

IgE-Dependent Mast Cell Activation Potentiates Airway Responses in Murine Asthma Models¹

Susanne I. Mayr,* Riaz I. Zuberi,* Min Zhang,* Jean de Sousa-Hitzler,[†] Karen Ngo,[†] Yasuko Kuwabara,* Lan Yu,*[‡] Wai-Ping Fung-Leung,[†] and Fu-Tong Liu^{2*‡}

We have studied murine models of asthma using FcεRIα-chain-deficient (FcεRIα^{-/-}) mice to investigate the role of IgE-dependent mast cell activation in these models. When mice were either 1) immunized once with OVA in alum i.p. and then challenged with OVA intranasally, or 2) repeatedly immunized with OVA in the absence of adjuvant and subsequently challenged with nebulized OVA, FcεRIα^{-/-} mice had significantly fewer eosinophils and lower IL-4 levels in their bronchoalveolar lavage fluid compared with wild-type mice. When mice were given anti-IL-5 antibody before OVA challenge in protocol 1, eosinophilic infiltration into the airways was significantly suppressed in both genotypes, but only FcεRIα^{-/-} mice showed significantly reduced airway hyperresponsiveness (AHR). In addition, when mice immunized and challenged with OVA also received a late OVA provocation at a higher concentration and were then exposed to methacholine, only wild-type mice developed a substantial increase in AHR. Since FcεRI is expressed mainly on mast cells in mouse airways, we conclude that IgE-dependent activation of this cell type plays an important role in the development of allergic airway inflammation and AHR in mice. The models used may be of value for testing inhibitors of IgE or mast cells for development of therapeutic agents for human asthma. *The Journal of Immunology*, 2002, 169: 2061–2068.

Human asthma is a chronic disease characterized by allergic airway inflammation with increased mucus production and lung epithelia remodeling, intermittent airway obstruction and hyperresponsiveness (AHR)³ (1). In recent years significant progress has been made in our understanding of cellular and molecular components involved in airway inflammation and AHR, especially through studies using murine models of asthma. The use of genetically engineered mice with altered expression or deletion of relevant gene products has been particularly useful. Various cellular and molecular components, such as cytokines, chemokines, cell adhesion molecules, as well as peptide and lipid inflammatory mediators, have been identified (2–4). However, the roles of IgE and mast cells in the allergic airway response in murine models remain controversial.

Mast cells have long been regarded as an important cell type involved in allergic inflammation. These cells are ordinarily distributed throughout the connective tissue, where they are often situated adjacent to blood and lymphatic vessels and beneath epithelial surfaces that are exposed to environmental Ags (5). Activation of these cells results in the release of spasmogenic and vasoactive mediators as well as chemical mediators that promote

infiltration of other leukocytes, including eosinophils, to sites of inflammation (6). Mast cells can signal naive and memory T cells to preferentially differentiate into Th2 cells (7) and induce IgE synthesis in B cells (8). It has also been reported that activation of mast cells in the airways of mice by anti-IgE Ab results in enhanced airway responsiveness to cholinergic stimulation by methacholine (9).

However, there are conflicting reports in the literature with regard to the role of mast cells in murine models of asthma, primarily based on studies in mast cell-deficient mice. While some reports have demonstrated that mast cell deficiency results in attenuated eosinophilic airway inflammation (10, 11), others have shown that this deficiency does not affect allergic airway inflammation and AHR (12–15). Recent studies have suggested that the extent to which mast cells contribute to airway inflammation and AHR in mice is highly dependent on the experimental model used to generate the airway response (16, 17).

The role of IgE in mast cell and basophil activation is critical in human allergic diseases (9). Recently, anti-IgE therapy currently in clinical trials has provided additional evidence for the role of IgE in both allergic rhinitis and asthma (18–21). Ag-specific IgE Abs bind mast cells via FcεRI. These cell surface-bound IgE molecules can subsequently become cross-linked upon binding to the Ag, resulting in mast cell activation and the release of a plethora of pro-inflammatory molecules. However, allergic airway inflammation and AHR can be elicited in mice in the absence of IgE (22, 23) or all classes of Abs (24). The existing literature suggests that a role for IgE or other classes of Abs in murine models of asthma is dependent on experimental variables. As an example, it has been proposed that IgE plays an important role in the development of airway inflammation and AHR only under experimental conditions in which eosinophilic inflammation is relatively low (25).

IgE-mediated mast cell activation is critically dependent on FcεRI. It appears that mice deficient in FcεRI may be well suited for delineation of the roles of mast cells and IgE in allergic airway inflammation. Since in mouse airways FcεRI is expressed only on

*Division of Allergy, La Jolla Institute for Allergy and Immunology, San Diego, CA 92121; [†]Pharmaceutical Research and Development, Johnson & Johnson, La Jolla, San Diego, CA 92130; and [‡]Department of Dermatology, University of California, Davis, School of Medicine, Sacramento, CA 95817

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² Address correspondence and reprint requests to Dr. Fu-Tong Liu, Department of Dermatology, University of California, Davis, 4860 Y Street, Suite 3400, Sacramento, CA 95817. E-mail address: flui@ucdavis.edu

³ Abbreviations used in this paper: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; BMDC, bone marrow-derived mouse mast cell; MCh, methacholine; Penh, enhanced pause.

mast cells, the phenotypic alterations detected in these mice could be connected to mast cells. Studies with these mice would obviously shed light on the role of IgE, since FcεRI is the major receptor for IgE. In addition, while the mast cell-deficient mice commonly used to study the role of mast cells in various disease models have defects other than the lack of mast cells, the defect of FcεRI-deficient mice is restricted to IgE-mediated mast cell/basophil responses. Also, IgE can exert its effects through both FcεRI and FcεRII, and thus the phenotype of IgE-deficient mice may reflect the functions of IgE mediated through either of these two receptors. The phenotype of FcεRI-deficient mice, on the other hand, should be related only to those mediated through FcεRI.

Thus, studies in FcεRI-deficient mice should complement earlier studies in mast cell-deficient mice and IgE-deficient mice and provide additional insights into the role of IgE-mediated mast cell activation in murine models of asthma. We now report that 1) FcεRI-deficient mice exhibit reduced airway eosinophilic inflammation and AHR when sensitized to the Ag systemically and challenged subsequently with the Ag through the airways; and 2) the sensitivity/threshold of this response are differentially regulated in FcεRI^{-/-} and wild-type mice.

Materials and Methods

Generation of FcεRIα^{-/-} mice

FcεRIα^{-/-} mice were produced by targeted disruption of the gene encoding the FcεRIα-chain. A 2.4-kb DNA fragment (knockout construct) containing part of exon 1 and complete exons 2 and 3 of the mouse FcεRI gene (26) was obtained by PCR of embryonic stem cell genomic DNA. The mouse FcεRIα-chain gene-specific primers used in PCR are 5'-CCCCTC GAGCCTAGCACTGCTGTTTCATGT-3' and 5'-CCCGCGGCCGCGCT TCATGGATTGTTTCAGAGC-3'. The mouse FcεRIα gene was disrupted by inserting the neomycin-resistant gene cassette of 1.2 kb into the BamHI site of exon 3. Mouse D3 embryonic stem cells from 129/SV mice were maintained in an undifferentiated state of growth on a feeder layer of mitomycin C-treated embryonic fibroblasts in DMEM supplemented with 100 U/ml leukemia inhibitory factor (Life Technologies, Gaithersburg, MD) and 15% FBS. Embryonic stem cells were transfected with the knockout construct DNA by electroporation, and transfected cells were selected in medium containing 300 μg/ml G418. Homologous recombination was detected by PCR as previously described (27).

Chimeric mice were generated from blastocysts injected with the selected embryonic stem cells. Mice carrying the disrupted FcεRI gene were obtained by breeding chimeric mice with C57BL/6J mice and screening for agouti offspring. The disrupted FcεRI gene in agouti mice was detected by Southern blot analysis of tail DNA (see Results). Mice heterozygous for the disrupted gene were interbred to homozygosity. The mice were then backcrossed for nine generations with BALB/cBYJ mice (The Jackson Laboratory, Bar Harbor, ME). The heterozygous animals were intercrossed to obtain homozygous FcεRIα^{-/-} and wild-type animals with the same genetic background. Confirmation of the disruption of the FcεRIα-chain gene was accomplished by PCR and Southern blot analysis. Experiments were performed on age- and sex-matched 8- to 12-wk-old mice. The animals were maintained in pathogen-free conditions and on chicken egg albumin-free diets.

Reagents

Acetyl-β-methacholine chloride (methacholine (MCh)) was purchased from Sigma-Aldrich (St. Louis, MO). Rat anti-IL-5 mAb was purified from the TRFK-5 hybridoma cell line provided by Dr. R. Coffman (DNAX-Research Institute of Molecular and Cellular Biology, Palo Alto, CA) (28). Monoclonal anti-DNP IgE was purified from ascites from hybridoma 26.82 as previously described (29).

Flow cytometric analysis

Bone marrow-derived mouse mast cells (BMMC) from naive FcεRIα^{-/-} and wild-type mice were generated by maintaining femoral bone marrow cells in vitro for 4–5 wk according to a published protocol (30). The cells were incubated first with 10 μg/ml anti-DNP IgE for 40 min and then with FITC-conjugated anti-mouse IgE (BD PharMingen, San Diego, CA) for another 30 min. Flow cytometric analysis of the stained cells was per-

formed with FACScan (BD Biosciences, Mountain View, CA) equipped with CellQuest software.

Hexosaminidase release assay

BMMC were plated at 2×10^6 /ml in 25-ml tissue culture flasks and sensitized with 0.5 μg/ml anti-DNP IgE overnight at 37°C in a CO₂ incubator. The cells were washed once and resuspended at 10×10^6 /ml. Then, 0.5-ml aliquots of the cell suspension were placed in Eppendorf tubes. The cells were then incubated with 0, 3, 10, 30, 100, and 300 ng/ml multivalent Ag DNP-BSA for 45 min. Afterward, the cells were centrifuged, and the supernatants were collected. The cell pellets were lysed with 0.1% Triton. All samples were kept at -20°C until tested for enzyme activity.

For quantitating β-hexosaminidase, 30 μl supernatants or Triton-lysed cell pellets were placed in wells of 96-well flat-bottom plates (Fisher Scientific, Los Angeles, CA) containing 20 μl of the substrate, 3 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma-Aldrich), in 0.1 M citrate buffer, pH 4.5. The assay was terminated 20 min later by the addition of 0.1 M sodium carbonate buffer, pH 10.0. The color developed was measured spectrophotometrically at 405 nm. The enzyme release was expressed as the percentage of the total β-hexosaminidase content of the cells after subtraction of unstimulated release (31).

Passive cutaneous anaphylaxis reaction

The ears and shaved dorsal skin of naive FcεRIα^{-/-} and wild-type mice were injected with either saline or dilutions of anti-DNP IgE hybridoma ascites intradermally. Three hours later, 200 μl DNP-BSA at 1 mg/ml was injected i.v., and 30 min later the mice were euthanized. Ear and skin biopsies were placed directly in 10% zinc formalin. The paraffin-embedded 5-μm sections were cut and hydrated, then stained with toluidine blue. The number of mast cells was counted in 20 100-mm² areas using an ocular grid. The morphology of the mast cells was graded as normal (<10% of cytoplasmic granules exhibiting fusion, staining alterations, or extrusion from the cell), moderately degranulated (10–50% of granules altered as described above), and extensively degranulated (>50% granules altered as described above) (32).

Sensitization protocol

Mice were immunized with OVA (chicken egg albumin grade V from Sigma-Aldrich) i.p. according to the following three protocols: 1) 10 μg OVA and 2 mg/ml aluminum hydroxide gel (alum) as adjuvant in 0.5 ml saline once, 2) 10 μg OVA in 0.5 ml pyrogen-free saline without adjuvant 11 times on alternate days, and 3) OVA-alum as described above, followed by a booster OVA-alum injection on day 14.

Airway Ag challenge

Mice immunized according to protocol 1 were challenged 14 days later intranasally with 10 μg OVA in 15 μl saline on 4 alternate days. Mice immunized according to protocol 2 were challenged with 1% aerosolized OVA in PBS 30 min each day for 6 consecutive days 14 days after the last immunization. Control mice were immunized in an identical fashion, but challenged with aerosolized PBS. The aerosol was generated in a Plexiglas chamber connected to a nebulizer (Ultra Neb Nebulizer, 120VAC, with a maximum aerosol output of 6 ml/min and an average particle size of <4 μm with an airflow of up to 30 lpm). Mice immunized according to protocol 3 were challenged with aerosolized OVA on 7 consecutive days 10 days after the last immunization. Seven days after the last Ag challenge, mice were exposed to OVA aerosol generated from a 5% OVA solution, while control mice received aerosolized PBS.

Anti-IL-5 Ab treatment

Mice immunized once with OVA in alum (sensitization protocol 1) were given 14 days later either 10 μg rat anti-IL-5 Ab or, as a control, purified normal rat IgG (Zymed, San Francisco, CA) i.p. in pyrogen-free saline. Two hours later the mice received 10 μg OVA intranasally. The anti-IL-5 treatment and intranasal Ag challenge were performed on 4 alternate days.

Determination of AHR

The airway response was measured in unrestrained animals using whole-body plethysmography (Buxco, Troy, NY), as described previously (33). Readings were obtained at baseline and after exposure to aerosolized saline or MCh (5–50 mg/ml). Data were collected for 5 min after 3 min of inhalation, and average values were expressed as the enhanced pause (Penh): $Penh = [(Te - Tr)/Tr] \times (PEP/PIP)$, where *Te* is the expiratory time (seconds), *Tr* is the relaxation time (time of the pressure decay to 36% of total box pressure during expiration), *PEP* is the peak expiratory pressure

(milliliters per second), and *PIP* is the peak inspiratory pressure (milliliters per second). Results are expressed as the percent increase in *Penh* following challenge with each concentration of MCh, where baseline *Penh* is expressed as 100%, or as *Penh* values. The airway response of mice receiving aerosolized Ag was measured 4 h after the last challenge; it was measured 24 h after the last challenge in mice that received the Ag intranasally.

Collection and analysis of bronchoalveolar lavage fluid (BALF)

BALF was collected by cannulating the upper part of the trachea and lavaging three times with 1 ml pyrogen-free PBS (85–90% of the 1-ml input volume was retrieved). The lavage fluid collected was centrifuged at $400 \times g$ for 5 min at 4°C, and the cells were separated from the fluid. The fluid was then centrifuged at $1000 \times g$ for 2 min at 4°C to remove cellular debris and then stored at -20°C until it was evaluated. The cells were resuspended in PBS containing 1% FBS, and the total number of viable cell was determined by trypan blue exclusion using a hemocytometer. Differential cell counts were determined with cytopspin preparations, followed by Wright-Giemsa staining (Fisher Scientific, Los Angeles, CA).

Cytokine and Ig quantifications by ELISA

IL-4. Ninety-six-well plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight with the capture Ab 11B11 (National Cancer Institute, Biological Response Modifier Program, Bethesda, MD) at 4°C and then blocked with the assay diluent (1% BSA in PBS containing 1% FBS and 0.05% Tween 20) for at least 15 min. BALF samples and rIL-4 standard (a gift from Dr. J. Ohara, Denver, CO) were placed in appropriate wells, and the plates were incubated for 2 h at room temperature. The bound cytokine was detected with biotinylated rat anti-mouse IL-4 (BD PharMingen) with an incubation period of 2 h at room temperature, followed by avidin-HRP (Bio-Rad, Richmond, CA) at 1/1000 dilution for 30 min at room temperature. The plates were developed by addition of the substrate *o*-phenylenediamine dihydrochloride (Sigma-Aldrich). The reaction was stopped by adding 25 μ l of 4 N sulfuric acid before reading the plates in an ELISA plate reader (Spectramax 250; Molecular Devices, Sunnyvale, CA) at 490 nm. The experimental values were read off a standard curve generated using the plate reader software.

Total IgE. The plates were coated with affinity-purified goat anti-mouse IgE Ab (29) overnight at 4°C and then blocked with 1% BSA in PBS for 2 h at room temperature. The samples and appropriate dilutions of a standard anti-DNP IgE preparation (29) in the assay diluent (1% BSA in PBS containing 0.05% Tween 20) were incubated overnight at 4°C. The bound IgE was detected after 1-h incubation with purified rat anti-mouse-IgE-HRP (ϵ -chain specific; Southern Biotechnology Associates, Birmingham, AL) at room temperature and subsequent addition of substrate as described above.

Histological studies

After BAL the lungs were perfused with 10 ml PBS via the right heart ventricle. The exsanguinated left lung was then removed from the chest cavity and fixed in 10% zinc formalin (10 ml). The lobes were sagittally sectioned, paraffin embedded, cut in 5- μ m sections, and stained with Wright-Giemsa to identify eosinophils in perivascular and peripheral areas of lung tissue, which were counted using a 10×10 -mm grid ($\times 10$) plus $\times 40$ magnification ($\times 400$ final magnification).

Statistical analysis

Values are expressed as the mean $\pm$ SEM. The data were compared using Student's two-tailed *t* test with Excel 5.0; *p* < 0.05 was considered significant.

Results

Generation of *FcεRIα*^{-/-} mice

FcεRI in rodents has a tetrameric structure consisting of noncovalently associated subunits, $\alpha\beta\gamma_2$ (34). The α subunit is responsible for the IgE-binding function of the receptor, while the β and γ subunits are both primarily intracellular and critically involved in transducing signals induced by cross-linking of the receptor. All three subunits are required for cell surface expression of *FcεRI* in mice (35). *FcεRIα*^{-/-} mice were generated by gene targeting according to standard protocol, with the *FcεRIα* gene being disrupted within exon 3. Confirmation of homologous recombination was achieved by Southern blot analysis. Briefly, mouse genomic DNA

was digested with *EcoRI*, *BglII* plus *HindIII*, or *BglII* plus *EcoRV*, and the genomic blots were hybridized with a 0.7-kb DNA probe located at the 3'-flanking region of the knockout construct (Fig. 1). As shown in Fig. 1, a 4-kb *EcoRI* DNA fragment was detected for the wild-type *FcεRIα* gene, and this DNA fragment was shortened to 2.5 kb in the disrupted gene due to the presence of an *EcoRI* site in the neomycin-resistant gene cassette. The *HindIII*-*BglII* DNA fragment (4 kb) and the *EcoRV*-*BglII* DNA fragment (2.5 kb) of the wild-type gene were increased to 5.7 and 3.7 kb, respectively, in the disrupted gene due to insertion of the neomycin-resistant gene cassette.

We noted that the skin from *FcεRIα*^{-/-} and wild-type mice have comparable numbers of mast cells when stained with toluidine blue, consistent with previous reports (36, 37). The number of mast cells per 100-mm² dermis was 7.9 ± 1.6 for wild-type mice and 9.2 ± 2.1 for *FcεRIα*^{-/-} mice. Since *FcεRI* is primarily expressed by mast cells in mice (35), we confirmed the functional deficiency of this receptor by studying mast cell responses in these mice. We found that there was no detectable IgE binding by BMMC from *FcεRIα*^{-/-} mice by flow cytometry, while such binding was readily detectable with BMMC from wild-type mice (Fig. 2A). In addition, the absence of functionality of *FcεRI* on BMMC from *FcεRIα*^{-/-} mice was demonstrated in vitro by a lack of IgE-dependent degranulation. *FcεRIα*^{-/-} and wild-type BMMC were first incubated with DNP-specific IgE and then with DNP-BSA. No β -hexosaminidase release was detected in BMMC from *FcεRIα*^{-/-} mice, while BMMC from wild-type mice released the enzyme in a dose-dependent manner (Fig. 2B). The results are not due to a defect in the synthesis of the enzyme, since total BMMC lysates from both genotypes contained comparable amounts of the enzyme (data not shown). Finally, *FcεRIα*^{-/-} mice

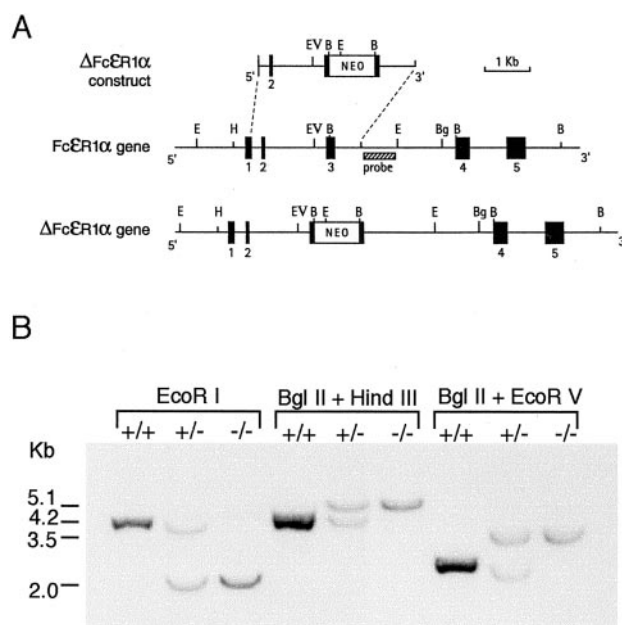


FIGURE 1. Generation of *FcεRIα*^{-/-} mice. **A**, Restriction maps of the construct used to transfect stem cells for homologous recombination (top), the endogenous intact *FcεRIα* gene (middle), and the disrupted *FcεRIα* gene after homologous recombination (bottom). EV, B, E, H, and Bg are restriction sites for *EcoRV*, *BamHI*, *EcoRI*, *HindIII*, and *BglII*, respectively. **B**, Southern blot analysis of *FcεRIα*^{-/-} mice. Mouse tail DNA was digested with restriction enzymes as indicated. Hybridization was performed using a 3'-flanking DNA probe (▨ in A). Southern blot patterns obtained from tail DNA of wild-type, heterozygous, and homozygous *FcεRIα*^{-/-} mice were as predicted from restriction maps shown in A.

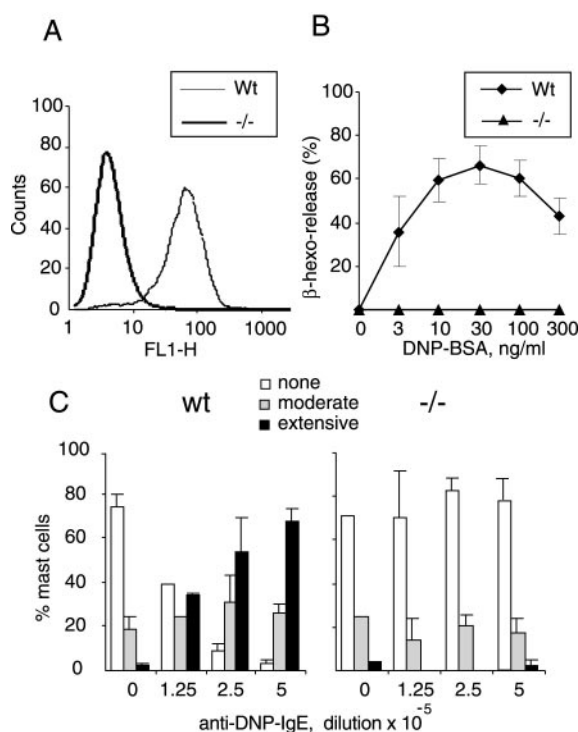


FIGURE 2. IgE receptor expression and mediator release from mast cells. **A**, Wild-type (---) and $Fc\epsilon RI\alpha^{-/-}$ (—) BMMC were incubated with 10 μ g/ml anti-DNP IgE, stained with FITC-conjugated anti-mouse IgE, and then analyzed by flow cytometry. **B**, Wild-type (wt) and $Fc\epsilon RI\alpha^{-/-}$ (—) BMMC were sensitized with anti-DNP IgE and then incubated with different concentrations of DNP-BSA. β -Hexosaminidase release was determined as described in *Materials and Methods*. Data are the mean $\pm$ SEM of BMMC preparations from three mice of each genotype. **C**, Different dilutions of anti-DNP IgE hybridoma ascites were injected intradermally into the ears of mice (one site per ear). Three hours later, 200 μ g DNP-BSA was injected i.v., and 30 min later the mice were euthanized, and tissue samples were collected. The samples were stained with toluidine blue, and the morphology of mast cells in the ear skin was evaluated as cells with none (normal mast cells; < 10% degranulation), moderate (10–50% degranulation), or extensive degranulation (>50% degranulation) and is expressed as the percentage of mast cells showing each state of degranulation. The data represent the mean $\pm$ SEM of three mice per genotype per group.

showed a defective IgE-dependent mast cell response *in vivo*. When sensitized with DNP-specific IgE s.c. and then challenged with the Ag DNP-BSA systemically, $Fc\epsilon RI\alpha^{-/-}$ mice did not exhibit an appreciable cutaneous reaction, in contrast to wild-type mice, which showed extensive dermal mast cell degranulation (Fig. 2C). Very few mast cells in the skin sections of control mice from both genotypes degranulated. Furthermore, the total numbers of mast cells in control vs Ag-challenged mice were comparable for both genotypes, suggesting that most, if not all, degranulated mast cells were detected by the method we used. The data were obtained from three mice per group for each genotype.

$Fc\epsilon RI\alpha^{-/-}$ mice exhibit reduced airway responses upon immunization to an Ag with adjuvant and then challenged with the Ag intranasally

When mice were immunized twice i.p. with OVA in alum and then challenged with nebulized OVA once a day for 6 consecutive days, both $Fc\epsilon RI\alpha^{-/-}$ and wild-type mice showed marked airway responses measured 4 h after the last Ag challenge, as gauged by the development of AHR and by the number of eosinophils as well as

the levels of IgE and IL-4 in BAL fluid. However, there was no significant difference between the two genotypes (data not shown). In mice, mast cells are distributed primarily peritracheally and not in lung parenchyma (38). Therefore, the extent of the contribution of mast cells in the airway response is probably dependent on where and how the Ag is administered. Indeed, when we challenged OVA-immunized mice with OVA intranasally 24 h after the last Ag challenge, we found a significant difference in certain airway responses between $Fc\epsilon RI\alpha^{-/-}$ mice and wild-type mice in three independently performed experiments. Both the number of eosinophils infiltrating the lungs (Fig. 3A), as well as the IL-4 levels in BALF were significantly lower in $Fc\epsilon RI\alpha^{-/-}$ mice than in wild-type mice (Fig. 3B), but IgE levels in BALF (Fig. 3C) and AHR (Fig. 3D) were very similar. In addition, $Fc\epsilon RI\alpha^{-/-}$ mice developed a significantly lower extent of eosinophil infiltration in perivascular areas in the lungs compared with wild-type mice (numbers of eosinophils in the lungs of $Fc\epsilon RI\alpha^{-/-}$ mice: Ag challenged, $38.43 \pm 12.8/100 \text{ mm}^2$; controls, $1.0 \pm 0.66/100 \text{ mm}^2$; wild-type mice: Ag challenged, $74.07 \pm 12.39/100 \text{ mm}^2$; controls, $2.33 \pm 2.3/100 \text{ mm}^2$).

We have performed experiments in which BAL fluid was obtained at 30, 60, 90, or 120 min after the last Ag challenge and noted that $Fc\epsilon RI\alpha^{-/-}$ mice consistently showed lower numbers of eosinophils and lower levels of IL-4 in BAL fluid (data not shown).

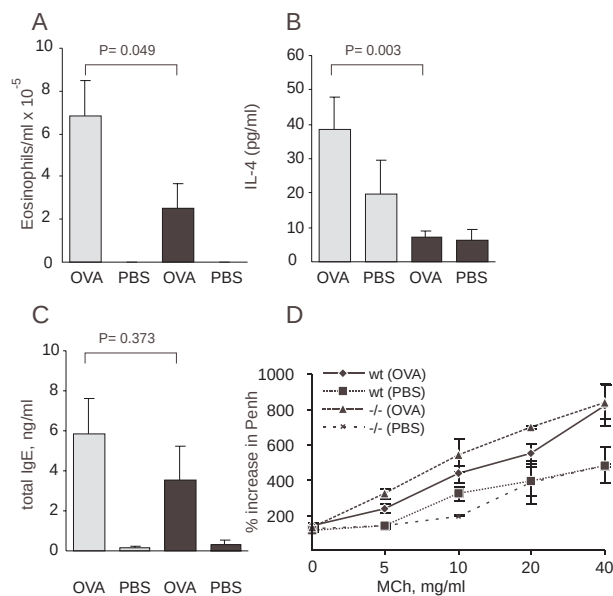


FIGURE 3. Comparison of airway inflammation induced in OVA-sensitized mice after intranasal challenge with OVA. Wild-type (□) and $Fc\epsilon RI\alpha^{-/-}$ (■) mice were immunized with OVA in alum and 14 days later were challenged with OVA or PBS intranasally on 4 alternate days. BALF was obtained 24 h after the last challenge and analyzed for the number of eosinophils (**A**) and levels of IL-4 (**B**) and total IgE (**C**). The number of eosinophils and IL-4 levels in BALF of OVA-challenged $Fc\epsilon RI\alpha^{-/-}$ mice were significantly reduced compared with those in wild-type mice ($p < 0.05$). Total IgE levels were not significantly different between $Fc\epsilon RI\alpha^{-/-}$ and wild-type mice. The development of AHR is shown as the percent increase in Penh values for both genotypes. Zero on the x-axis represents the saline challenge (**D**). There is no difference in the baseline Penh value between the groups. Data represent the mean $\pm$ SEM of three separate experiments (each experiment included three mice per group for each genotype).

FcεRIα^{-/-} mice exhibit significantly attenuated airway responses when immunized with an Ag in the absence of adjuvant and challenged with the Ag through the airways

Williams et al. (16) reported recently that mast cell-deficient mice exhibit reduced airway responses compared with wild-type mice only when the mice are sensitized with OVA in the absence of alum and then challenged with OVA through the airways. We reasoned that since FcεRI is expressed primarily by mast cells, such a protocol would more probably reveal phenotypic defects in FcεRIα^{-/-} mice. We sensitized both genotypes with OVA 11 times in the absence of adjuvant every other day and then, 14 days later, challenged the mice with nebulized OVA once a day for 6 consecutive days. The BALF from FcεRIα^{-/-} mice collected 4 h after the last Ag challenge contained a significantly lower number of eosinophils than wild-type mice (Fig. 4A). Further, BALF from FcεRIα^{-/-} mice contained a significantly lower amount of IL-4 compared with that from wild-type mice (Fig. 4B). The two genotypes, however, showed no difference in their IgE levels in BALF and AHR (Fig. 4, C and D). The data were generated from three independently performed experiments.

Anti-IL-5 Ab treatment results in a significant reduction in both airway eosinophil infiltration and AHR in FcεRIα^{-/-} mice

The causal relationship between eosinophils and AHR remains controversial. Treatment of mice with anti-IL-5 Ab, which results in a nearly complete suppression of the airway eosinophil re-

sponse, does not always result in a reduction in AHR (see *Discussion*). We believe that the effect of reduction in eosinophil infiltration may have a more pronounced effect on AHR when the mast cell response is blunted, such as in FcεRIα^{-/-} mice. To test this hypothesis, mice were immunized with OVA in alum and then challenged with OVA intranasally four times, while they also received rat anti-IL-5 mAb or normal rat IgG. The responses were measured 24 h after the last Ag challenge. Consistent with our other experiments, described above, BALF from rat IgG-treated FcεRIα^{-/-} mice contained significantly lower number of eosinophils than that from comparably treated wild-type mice (Fig. 5A). As expected, eosinophil numbers decreased dramatically in the anti-IL-5 Ab-treated groups for both genotypes (Fig. 5A). Also consistent with the results described above, FcεRIα^{-/-} mice were not defective in the development of AHR and, in fact, showed higher AHR than wild-type mice in the absence of anti-IL-5 treatment (Fig. 5B, rat IgG treated groups). However, the anti-IL-5 Ab treatment caused a substantial reduction in AHR in FcεRIα^{-/-} mice only (Fig. 5B), suggesting a greater dependency of the bronchial response on eosinophil infiltration in these mice. The data were generated from three independently performed experiments.

FcεRIα^{-/-} mice develop significantly lower AHR in response to Ag provocation

Previously we showed that when mice were immunized with Ag systemically and subsequently challenged by the same Ag through the airways, Ag-specific IgE remained detectable in the airways for >2 wk after the last airway challenge (39). Thus, the airway mast cells should remain sensitized by Ag-specific IgE even after the airway inflammation has subsided and should respond when the animals are provoked with the same Ag through the airways at a later time point. We reasoned that under these conditions the airway response is likely to be more dependent on mast cells, and thus a reduced airway response is more likely to be noted in FcεRIα^{-/-} mice. Therefore, mice were immunized twice with OVA, challenged 10 days later with nebulized OVA (1%) for 7

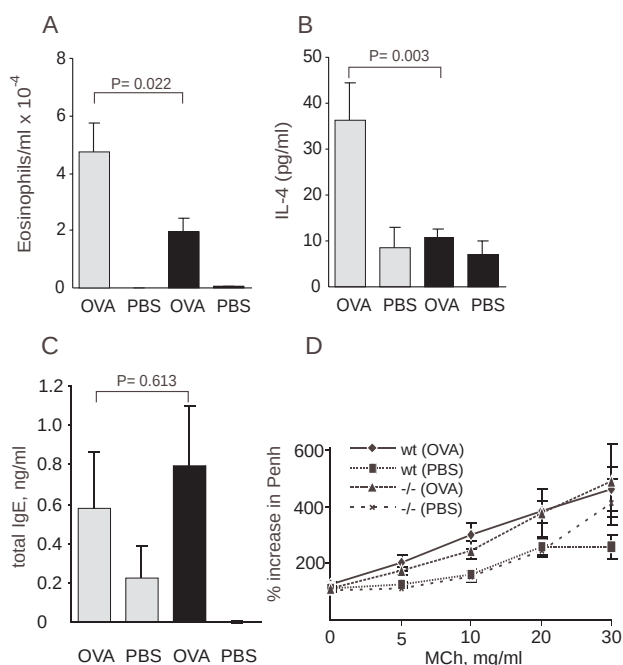


FIGURE 4. Comparison of airway inflammation induced in mice sensitized to OVA in the absence of adjuvant. Wild-type (□) and FcεRIα^{-/-} (■) mice were sensitized 11 times with OVA without adjuvant and 14 days later were challenged with nebulized OVA on 6 consecutive days. BALF was collected 4 h after the last aerosol and analyzed for the number of eosinophils (A) and levels of IL-4 (B) and total IgE (C). The levels of eosinophils and IL-4 were significantly lower in the OVA-treated groups of FcεRIα^{-/-} mice compared with wild-type mice. Development of AHR to MCh challenge was measured in unrestrained, conscious mice as Penh values. The percent increase in experimental Penh values for both genotypes is shown (D). There is no difference in the baseline Penh value between groups. Data represent the mean ± SEM of three separate experiments (each experiment included three mice per group for each genotype).

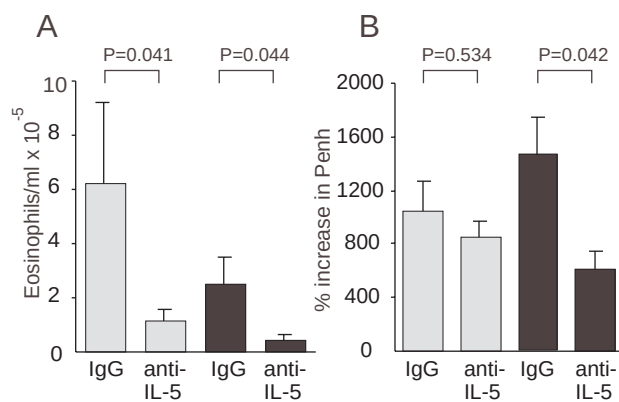


FIGURE 5. The effect of anti-IL-5 Ab treatment on airway responses. Wild-type (□) and FcεRIα^{-/-} (■) mice were immunized with OVA in alum once and 14 days later were treated on 4 alternate days with rat anti-IL-5 mAb or control rat IgG i.p., followed by intranasal OVA challenge. AHR was measured, and BALF was obtained 24 h after the last Ag challenge. A, Levels of eosinophils in BALF. In both genotypes the decrease in the number of eosinophils by anti-IL-5 treatment was significant compared with that in the IgG-treated groups. B, Percent increase in Penh in response to challenge with 20 mg/ml MCh. The percent increase in Penh in PBS-challenged mice (controls) was 400 ± 25% over baseline Penh (100%). There was no difference in the baseline Penh value between groups. Data represent the mean ± SEM of three separate experiments (each experiment included three mice per group for each genotype).

consecutive days, and 7 days after the last Ag challenge were exposed to OVA aerosol generated from a 5% OVA solution. Immediately afterward, AHR to MCh challenge was measured repeatedly over time. As shown in Fig. 6, a significant increase in Penh was already detectable in wild-type mice in the first measurement (10 min) after the 5% OVA provocation, and the Penh value reached a peak 1 h after provocation. In significant contrast, $Fc\epsilon RI^{-/-}$ mice did not show such an increase in Penh during the entire period of observation. Their response was, in fact, similar to that of mice that were sensitized but not challenged by the Ag, which showed only a slow increase in Penh in response to the MCh challenge following the Ag provocation. All groups returned to a similar low Penh 2 h after the 5% OVA provocation. The data were generated from two independently performed experiments.

Discussion

We report herein that $Fc\epsilon RI^{-/-}$ mice develop significantly lower allergic airway inflammatory response and AHR compared with wild-type mice when immunized with OVA systemically and then challenged by the same Ag through the airways. Firstly, when mice were immunized with OVA in alum and challenged with the Ag through the intranasal route or were immunized with OVA in the absence of adjuvant and challenged with nebulized Ag, $Fc\epsilon RI^{-/-}$ mice developed a significantly lower degree of eosinophilic airway inflammation compared with wild-type mice. Secondly, anti-IL-5 treatment before airway OVA challenge caused a significant decline in AHR in $Fc\epsilon RI^{-/-}$ mice, but not in wild-type mice. Thirdly, in an OVA provocation protocol, the wild-type mice with intact mast cell function responded with a higher AHR, while $Fc\epsilon RI^{-/-}$ mice failed to respond to this Ag provocation, essen-

tially demonstrating a background level AHR. Because in mice $Fc\epsilon RI$ is the major receptor for IgE on mast cells and basophils (35), our findings indirectly imply a role for IgE-mediated mast cell/basophil activation in the allergic airway response in murine models of asthma.

It has been reported that germinal centers are formed in mice in the parenchyma of inflamed lungs following airway Ag challenge. These contain IgG- and IgE-producing plasma cells, which have been shown to produce the corresponding isotypes when isolated from the lungs and cultured in vitro (40). The locally produced IgE as well as circulating IgE are likely to sensitize mast cells in the airways. Because mast cell activation has been shown to be a source of induction of eosinophil infiltration as well as AHR (9, 41), it is conceivable that mast cells play an important role in the airway response in murine models of asthma. Indeed, there are studies supporting the role of mast cells in these models (10, 11). However, there are other reports in the literature that question mast cell participation in this response (12–15). Recently, strong evidence has been presented that ties the role of mast cells in murine models of asthma to the experimental protocols used. Thus, Kobayashi et al. (17) noted that mast cell-deficient mice developed reduced AHR compared with normal congenic mice only under certain experimental conditions. They proposed that AHR could be induced by different mechanisms, and only certain protocols would enlist mechanisms that involve IgE-mediated mast cell activation. Likewise, Williams and Galli (16) noted that while mast cell-deficient mice developed airway eosinophilia and AHR comparable to normal congenic mice, only the former developed significantly reduced responses when the mice were immunized with OVA in the absence of adjuvant. Therefore, our finding that $Fc\epsilon RI^{-/-}$ mice exhibit reduced airway responses only under certain experimental conditions is in accord with these previous reports. It has to be noted, however, that Kobayashi et al. (17) described a contribution of mast cell activation to AHR, but not to eosinophil recruitment, whereas we found a $Fc\epsilon RI$ -dependent contribution to eosinophil recruitment, but not to AHR. Regardless of these differences, our findings together with these recent reports strongly support the importance of IgE-dependent mast cell activation in airway inflammation in murine models of asthma.

The reduced airway inflammatory response (eosinophil infiltration and IL-4 production) in $Fc\epsilon RI^{-/-}$ mice was observed only when mice were immunized with OVA in alum and then challenged with OVA administered intranasally and not when equally immunized mice were challenged with nebulized OVA. The results suggest that IgE-dependent mast cell activation contributes to the airway inflammation through a local effect in the airways and not in the phase of systemic sensitization to the Ag. In the airways, mast cell activation may lead to enhanced eosinophil infiltration through secreted mediators that promote the diapedesis of eosinophils by acting on these cells directly or on the blood vasculature. With regard to the reduced IL-4 production in $Fc\epsilon RI^{-/-}$ mice, the results are consistent with the fact that this cytokine is produced by mast cells. Alternatively, mast cells are known to regulate T cell functions (42), and thus the diminished mast cell activation in $Fc\epsilon RI^{-/-}$ mice could result in a down-regulated T cell response in the airways and consequently reduced Th2 cytokine production.

Our finding that anti-IL-5 Ab treatment caused a significant reduction in AHR only in $Fc\epsilon RI^{-/-}$ mice and not in wild-type mice sheds light on the relationship between infiltration of eosinophils and induction of AHR. Presently, the participation of eosinophils in the development of AHR is not clear. Studies by Nagai et al. (14) and Corry et al. (43) showed that the anti-IL-5 Ab treatment inhibited airway eosinophilia, but not AHR. Kobayashi et al. (17)

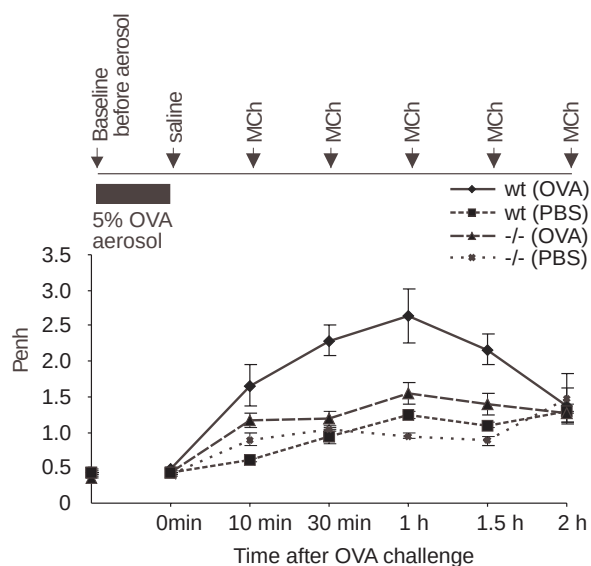


FIGURE 6. Comparison of AHR induced in mice after airway OVA provocation. Wild-type and $Fc\epsilon RI^{-/-}$ mice were sensitized to OVA in alum on days 1 and 14. Ten days later they were challenged by aerosol for 30 min with 1% OVA once a day for 7 consecutive days. One week later animals were exposed to 5% OVA or PBS aerosol for 20 min. Directly thereafter, mice were challenged with MCh (10 mg/ml) at 10, 30, and 60 min and 1.5, 2, 2.5, and 3 h, then every hour during the next 8 h, and AHR was measured at each time point. Results up to 2 h after 5% OVA challenge are shown; there is a significant difference between wild-type (OVA) and $Fc\epsilon RI^{-/-}$ (OVA) mice in the 30 min, 1 h, and 1.5 h points ($p < 0.05$). There was no significant change in Penh after 2 h (data not shown). Data represent the mean Penh $\pm$ SEM of two separate experiments (each experiment included three mice per group for each genotype).

also described markedly inhibited airway eosinophilia without a concomitant reduction in AHR in mice treated with anti-IL-5 Ab. However, there are studies showing that IL-5-deficient mice, which do not develop eosinophilia, also fail to develop AHR, supporting a causal relationship between eosinophilic airway inflammation and AHR (44). Hogan et al. (45) proposed that the contribution of eosinophils to the development of AHR in mice might sometimes be masked by coexisting pathways that operate independently of eosinophils. We propose that IgE-dependent mast cell activation may represent such a pathway, and that in its absence, as in the case of FcεRI $\alpha^{-/-}$ mice, a positive correlation between eosinophilia and AHR can be more easily demonstrated (Fig. 5). Our results also indicate that there is a synergy between mast cells and eosinophils in the induction of AHR in murine models of asthma. This suggests that mast cells and eosinophils secrete different mediators that may activate bronchial smooth muscle through distinct pathways, and these pathways cooperate synergistically in inducing heightened bronchial responsiveness.

We were able to show that IgE-dependent mast cell activation can clearly contribute to the development of AHR in experiments in which mice were given a late airway Ag provocation with 5% OVA, and the MCh challenge was administered immediately thereafter. Previously Martin et al. (9) showed with mast cell-deficient mice and normal congenic mice that IgE-mediated activation of mast cells enhances pulmonary responsiveness to cholinergic stimulation. The most likely explanation for our finding is that the observed increase in Penh in OVA-provoked wild-type mice represents a mast cell-dependent reaction induced by the Ag, since in both treatment groups of FcεRI $\alpha^{-/-}$ mice and in wild-type mice that did not receive OVA provocation (PBS control), the Penh value did not achieve the same level as in the test mice (Fig. 6). The results support the idea that IgE-dependent mast cell activation can result in AHR, probably through certain mediators that alone are not potent enough to cause a detectable bronchial response under our experimental conditions. Mice develop bronchoconstriction in response to MCh challenge because of the stimulation of smooth muscles via muscarinic M3 receptors. This mechanism is effective in connection with mast cell degranulation after the Ag provocation. The localization of mast cells in lungs in mice is mainly in the tracheal and main bronchial submucosal areas (38). Thus, the release of mast cell mediators triggered by Ag provocation and inhaled MCh takes place at the effector sites. We think that in our model FcεRI $\alpha^{-/-}$ mice only show the smooth muscle contraction through MCh provocation, whereas the wild-type mice are highly sensitized and respond with a mast cell-dependent reaction after Ag provocation plus cholinergic stimulation of smooth muscles.

In humans FcεRI is expressed on cell types other than mast cells and basophils, suggesting that this receptor may have broader functions than previously envisioned. In particular, the expression of FcεRI on monocytes and Langerhans cells that are classically considered APC suggests a possible role of this receptor in IgE-dependent Ag processing and presentation. In fact, peripheral monocytes and dendritic cells have been shown to take up Ags through FcεRI in an IgE-dependent fashion and to present these Ags to T cells (46, 47). The role of FcεRI in IgE-dependent Ag presentation by mast cells to T cells has also been demonstrated (48). However, in this study we did not observe any significant difference in IgE production between FcεRI $\alpha^{-/-}$ mice and wild-type mice. These results are consistent with the lack of expression of this receptor in classical APC in mice and also suggest that mast cell FcεRI does not play an important role in Ag presentation under our experimental conditions.

Our results show that FcεRI is important in the development of allergic airway inflammation. Hence, this receptor could be a useful target for the treatment of human asthma. Our study demonstrates that the role of FcεRI in allergic airway inflammation is strongly dependent on how mice are exposed to Ag. By using other experimental conditions, an even more pronounced amplification of the FcεRI participation in the allergic response could be achieved. Our experimental approaches provide important information for both mechanistic investigations as well as further testing of mast cell and IgE inhibitors. Furthermore, this report points out that mechanisms of asthma are multivariant and complicated, and therefore the treatment of this disease may require combinations of drugs targeting different components of the allergic response pathways. In addition, FcεRI $\alpha^{-/-}$ mice provide one important tool for testing treatments that are targeted at cell types other than mast cells and molecules other than IgE and FcεRI.

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