

Amphiregulin Is Not Essential for Ovalbumin-Induced Acute Airway Inflammation in Mice

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ABSTRACT

Background: The number of amphiregulin (AR)-positive mast cells in the bronchial mucosa and the levels of AR in sputum from asthmatic patients have been reported to be increased. In addition, AR can promote mucin gene expression in human epithelial cells, suggesting that AR contributes to the pathogenesis of allergic asthma.

Methods: To elucidate the role of AR in the pathogenesis of asthma, we immunized AR-deficient mice with ovalbumin (OVA) and then induced airway inflammation in them after OVA inhalation. The OVA-induced airway inflammation was assessed on the basis of the lung histology, number of leukocytes in the bronchoalveolar lavage (BAL) fluid, Th2 cytokine levels in the BAL fluid and OVA-specific IgG1 and IgE levels in the serum and compared between AR-sufficient and -deficient mice.

Results: The OVA-induced airway inflammation was comparable in the AR-sufficient and -deficient mice.

Conclusions: Amphiregulin is not essential for induction of acute airway inflammation by OVA in mice.

KEY WORDS

amphiregulin, asthma, EGF, IgE, mast cells

INTRODUCTION

Allergic asthma is considered to be a typical Th2-type cytokine-mediated chronic pulmonary disease.^{1,2} Th2-cell-derived IL-4 and IL-13 are essential for IgE class-switching and production by B cells. Subsequently, IgE provokes antigen-specific mast cell activation to induce degranulation and cytokine secretion, including Th2-type cytokines such as IL-4 and IL-13.^{1,2} IL-5 and IL-13, and IL-9, are key cytokines for inducing accumulation and activation of eosinophils and mast cells, respectively, in sites of inflammation.^{1,2} Therefore, these Th2-type cytokines orchestrate the activation of various types of immune cells, leading to exacerbation of airway inflammation and airway hyperresponsiveness. In particular, Th2 cells and mast cells, as sources of Th2-type cytokines, are deeply involved in the pathogenesis of asthma.

We previously demonstrated that human mast cells

can produce amphiregulin (AR), which is a member of the EGF family of growth factors/cytokines, after IgE-FcεRI crosslinking.³ AR is known to be physiologically/pathologically involved in mammary gland development, blastocyst implantation, bone formation and nerve regeneration, and to contribute to the progression of breast cancer.^{4,5} In addition, AR can activate keratinocytes and epidermal hyperplasia, suggesting that it is involved in the pathogenesis of psoriasis.^{5,6} However, the precise contributions of AR to the immune responses are poorly understood. Regarding this, we reported for the first time that the number of AR-expressing mast cells was increased in bronchial mucosa from patients with asthma.³ Moreover, AR promoted expression of the mucin gene such as MUC2 and MUC5AC in human epithelial cells³ and proliferation of human lung fibroblasts.⁷ In addition to mast cells, it was shown that Th2 cells, but not Th1 cells, expressed AR, and that Th2-cell-derived AR en-

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hanced resistance to nematode infection.⁸ These observations suggest that AR produced by mast cells and/or Th2 cells potentially contributes to the development of immune responses to infections and allergies. In support of this notion, Kim *et al.*⁹ very recently reported that the levels of AR in the sputum from patients with asthma were increased and correlated with an increased number of eosinophils in the sputum. However, the precise role of AR in the pathogenesis of asthma remains to be elucidated. Therefore, in the present study, we used AR-deficient mice to investigate the contribution of AR in the development of allergen-induced airway inflammation.

METHODS

MICE

Amphiregulin-deficient (AR^{-/-}) mice on a 129 × B6 mixed background were originally generated as described elsewhere.¹⁰ Littermates (AR^{+/-} and AR^{-/-} mice) were used in all experiments. By ELISA, the levels of AR in lung homogenates were comparable between AR^{+/-} and AR^{-/-} mice (data not shown). The mice were housed under specific pathogen-free conditions at the National Research Institute for Child Health and Development, and the animal protocols were approved by the Institutional Review Board of the National Research Institute for Child Health and Development.

OVA-INDUCED AIRWAY INFLAMMATION

OVA-induced airway inflammation was established in the mice as described elsewhere.¹¹ Briefly, mice were immunized intraperitoneally with 10 µg OVA (Grade V, SIGMA-Aldrich, St Louis, MO, USA) in 0.2 ml PBS on days 1, 3, 5, 7, 9, 11 and 13. On days 41, 44 and 47, the animals were treated intranasally with 200 µg OVA in 20 µl PBS or with 20 µl PBS alone.

HISTOLOGY

At 24 hours after the last OVA or PBS inhalation, lungs were harvested and fixed in Carnoy's fluid. The fixed lungs were embedded in paraffin, and 5-µm-thick serial sections were stained with hematoxylin and eosin (H&E).

BAL FLUID

At 24 hours after the last OVA or PBS inhalation, BAL fluid and cells were collected as described previously.¹² The total cell number in the BAL fluids was counted, and cytospin specimens were prepared to quantify the proportions of the different cell types after staining with Hansel's stain.

CYTOKINE ELISA

IL-4, IL-5, eotaxin, TSLP and AR levels in the BAL fluids were measured using mouse IL-4 and IL-5 ELISA kits (eBioscience, San Diego, CA, USA) and mouse eotaxin, TSLP and amphiregulin and human/mouse

TGF-β1 ELISA kits (R&D Systems, Minneapolis, MN, USA).

OVA-SPECIFIC Ig DETECTION

At 24 hours after the last OVA or PBS inhalation, blood was collected, and the serum was separated and stored at -80°C until assay. OVA-specific IgG1 and IgE levels in sera were determined as described elsewhere.¹³

STATISTICAL ANALYSES

Data show the mean + SEM and were evaluated for statistical significance using the two-tailed Student's *t* test.

RESULTS

AMPHIREGULIN IS NOT ESSENTIAL FOR THE DEVELOPMENT OF IgE- AND MAST CELL-MEDIATED OVA-INDUCED AIRWAY INFLAMMATION

It is known that the requirement for mast cells and IgE in the development of mouse airway inflammation induced by OVA differs as a result of whether the sensitization with OVA is performed in the presence or absence of aluminum hydroxide (alum). That is, mast cells and IgE are required for sensitization in the absence of alum, but not in the presence of alum.¹⁴ Since we reported that AR is produced by mast cells after IgE-FcεRI crosslinking, we used AR-deficient mice to investigate the role of AR in IgE- and mast cell-mediated OVA-induced airway inflammation. After OVA sensitization without alum, mice were treated intranasally with OVA or PBS. At 24 hours after the last OVA challenge, the numbers of total cells, eosinophils, macrophages, lymphocytes and neutrophils in the BAL fluid were comparable between AR^{+/-} and AR^{-/-} mice (Fig. 1). Consistent with those BAL cell profiles, airway inflammation such as leukocyte recruitment, goblet and epithelial cell hyperplasia and mucus secretion assessed on the basis of the lung histology was also similar in the AR^{+/-} and AR^{-/-} mice after the OVA challenge (Fig. 2). These observations indicate that AR is not essential for IgE- and mast cell-mediated OVA-induced airway inflammation in mice.

AMPHIREGULIN-DEFICIENT MICE SHOWED IDENTICAL LEVELS OF Th2 CYTOKINES IN BAL FLUIDS DURING MURINE ASTHMA

We next examined the contribution of AR to the production of Th2 cytokines such as IL-4, IL-5 and IL-13 and AR-receptor expressing epithelial cell-derived cytokines such as eotaxin, TGF-β1 and TSLP during IgE- and mast cell-mediated OVA-induced airway inflammation. At 24 hours after the last OVA inhalation, the levels of IL-4 and eotaxin were significantly increased in BAL fluid from OVA-challenged mice in comparison with PBS-treated mice (Fig. 3). However,

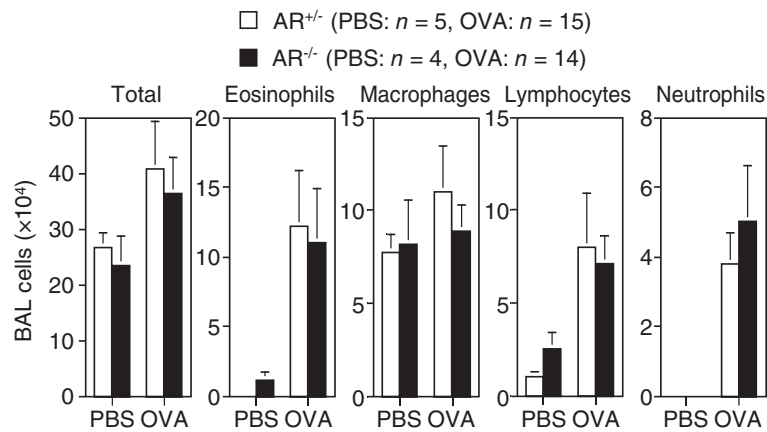


Fig. 1 Number of BAL cells in amphiregulin-deficient mice during OVA-induced airway inflammation. OVA-sensitized mice were treated intranasally with OVA or PBS alone. At 24 hours after the last OVA or PBS inhalation, BAL fluids were collected. Then the number and types of cells in the BAL fluids were determined. Open columns = AR^{+/-} mice (PBS: *n* = 5; OVA: *n* = 15), and closed columns = AR^{-/-} mice (PBS: *n* = 5; OVA: *n* = 14). Data show the mean + SEM.

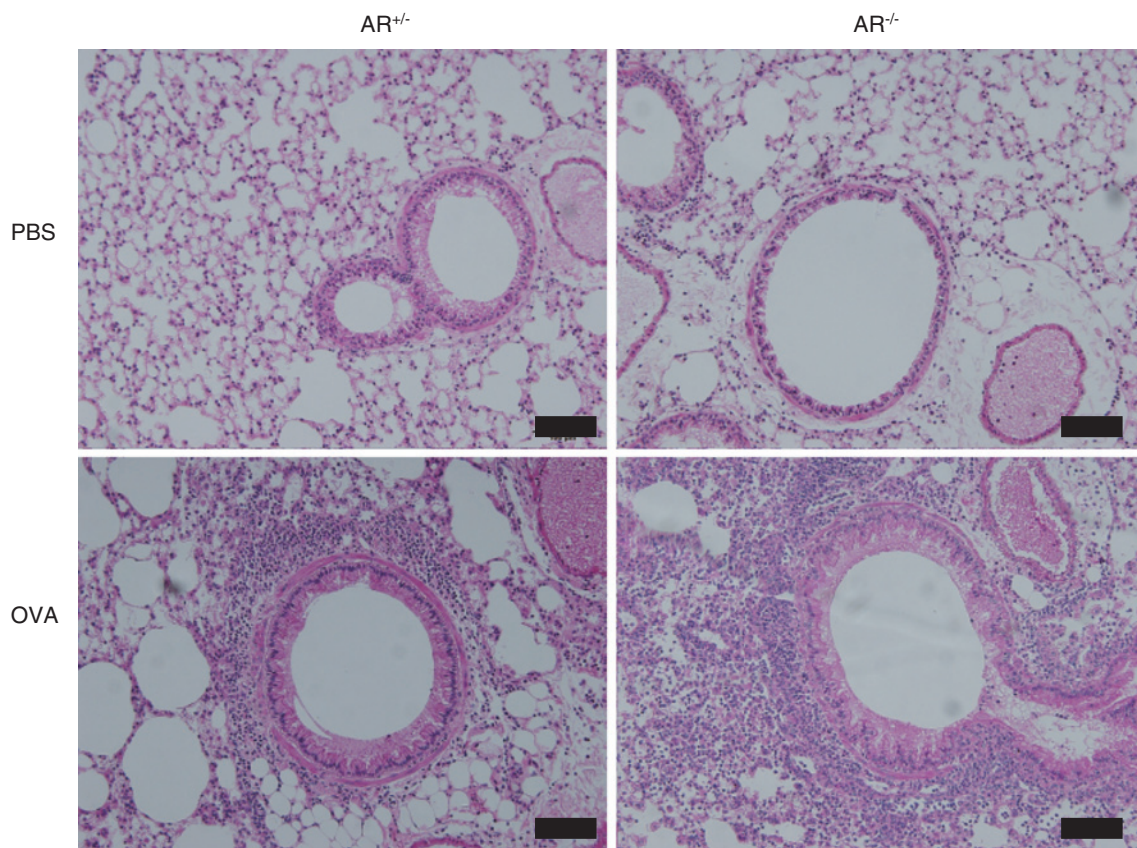


Fig. 2 Airway inflammation was similarly observed in amphiregulin-deficient mice during OVA-induced airway inflammation. At 24 hours after the last OVA or PBS inhalation, lungs were harvested from mice as shown in Figure 1. The sections of lungs were stained with H & E. Scale bars = 100 μ m. Data show a representative result.

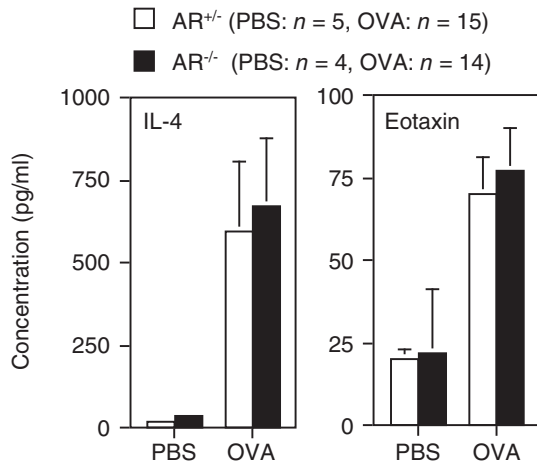


Fig. 3 IL-4 and eotaxin levels in BAL fluids from amphiregulin-deficient mice during OVA-induced airway inflammation. At 24 hours after the last OVA or PBS inhalation, BAL fluids were collected from mice as shown in Figure 1. IL-4 and eotaxin levels in the BAL fluids were determined by ELISA. Open columns = AR^{+/-} mice (PBS: *n* = 5; OVA: *n* = 15), and closed columns = AR^{-/-} mice (PBS: *n* = 5; OVA: *n* = 14). Data show the mean + SEM.

the amounts of AR, IL-5, IL-13, TGF- β 1 and TSLP in the BAL fluids were below the limit of detection by ELISA in the tested settings (data not shown). We found that the levels of IL-4 and eotaxin in BAL fluids were indistinguishable between the OVA-challenged AR^{-/-} mice and the OVA-challenged AR^{+/-} mice (Fig. 3), indicating that AR is not necessary for IL-4 and eotaxin production during IgE- and mast cell-mediated OVA-induced airway inflammation in mice.

OVA-SPECIFIC Ig PRODUCTION IS EQUIVALENT IN AMPHIREGULIN-DEFICIENT MICE DURING MURINE ASTHMA

OVA-specific IgE is important for the pathogenesis of the murine asthma model. At 24 hours after the last OVA inhalation, the levels of OVA-specific IgG1 and IgE were markedly increased in sera from OVA-challenged mice compared with sera from PBS-treated mice (Fig. 4). However, the Ig levels were not statistically different between AR^{+/-} and AR^{-/-} mice (Fig. 4). These observations indicate that AR is not responsible for Th2-cytokine-dependent IgG1 and IgE production during IgE- and mast cell-mediated OVA-induced airway inflammation in mice.

DISCUSSION

Amphiregulin, a member of the EGF family of growth factors/cytokines, was first identified in supernatants from breast cancer cell lines stimulated with PMA.¹⁵ AR has dual functions: it both enhances and inhibits the growth of certain cell lines.^{4,5} In addition to its

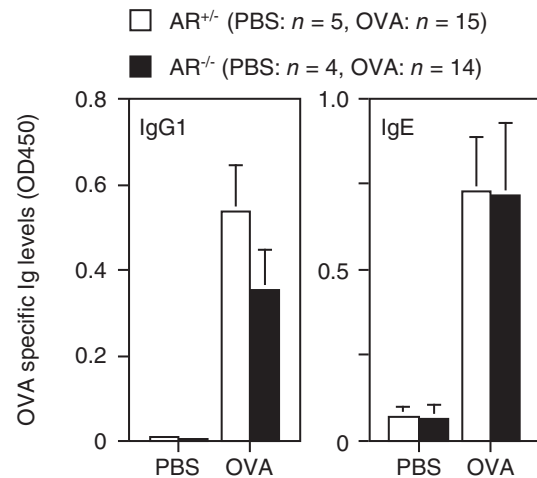


Fig. 4 Serum OVA-specific IgG1 and IgE levels in amphiregulin-deficient mice during OVA-induced airway inflammation. At 24 hours after the last OVA or PBS inhalation, sera were collected from mice as shown in Figure 1. OVA-specific IgG1 and IgE levels in the sera were determined by ELISA. Open columns = AR^{+/-} mice (PBS: *n* = 5; OVA: *n* = 15), and closed columns = AR^{-/-} mice (PBS: *n* = 5; OVA: *n* = 14). Data show the mean + SEM.

role as a growth factor, AR also acts as a cytokine in immune systems.⁸ Indeed, Th2-cell-derived AR is crucial for host defense against nematode infection.⁸ Moreover, IgE-Fc ϵ RI-stimulated mast cells produce AR, and AR enhances mucin gene expression in human epithelial cells and proliferation of human lung fibroblasts.^{3,7} These observations suggest that AR may contribute to the pathogenesis of Th2 cell- and mast cell-mediated immune responses, such as nematode infection, and allergic disorders. In support of this, we previously showed that the number of AR-expressing mast cells is increased in bronchial mucosa from patients with asthma.³ In addition, AR levels are increased in the sputum from asthmatic patients.⁹ However, in the present study, we showed that even AR-deficient mice similarly developed Th2 cytokine- and mast cell-mediated airway inflammation in response to OVA. That is, OVA-induced airway inflammation accompanied by eosinophilia, OVA-specific IgG1 and IgE production, and IL-4 and eotaxin production was equivalently observed even in AR-deficient mice. These observations indicate that AR is not essential for OVA induction of Th2- and mast cell-mediated airway inflammation. However, the precise role of AR in the pathogenesis of allergic asthma may not be fully explained by the animal model used in our study, in which treatment with steroids can result in attenuation of acute airway inflammation. Accordingly, we originally identified AR as an upregulated gene in IgE-stimulated human mast cells even in the presence of glucocorticoids.³

These observations suggest that AR may be involved in the pathogenesis of steroid-resistant chronic asthma associated with airway remodeling rather than the pathogenesis of steroid-responsive acute asthma. Therefore, the role of AR in the pathogenesis of chronic airway inflammation should be investigated by using AR-deficient mice in a future study.

In summary, we demonstrated that AR is not essential for induction of acute airway inflammation by OVA in mice.

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