, mucus hypersecretion, AHR, and plasma cells within lung infiltrates [4]. In contrast, there is a reduced booster response for serum IgE and a decrease in lung eosinophil infiltration [4]. The apparent inverse correlation between eosinophilia and plasma cells within lung infiltrates within 4 weeks of repeated aerosol antigen challenges suggests a potential involvement of B cells and/or immunoglobulin (Ig). The role of B cells, plasma cells, and Ig in allergic asthma is not entirely clear despite the presence of plasma cells in lungs of humans and mice with asthma [15–19] and the ability to induce allergic asthma in mice deficient in IgE [20] and B cells [21–23]. B cells have been found to play a critical role in inhalation tolerance [14], but have been shown to be unnecessary in other models of immune tolerance [24–27]. These contrasting observations indicate that the role of B cells in immune suppression remains controversial.

The purpose of this study was to determine whether B cells played a role in suppression of lung eosinophilic inflammation upon repeated antigen challenges in mice with established asthma. Using a model to induce overt disease and then subsequent suppression, we compared the effects of repeated aerosol antigen exposure in B-cell-deficient and B-cell-sufficient mice. We now report that repeated exposure to ovalbumin (OVA) inhibits lung eosinophilic inflammation and mucus hypersecretion in the presence and absence of B cells. Furthermore, we established that repeated aerosol antigen exposure of wild-type mice enhanced serum antigen-specific IgG1 and bronchoalveolar lavage (BAL) fluid IgE and IgG1 but only maintained serum IgE levels. Additionally, repeated aerosol challenges induced chronic lung damage characterized by smooth muscle hypertrophy of lung arterioles in mice lacking B cells.

Materials and Methods

Mice

BALB/c, C57Bl/6 (B6), and B6. μ MT mice aged six to eight weeks were maintained in the VIRCC facility and provided OVA-free food and water ad libitum. All experiments complied with the requirements of the Animal Care Committee of the University of Vienna.

Immunization

Mice were immunized with 25 μ g of OVA (Sigma Chemical Co., St. Louis, Mo., USA) or PBS in 2 mg of alum intraperitoneally on day 0 and day 5. All mice were challenged 1 week later with nebulized 1% OVA in PBS in a Plexiglas chamber by a KLAVA ultrasonic nebulizer for 60 min twice daily on days 12 and 13 to induce acute onset disease. Following acute disease we treated the mice with weekly aerosol challenges, with 1% OVA for 60 min twice daily, once a week for up to 6 weeks.

Bronchoalveolar Lavage

The tracheas of lethally anesthetized mice were cannulated and lavaged with three 0.8-ml aliquots of PBS. The BAL fluid was centrifuged, and cell pellets were resuspended in PBS. Total leukocytes were counted with a hemocytometer. Cytospin slides were prepared with BAL cells and stained with May-Gruenwald-Giemsa to determine the cell differential.

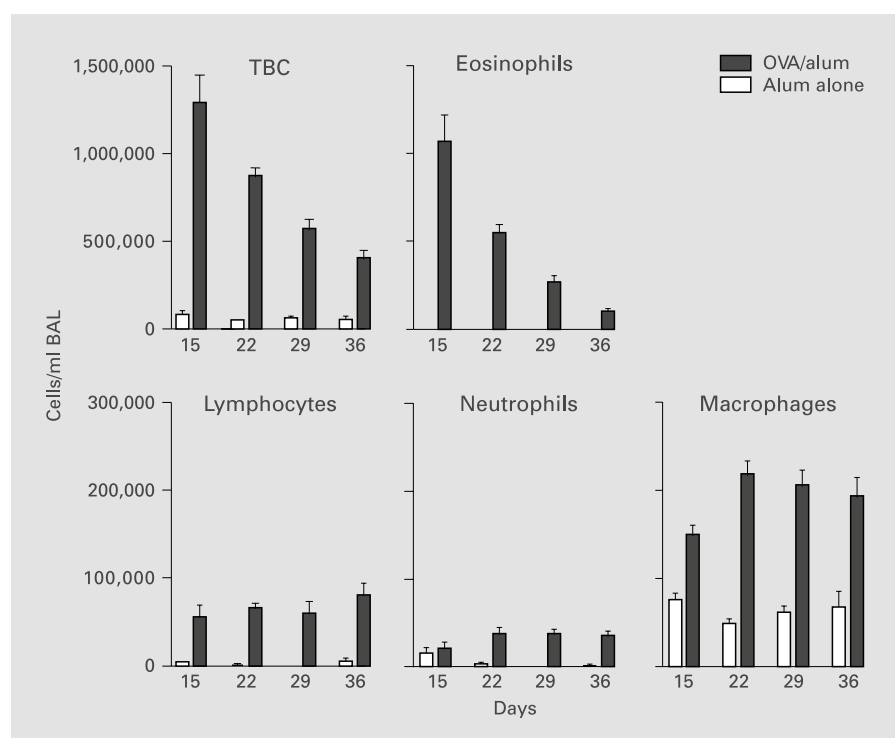
Lung Inflammation and Mucus Production

Following BAL, the pulmonary arteries were perfused with 10% formalin. Paraffin-embedded lung sections of 3–4 μ m were stained with hematoxylin and eosin (HE) for morphological evaluation and with periodic acid-Schiff stain (PAS) for mucopolysaccharide staining.

Serum Immunoglobulin

For the measurement of OVA-specific IgG1, ELISA plates were coated with OVA at 10 μ g/ml overnight at 4°C. The plates were washed and blocked with 2% BSA-0.05% Tween 20 for 2 h at 37°C.

Fig. 1. Repeated aerosol OVA challenges suppress BAL eosinophilia. Kinetics of total cell counts (TBC) and individual cell counts per milliliter of BAL fluid in BALB/c mice on days 15, 22, 29, and 36 compared to mice treated with alum alone. Data presented are from two independent experiments (n = 5–6).



Titrated sera or BAL fluid were incubated for 2 h at room temperature. After washing, biotinylated anti-IgG1 detection mAb was added and plates were incubated for 1 h. Europium-streptavidin was added to each well after the plates were washed. 100 μ l of enhancement solution was added (Delfia, Wallac Oy, Turku, Finland) and Eu^{3+} release was measured by fluorometry at 340 nm excitation and 614 nm emission. Pooled samples from mice with acute disease were used to generate the standard curves. In similar assays, OVA-specific IgE, plates were coated with anti-mouse IgE (LO-ME-3, Serotec, UK) and detected with biotinylated OVA.

Statistical Analysis

Groups were compared by the unpaired Student t test. Values of p were considered significant at <0.05 . Results for all measurements are expressed as the mean $\pm$ SEM.

Results

Repeated Aerosol OVA Challenge Suppresses Lung Eosinophilia

To establish the kinetics of the inflammatory response upon repeated OVA aerosol exposures in wild-type mice with acute allergic asthma, we evaluated the BAL samples from individual BALB/c mice 48 h after each series of challenges. Total airway inflammatory response is measured by counting TBC (total BAL fluid cells), which peaked after the first aerosol challenge and diminished

with each aerosol challenge (fig. 1). By day 36, TBC counts were reduced to 31% of those of acute onset disease on day 15. Differential cell counts revealed that eosinophils had decreased by 91% by day 36 compared to peak counts on day 15. In contrast, neutrophils, lymphocytes and macrophages were significantly increased upon antigen challenges compared to alum-alone controls ($p < 0.05$) and to an irrelevant antigen (data not shown). These data demonstrate that repeated aerosol antigen challenges specifically suppress lung eosinophilia.

Airway Eosinophilia Is Suppressed upon Repeated OVA Aerosol Challenge in B-Cell-Deficient Mice

To test whether the reduction of eosinophils correlates with increasing numbers of plasma cells in the lungs [4], we investigated the effects of repeated aerosol-OVA challenge in μ MT mice. As these mice are established on a B6 background, we compared repeated aerosol challenge experiments in B6 wild-type mice. After 3 weekly aerosol treatments (day 29), μ MT mice developed a similar pattern of BAL fluid inflammation as wild-type controls (fig. 2). However, BAL fluid eosinophils in μ MT mice were 66% less at this time point than in B6 mice. Additionally, BAL fluid neutrophils and lymphocytes were lower in μ MT than in B6 mice by 70 and 47%, respective-

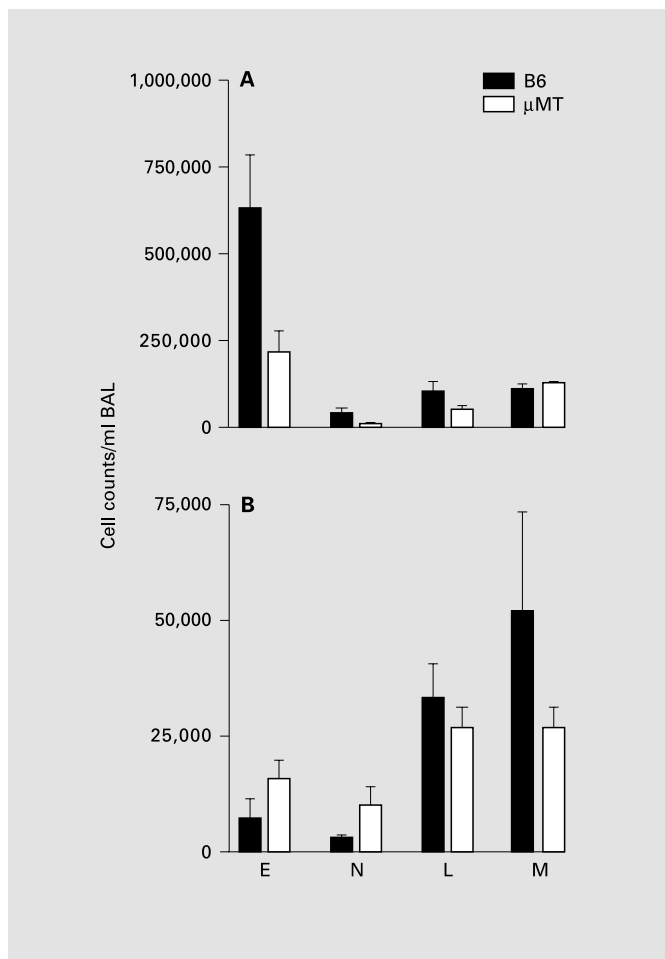


Fig. 2. Repeated aerosol OVA challenges suppress BAL eosinophilia in B6 and μ MT mice. Individual leukocyte cell counts in BAL fluid from B6 and μ MT mice on days 29 (**A**) and 45 (**B**). Values are expressed as mean $\pm$ SEM. L = Lymphocytes; N = neutrophils; E = eosinophils; M = macrophages. Data presented are from two independent experiments (n = 6).

ly. BAL fluid cell counts for all mice were significantly higher than negative controls ($p < 0.05$, data not shown). After 3 additional weeks of OVA aerosol (day 45), BAL fluid eosinophils in μ MT and B6 mice were lower than on day 29 by 92.6 and 98.8%, respectively. Macrophages, lymphocytes, and neutrophils were higher than negative controls (data not shown), but were significantly reduced in μ MT and B6 mice by day 45. There were no significant differences between cell counts between μ MT and B6 mice on day 45. These data show a dramatic reduction in airway eosinophils and a significant reduction in all cell types after 6 weeks of aerosol OVA in the presence and absence of B cells.

Parenchymal Inflammation Is Suppressed upon Repeated OVA Aerosol Challenge in Wild-Type and μ MT Mice

Inflammatory infiltrates during overt disease in HE lung sections from B6 mice were characterized by a high numbers of lymphocytes, macrophages, large numbers of plasma cells, and few eosinophils on day 29 (fig. 3A). Lung sections from μ MT mice revealed infiltrates containing many lymphocytes, macrophages, an obvious absence of plasma cells and few eosinophils (fig. 3B). By day 45 there were no eosinophils in B6 lung infiltrates (fig. 3C) but a few eosinophils could still be observed in μ MT lungs (fig. 3D). Inflammatory infiltrates in B6 lung sections were almost completely resolved (fig. 3C) and markedly reduced in μ MT mice by day 45 (fig. 3D). Taken together, these data illustrate that repeated OVA aerosol challenges induce suppression of lung eosinophilia in wild-type B6 and μ MT mice.

Hypersecretion of Mucus Diminishes with Repeated Aerosol Challenge

Mucus hypersecretion is another feature of allergic asthma related to Th2-type immunity in mouse models of allergic asthma. To compare the relative mucus production in mice treated with repeated aerosol OVA challenges, we stained paraffin-embedded sections with PAS. B6 and μ MT mice had very few mucus-producing cells and no goblet cell hyperplasia by day 45 (fig. 3E, F) compared to high amounts of mucus and goblet cell hyperplasia during acute and overt disease [4]. Mucus production was not observed in negative controls (data not shown). These results show diminished mucus production upon repeated aerosol antigen challenge in B6 and μ MT mice, which appears to correlate with reduced lung inflammation.

Repeated Aerosol OVA Challenge Induces Lung Microvascular Damage in μ MT Mice

Airway remodeling is a feature of chronic allergic asthma. We have not observed changes consistent with chronic lung damage in HE lung sections from wild-type mice (fig. 3A–C). In contrast, chronic exposure to aerosol antigen induced hypertrophy of vascular smooth muscle in μ MT mice by day 29 (fig. 3B) and day 45 (fig. 3D, 4), which was associated with areas of cellular infiltration. No other evidence of airway remodeling was apparent. These data demonstrate lung microvascular changes secondary to repeated aerosol antigen in the absence of B cells.

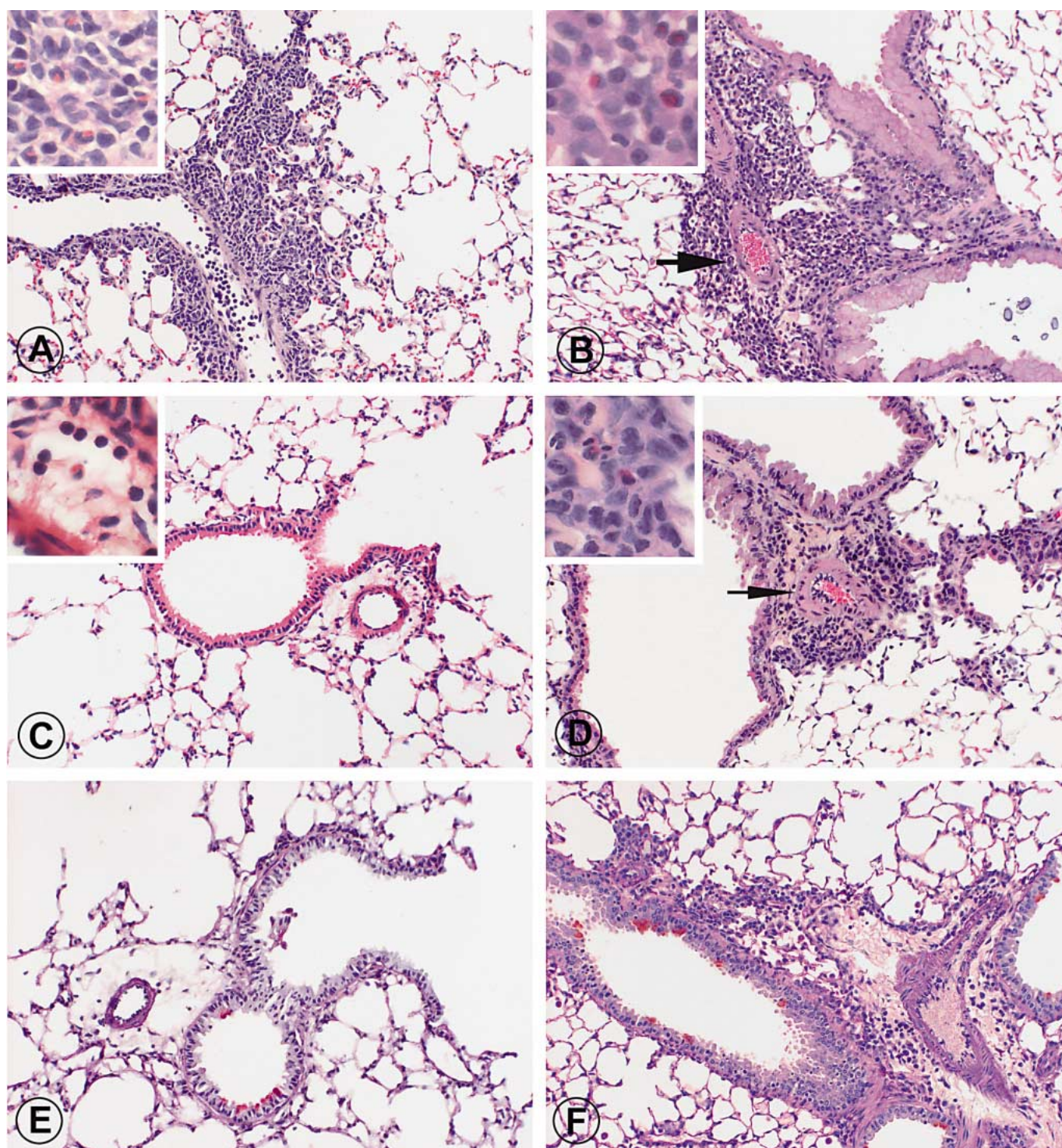


Fig. 3. Repeated aerosol OVA challenges suppress eosinophilia and mucus hypersecretion in B6 and μ MT mice. Representative photomicrographs of HE-stained whole lung sections from B6 mice on day 29 (**A**) and day 45 (**B**), and μ MT mice on day 29 (**C**) and day 45 (**D**). PAS-stained sections from B6 mice (**E**) and μ MT mice (**F**) on day 45. Objective lens $\times 100$ for low-power field and $\times 400$ for high-power field inserts. Arrows indicate smooth muscle hypertrophy. High-power field inserts reveal eosinophils, lymphocytes and plasma cells within infiltrates.

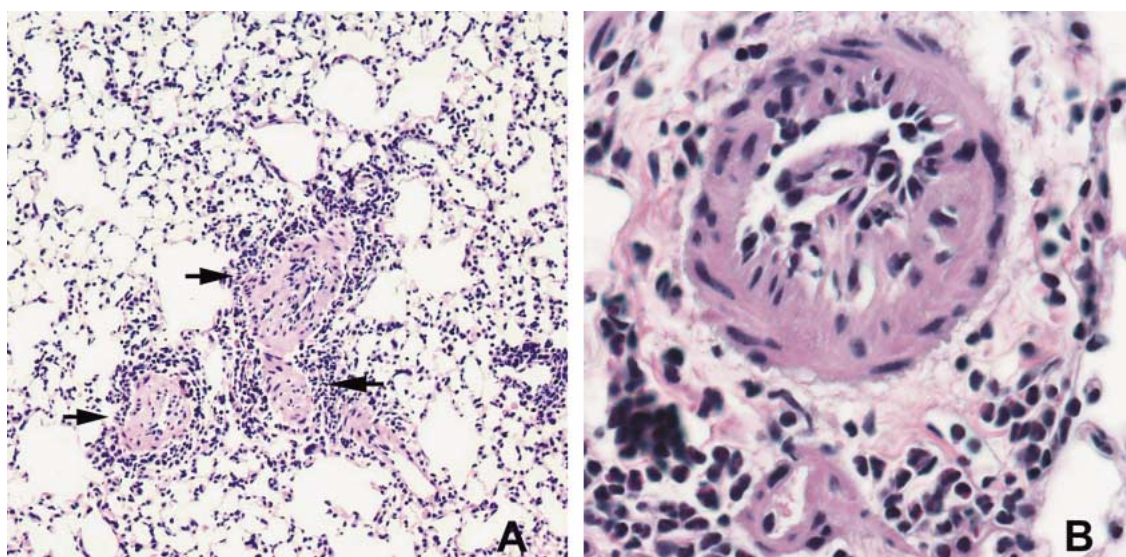


Fig. 4. Repeated aerosol OVA challenge induces vascular smooth muscle hypertrophy. Representative photomicrographs of HE-stained lung sections from μ MT mice on day 45. Objective lens $\times 100$ (**A**) and $\times 400$ (**B**). Arrows indicate vascular smooth muscle hypertrophy.

OVA-Specific Serum IgE and IgG1 Responses upon Repeated Aerosol Challenge

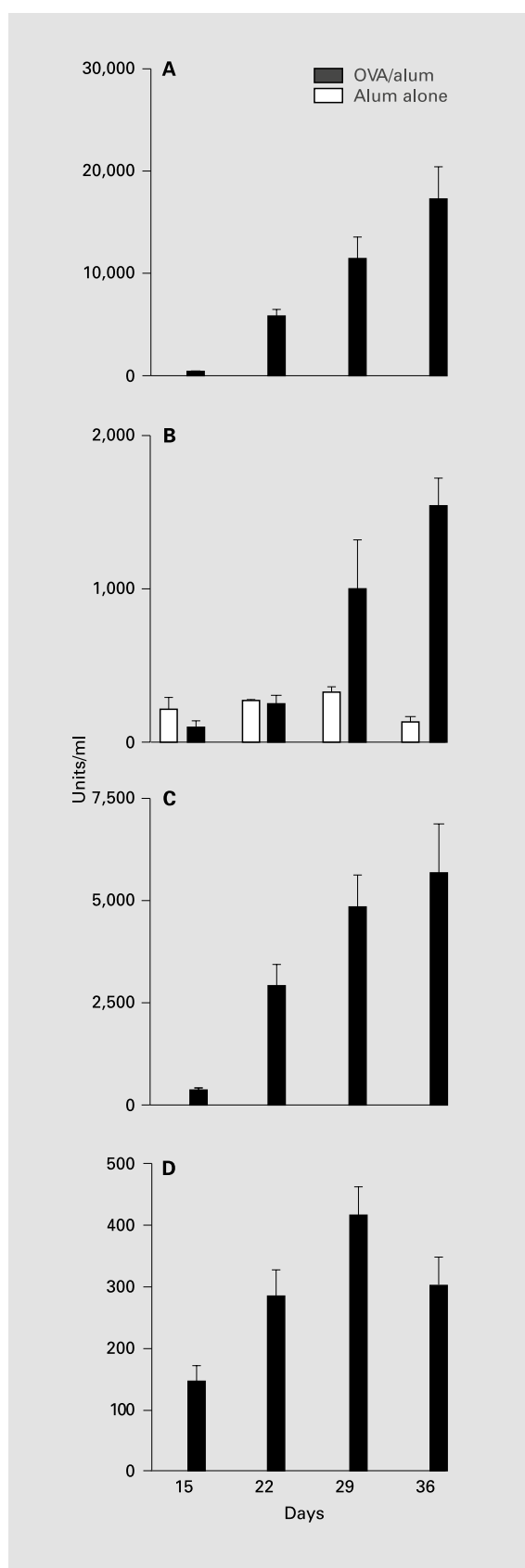
Antigen-specific serum IgE has been reported to diminish upon repeated aerosol challenges [2, 3]. Therefore, we sought to confirm this result and to determine whether IgG1 was also affected. We tested BAL and serum concentrations of OVA-specific IgE and IgG1 in BALB/c mice and found that levels were significantly elevated compared to negative controls ($p < 0.05$) (fig. 5). As previously reported, serum levels of OVA-specific IgE, while elevated and maintained above background, were not boosted at day 36. In contrast, BAL IgE levels were boosted upon aerosol challenge. Moreover, serum and BAL IgG1 were significantly elevated compared to negative controls ($p < 0.05$) and continued to rise following aerosol challenges.

Discussion

Upon repeated aerosol antigen challenge, mice initially develop overt allergic asthma characterized by lung inflammatory lesions consisting predominantly of lymphocytes, macrophages, high numbers of plasma cells, mucus hypersecretion, and high levels of airway and serum IgG1 and IgE. Once a critical threshold is reached, continued antigen exposure eventually leads to suppression of lung

eosinophilia, inflammatory infiltrates, and reduced mucus production while boosting antigen-specific Ig. This suggests that eosinophilic lung inflammation and mucus hypersecretion are dissociated from antibody production, which is further underscored by the findings that aerosol antigen-induced suppression occurs in B-cell-deficient mice.

Our data confirm previous studies in which suppression of eosinophilic inflammation upon repeated aerosol challenges has been reported [2, 3, 5]. Our kinetic experiments revealed that there was a rapid reduction of eosinophils while lymphocytes, macrophages, and neutrophils were maintained at increased numbers until day 36. However, these infiltrates soon diminished and eventually parenchymal inflammation resolved. The mechanism underlying suppression of inflammation is unclear. One explanation may be a reduction of Th2 cytokines or a switch from a dominant Th2 to a Th1 response. Reduced Th2 cytokines in BAL fluid and from cultured splenocytes associated with elevated BAL fluid IL-12 [3] and lower BAL fluid levels of IL-4 and IL-5 [5] have been previously demonstrated. However, the reduction in Th2 cytokines may be associated with the reduction in lung inflammation, but is sufficient for IgG1 and IgE production. This suggests Th2-mediated lung eosinophilic inflammation and Th2-isotype production are differentially regulated.



Mice lacking B cells developed overt disease and then progressive suppression of lung eosinophilia upon repeated aerosol antigen challenges. These data support previous reports showing that CD4 Th2 cell immune responses [28, 29] and, in particular, Th2-mediated allergic inflammation [21–23, 30] are successfully generated in μ MT mice. In addition, our data demonstrate that the generation of immune suppression in B-cell-deficient mice proceeds normally, as previously shown in other experimental systems [24–27]. We observed that reduction of eosinophils within lung infiltrates and overall inflammation was more evident in wild-type compared with μ MT mice. The reason for this difference is unclear. Our results demonstrate that B cells and Ig are neither necessary for the induction of overt disease nor for suppressing eosinophilic lung inflammation upon continued aerosol antigen exposure.

Repeated aerosol allergen challenges have been shown to suppress IgE production in animal models of allergic asthma [2, 3, 8–12, 31]. We observed a minimal reduction in the serum IgE antigen-dependent booster response. However, IgG1 in BAL fluid and serum and BAL fluid IgE were boosted following each antigen challenge. These data confirm a previous study showing that IgE levels remained constant and IgG1 and IgG2a continued to increase with repeated antigen exposure [5]. The reason for antigen-dependent increases of BAL fluid IgE and BAL fluid and serum IgG1 and a lack of IgE booster response in the serum is not clear. However, it may reflect differences in half-life between serum and BAL fluid and isotypes or differential regulation of isotypes, which has recently been shown [31]. Reports demonstrating serum IgE responses upon repeated aerosol challenge have been contradictory [3, 5] and probably relate to different protocols used to generate and maintain disease. Nevertheless, our data in B-cell-deficient mice suggest that the effect on Ig of repeated aerosol challenges is distinct and unrelated to the effect on inflammation and mucus production.

Airway remodeling, including subepithelial fibrosis, hypertrophy and hyperplasia of smooth muscle, and

Fig. 5. Repeated aerosol OVA challenge maintains elevated levels of OVA-specific IgG1 and IgE. OVA-specific BAL IgG1 (A) and IgE (B), and OVA-specific serum IgG1 (C) and IgE (D) were measured 48 h after the last aerosol challenge in BALB/c mice. Data are presented as averages of units/ml $\pm$ SEM. Results are from two independent experiments with more than 12 mice per group. * $p < 0.05$ for OVA compared with PBS/alum mice.

neovascularization, has been described in chronic models of allergic asthma [3, 32–40]. We did not observe fibrosis on HE-stained sections but observed prominent smooth muscle hypertrophy and apparent hyperplasia in μ MT mice. Angiogenesis and microvascular remodeling are features of chronic asthma [41]. The pathogenesis is unknown but factors including increased vascular endothelial growth factor and angiopoietin 1 [41], platelet dysfunction [42, 43], smooth muscle dysfunction [44], and endothelial cell dysfunction [45], abnormal TGF signaling [46], vasoconstrictor and vasodilator imbalance [44], coagulation abnormalities [47], monoclonal endothelial cell proliferation [48, 49], and inflammation [50] have been implicated. The mechanism underlying vascular remodeling in μ MT mice is not known, but may provide novel insights into disease pathogenesis.

In conclusion, we demonstrated that lung inflammation and mucus hypersecretion were suppressed by repeated aerosol antigen exposures independent of B cells and antibody. Additionally, in the absence of B cells, we

observed extensive and prominent vascular smooth muscle hypertrophy suggesting an apparent protective role for B cells. Whether the factors governing antigen-induced suppression in the mouse model are similar to those involved in allergen immunotherapy is unknown. However, understanding the mechanisms underlying repeated aerosol antigen suppression may reveal pharmacological targets leading to the development of improved immunosuppressive therapy in allergic disease.

Acknowledgements

We thank Drs. Carsten Schmidt-Weber and Christoph Burkart for discussion and critical reading of the manuscript. This work was funded in part by the Novartis Research Institute, Vienna, Austria, and grants from the Hochschuljubiläumsstiftung der Stadt Wien (H-207/2000) and the Austrian Science Foundation (P-14198MED). Paiboon Jungsuwadee is a scholar of the Austrian Exchange Service.

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