

Mast Cells, FcεRI, and IL-13 Are Required for Development of Airway Hyperresponsiveness after Aerosolized Allergen Exposure in the Absence of Adjuvant¹

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In certain models of allergic airway disease, mast cells facilitate the development of inflammation and airway hyper-responsiveness (AHR). To define the role of the high affinity IgE receptor (FcεRI) in the development of AHR, mice with a disruption of the α subunit of the high affinity IgE receptor (FcεRI^{-/-}) were exposed on 10 consecutive days to nebulized OVA. Forty-eight hours after the last nebulization, airway responsiveness was monitored by the contractile response of tracheal smooth muscle to electrical field stimulation (EFS). After the 10-day OVA challenge protocol, wild-type mice demonstrated increased responsiveness to EFS, whereas similarly challenged FcεRI^{-/-} mice showed a low response to EFS, similar to nonexposed animals. Further, allergen-challenged FcεRI^{-/-} mice showed less airway inflammation, goblet cell hyperplasia, and lower levels of IL-13 in lung homogenates compared with the controls. IL-13-deficient mice failed to develop an increased response to EFS or goblet cell hyperplasia after the 10-day OVA challenge. We transferred bone marrow-derived mast cells from wild-type mice to FcεRI^{-/-} mice 1 day before initiating the challenge protocol. After the 10-day OVA challenge, recipient FcεRI^{-/-} mice demonstrated EFS-induced responses similar to those of challenged wild-type mice. Transferred mast cells could be detected in tracheal preparations. These results show that FcεRI is important for the development of AHR after an aerosolized allergen sensitization protocol and that this effect is mediated through FcεRI on mast cells and production of IL-13 in the lung. *The Journal of Immunology*, 2004, 172: 6398–6406.

A feature of allergic disease is the increased production of IgE in response to common environmental allergens, and numerous studies have shown a significant relationship between sensitization to various allergens and allergic asthma (1, 2). In patients with asthma, exposure to allergen increases airway inflammation and airway hyperresponsiveness (AHR)³ (3, 4) even with small amounts of allergen, indicating a local amplification mechanism contributing significantly to inflammation and development of AHR in this setting. One of the cell populations responding to low doses of allergen and amplifying the response are tissue mast cells, which release a number of potent mediators of inflammation in an Ab-dependent mechanism (5). In asthmatics, mast cells have been described in increased numbers in the airway

wall, and recent reports have found these cells infiltrating airway smooth muscle (6).

Mast cells may recognize allergen through the high affinity IgE receptor (FcεRI), and IgE is thought to build a complex with allergen binding to this receptor. Allergen-cross-linking of receptors leads to the activation of mast cells, with the release of numerous mediators and cytokines (7–9). However, the role of mast cells in the development of AHR is somewhat controversial. When studying mechanisms of allergic airway disease in animals models, mast cell-deficient mice have variable decreases in eosinophil numbers (10, 11) after allergen challenge, but systemically sensitized and challenged mast cell-deficient mice are capable of developing a Th2 response, airway inflammation, and AHR similar to wild-type control mice (12–15). In most of these studies allergen sensitization was achieved by systemically injecting the allergen in combination with an adjuvant, whereas other studies, using less potent sensitization protocols, demonstrated a more obvious role for mast cells in the development of AHR (16, 17).

To characterize the role of mast cells and FcεRI in the development of AHR, we used an allergen challenge protocol in which wild-type, mast cell-deficient, and FcεRI-deficient mice were challenged repeatedly with allergen via the airways without the use of an adjuvant (18). In this model allergen exposure leads to dysfunction of muscarinic receptor 2, which results in an increase in acetylcholine release after electrical field stimulation (EFS) (19). After exclusive aerosolized allergen exposure, allergen-specific IgE and IgG1 are elevated in the serum of exposed mice (20), and the presence of B cells is essential for the development of AHR in this model (21). In the present study we show that after repeated

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³ Abbreviations used in this paper: AHR, airway hyperresponsiveness; BMMC, bone marrow-derived mast cell; EFS, electrical field stimulation; ES₅₀, frequency resulting in 50% of the maximal contraction; EU, ELISA unit; GFP, green fluorescent protein; MBP, major basic protein; MCh, methacholine; PAS, periodic acid-Schiff; sIL-13, soluble IL-13; UBI, human ubiquitin C promoter.

airway challenge, mast cell- and FcεRI-deficient mice fail to develop increased reactivity to EFS, a response that could be reconstituted by transfer of FcεRI-sufficient mast cells before the airway challenge.

Materials and Methods

Animals

Female BALB/cByJ mice, C57BL/6 mice, mast cell-deficient mice ([WB/Rej-*kit*^{W/+} × C57BL6J-*kit*^{W-v/+}]*F*₁⁻ [W/W^v] mice) and congenic WBB6F₁ normal mice from 8 to 12 wk of age were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice with a disruption of the α subunit of the high affinity IgE receptor (FcεRI^{-/-}; BALB/c background; provided by Drs. D. Dombrowicz and J. P. Kinet, Harvard Medical School, Boston, MA) (22, 23), IL-13-deficient mice (IL-13^{-/-}; BALB/c background; provided by Dr. D. Umetsu, Stanford University, Stanford, CA) (24), and CD57BL/6 mice that express a transgene for green fluorescent protein (GFP) under control of a human ubiquitin C promoter (UBI-GFP) (25) (provided by Dr. P. Marrack, National Jewish Medical and Research Center, Denver, CO) were maintained and bred in the animal facility. All mice were maintained on OVA-free diets. All experimental animals used in this study were under a protocol approved by the institutional animal care and use committee of National Jewish Medical and Research Center.

Experimental protocols

Experimental groups consisted of three or four mice per group, and each experiment was performed at least twice. Mice were exposed to OVA (Sigma-Aldrich, St. Louis, MO) as described previously (18). Briefly, a solution of 1% OVA in 0.9% saline or endotoxin-depleted 1% OVA solution was delivered by ultrasonic nebulization for 20 min daily over 10 consecutive days in a closed chamber. Control mice were exposed to 0.9% saline alone following the same protocol. AHR was assessed 48 h after the last nebulization.

Administration of the soluble IL-13Rα2-IgGFC fusion protein (sIL-13Rα2-Fc)

Murine IL-13Rα2-hIgG fusion protein was prepared as previously described (26). In the 10-day OVA challenge protocol, IL-13Rα2-hIgG fusion protein was administered by i.p. injection (300 μg/mouse) 1 h before OVA challenge on days 1, 4, 7, and 10. As a control, the same amount of human IgG was injected at the identical time points.

Detection of endotoxin and removal of endotoxin from OVA solution

Endotoxin concentrations in OVA solution were assessed by *Limulus* amoebocyte lysate (Charles Rivers Breeding Laboratories, Charleston, SC). Endotoxin depletion of OVA solutions was performed following previously described protocols (27, 28). Briefly, OVA solution (30 mg/ml in endotoxin-free saline) was mixed with 1% Triton X-114 (Sigma-Aldrich) by vigorously vortexing and was placed in an ice bath. After vortexing the chilled samples, the tubes were warmed at 37°C for 5 min to allow two phases to form. Samples were then centrifuged (10 min, 7000 rpm). After centrifugation, the detergent layer was found as an oily droplet at the bottom of the tube. The upper aqueous layer was removed carefully. Residual detergent in the aqueous phase was removed by treatment with endotoxin-free Bio-Beads SM-4 (Bio-Rad, Hercules, CA) as described previously (28). The resultant solution contained little endotoxin (1.8 ELISA unit (EU)/mg protein).

Transfer of bone marrow-derived mast cells (BMMC)

Mast cells were derived from bone marrow as previously described (29). Bone marrow was obtained from wild-type mice, FcεRI^{-/-} mice, IL-13^{-/-} mice, and UBI-GFP mice and was cultured in IMDM (Life Technologies, Grand Island, NY) supplemented with 5% FBS (Summit Biotechnology, Fort Collins, CO), 50 μM 2-ME (Life Technologies), 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 0.5 μg/ml amphotericin B, IL-3 obtained from medium conditioned by X63-AG8-653 myeloma cells transfected with a vector expressing IL-3 (30), and Kit ligand from medium conditioned by CHO cells (provided by S. Webb, National Jewish Medical and Research Center, Denver, CO). After 4 wk of culture, >98% of non-adherent cells contained granules that stained positively with toluidine blue, and >98% expressed c-Kit on their surface as determined by FACS analysis using anti-c-Kit mAb. Wild-type or FcεRI^{-/-} mice were injected with 5 × 10⁶ BMMC i.v. either 1 day or 20 wk before starting the airway challenges and received either 10-day treatment with OVA or saline ne-

bulization. C57BL/6 mice received 5 × 10⁶ BMMC from UBI-GFP mice i.v. 1 day before initiating the airway challenges with either saline or OVA.

Assessment of airway smooth muscle responsiveness

Forty-eight hours after the last airway challenge, AHR was monitored as airway smooth muscle responsiveness to EFS, measured as described previously (18). Tracheas were removed, and 0.5-cm preparations were placed in Krebs-Henseleit solution suspended by triangular supports transducing the force of contractions. EFS with an increasing frequency from 0.5 to 30 Hz was applied, and the contractions were measured. The duration of the stimulation was 1 ms. Frequencies resulting in 50% of the maximal contraction (ES₅₀) were calculated from linear plots from each individual animal and were compared between the groups. In addition, to assess postsynaptic cholinergic sensitivity of the tissues, cumulative dose-response curves to methacholine (MCh) were performed in half-log increments using concentrations ranging from 10⁻⁸ to 10⁻⁴ M. Results in the control and experimental groups at each time point were expressed in terms of the maximal contractile response as well as the EC₅₀ for MCh.

Histology

Lungs were fixed by inflation (1 ml) and immersion in 10% formalin. Cells containing eosinophilic major basic protein (MBP) were identified by immunohistochemical staining as previously described using rabbit anti-mouse MBP (provided by Dr. J. J. Lee, Mayo Clinic, Scottsdale, AZ). The slides were examined in a blinded fashion with a Nikon microscope (Melville, NY) equipped with a fluorescein filter system. The numbers of peribronchial eosinophils in the tissues were evaluated using IPLab2 software (Signal Analytics, Vienna, VA) for the Macintosh, counting six to eight different fields per animal. For detection of mucus-containing cells in formalin-fixed airway tissue, sections were stained with periodic acid-Schiff (PAS) and H&E and were quantitated as previously described (31). Tracheal sections were stained with 1% toluidine blue (Sigma-Aldrich) to detect metachromatic mast cell cytoplasmic granules. To maximize the ability to detect mast cells stained with toluidine blue, lung tissue was fixed in formalin for <24 h. Nonetheless, this may result in some underestimation of mast cell numbers. Tracheal sections from C57BL/6 mice receiving BMMCs derived from UBI-GFP mice were deparaffinized and mounted using mounting medium with 4',6-diamido-2-phenylindole hydrochloride (Vector Laboratories, Burlingame, CA).

Assessment of IL-13 levels in lung homogenates

Lungs were weighed and completely homogenized in dilution buffer (PBS, 0.1% Triton X (Sigma-Aldrich), and proteinase inhibitor (BD Pharmingen, San Diego, CA)) using a tissue homogenizer (Tissue Tearor; BioSpec, Bartlesville, OK) and then centrifuged at 14,000 rpm for 30 min. Supernatants were collected and frozen at -80°C until further analysis. Total protein content was measured by colorimetric assay (DC Protein Assay; Bio-Rad), and levels of IL-13 were assessed by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's directions.

Measurement of total and OVA-specific Ab

Serum levels of total IgE and OVA-specific IgE and IgG1 were measured by ELISA as previously described (31). Briefly, 96-well plates (Immunon 2; Dynatech, Chantilly, VA) were coated with either OVA (5 μg/ml) or purified anti-IgE (02111D; BD Pharmingen). After addition of serum samples, a biotinylated anti-IgE Ab (02122D; BD Pharmingen) was used as the detecting Ab, and the reaction was amplified with avidin-HRP (Sigma-Aldrich). IgG1 was detected using alkaline phosphatase-labeled anti-IgG1 (02003E; BD Pharmingen). The OVA-specific Ab titers of the samples were related to pooled standards that were generated in the laboratory and expressed as ELISA units per milliliter. Total IgE levels were calculated by comparison with known mouse IgE standards (BD Pharmingen). The limit of detection was 100 pg/ml for total IgE.

Statistical analysis

ANOVA was used to determine the levels of difference between all groups. Comparisons for all pairs were performed using Tukey-Kramer honestly significant difference test, and *p* values for significance were set at 0.05. Values for all measurements were expressed as the mean ± SEM.

Results

Aerosolized exposure to OVA increases total IgE and OVA-specific IgE and IgG1 in FcεRI^{-/-} mice

Mice exposed to 10-day OVA challenge (in the absence of adjuvant) demonstrate increased serum levels of OVA-specific IgE and

Table I. Levels of serum Igs^a

	BALB/c 10 days saline	BALB/c 10 days OVA	BALB/c 10 days OVA hIgG	BALB/c 10 days OVA sIL-13Rα2	FcεRI ^{-/-} 10 days saline	FcεRI ^{-/-} 10 days OVA	IL-13 ^{-/-} 10 days saline	IL-13 ^{-/-} 10 days OVA
Total IgE (ng/ml)	15.4 ± 0.6	43.4 ± 3.1	52.3 ± 7.2	50.5 ± 5.3	18.5 ± 6.2	54.3 ± 10	13.6 ± 1.2	39.7 ± 9.1
OVA-specific IgE (EU/ml)	<5	70.1 ± 10.4	63 ± 23	59 ± 19.5	<5	85 ± 13.9	<5	49.2 ± 14.9
OVA-specific IgG1 (EU/ml)	<5	82.8 ± 14.2	54.3 ± 24.5	49.4 ± 15.2	<5	78.5 ± 10.9	<5	35.3 ± 14.9

^a Mice were challenged as described in *Materials and Methods*. Serum levels of Igs were assessed 48 h after the last challenge. Mean values ± SEM are given; FcεRI^{-/-}, mice deficient of the high affinity IgE receptor; IL-13^{-/-}, mice deficient of IL-13; hIgG, mice treated with control Ab; sIL-13Rα2, mice treated with soluble IL-13Rα2 fusion protein; EU, ELISA units.

IgG1 (32). To assess the effect of 10-day OVA nebulization exposure on Ig production in FcεRI^{-/-} mice, we measured serum levels of total IgE and OVA-specific IgE and IgG1. OVA-challenged FcεRI^{-/-} mice showed increased serum levels of total IgE and OVA-specific IgE and IgG1, which were similar to those in OVA-challenged wild-type mice and significantly higher ($p < 0.05$) than those in saline-challenged FcεRI^{-/-} and wild-type mice (Table I).

FcεRI^{-/-} mice fail to develop AHR after 10-day OVA challenge

To assess whether mast cells play a role in the development of AHR in this model, we challenged mast cell-deficient mice on 10 consecutive days with OVA. Indeed, OVA-challenged, mast cell-deficient mice demonstrated no significant difference in ES₅₀ compared with the saline-challenged, mast cell-deficient mice (Fig. 1A), whereas the respective 10-day OVA-challenged, wild-type mice showed significantly ($p < 0.05$) lower ES₅₀ values than the saline-exposed wild-type mice (Fig. 1A).

Similar results were found for FcεRI^{-/-} mice. After 10-day OVA nebulization, wild-type mice showed a significantly lower ES₅₀ than saline-challenged animals, whereas OVA-challenged FcεRI^{-/-} mice showed a response to EFS similar to that of saline-exposed wild-type or FcεRI^{-/-} mice (Fig. 1B). Overall responses to carbachol and maximal generated force were not statistically different between any of the groups (data not shown).

Lung inflammation and goblet cell hyperplasia after 10-day OVA nebulization

Following this protocol, few inflammatory cells were detectable in the bronchoalveolar lavage fluid. Lung tissue inflammation and, in

particular, eosinophil accumulation were assessed using immunohistochemistry with anti-MBP. After the 10-day OVA challenge, wild-type mice demonstrated a modest increase in peribronchial eosinophil numbers compared with the saline-challenged control mice (Fig. 2). In OVA-challenged FcεRI^{-/-} mice, no such increase in tissue inflammation was found (Fig. 2). To assess goblet cell hyperplasia, slides were stained with PAS. After 10-day OVA nebulization, wild-type mice demonstrated PAS-positive cells (mean ± SEM; 15.2 ± 2.3 PAS-positive cells/mm of basement membrane; $p < 0.001$ compared with other groups), whereas saline-challenged wild-type and saline- and OVA-challenged FcεRI^{-/-} mice showed no PAS-positive cells (Fig. 3).

Endotoxin has been shown to directly induce the expression of IL-13 in mast cells (33, 34) and therefore might induce increased airway responsiveness and goblet cell hyperplasia. To address potential contamination of the nebulized OVA with endotoxin on the development of increased airway reactivity and goblet cell hyperplasia, mice were exposed for 10 days to 1% OVA (endotoxin concentration, 125.8 EU/mg OVA) or endotoxin-depleted 1% OVA (endotoxin concentration, 1.8 EU/mg OVA). After 10-day OVA exposure, mice receiving the endotoxin-depleted OVA developed similar increases in airway reactivity (Fig. 4A) and similar exposure of goblet cells (Fig. 4B) compared with mice receiving the untreated OVA.

IL-13 levels in lung homogenates are not increased after allergen challenge in FcεRI-deficient mice

IL-13 has been shown to be an important mediator in the development of AHR and goblet cell hyperplasia (35–38). To assess whether IL-13 is up-regulated in the 10-day challenge model, we

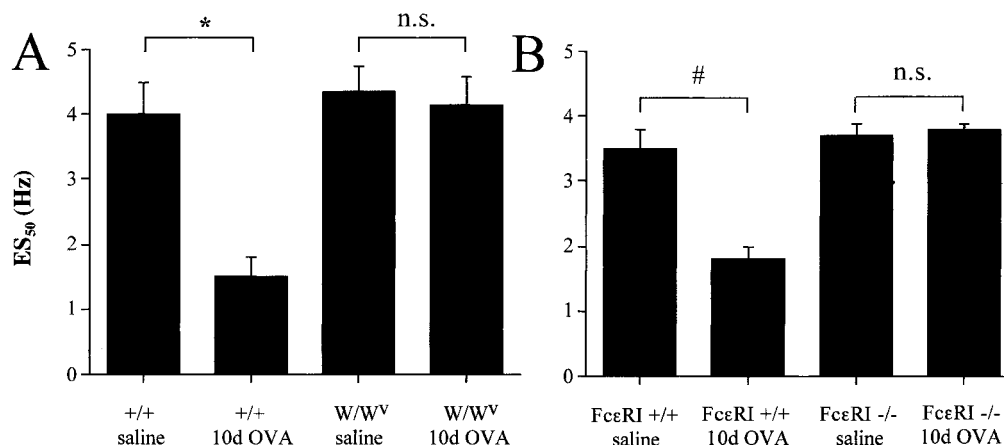


FIGURE 1. Airway responsiveness in mast cell-deficient mice (A) and FcεRI-deficient mice (B). Mast cell-deficient mice were exposed to either 10-day saline (W/W^v saline; $n = 8$) or 10-day OVA (W/W^v 10 day OVA; $n = 10$) as were the wild-type control mice (+/+ saline, $n = 8$; +/+ 10 day OVA, $n = 8$, respectively; data from two independent experiments). Similarly FcεRI-deficient mice received either 10-day saline (FcεRI^{-/-} saline; $n = 12$) or 10-day OVA (FcεRI^{-/-} 10 day OVA; $n = 16$) as did the wild-type controls (FcεRI^{+/+} saline; $n = 12$; FcεRI^{+/+} 10 day OVA, $n = 18$, respectively; data from four independent experiments). The mean ± SEM are given. *, $p < 0.01$ compared with +/+ saline, W/W^v saline, and W/W^v 10 day OVA; #, $p < 0.01$ compared with FcεRI^{+/+} saline, FcεRI^{-/-} saline, and FcεRI^{-/-} 10 day OVA.

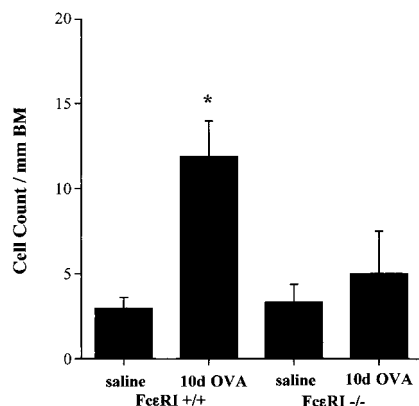


FIGURE 2. Role of FcεRI in eosinophil inflammation in the lung after allergen exposure. Peribronchial eosinophil inflammation was quantified in lung tissue stained with an Ab to MBP, and results are expressed as the number of MBP-positive cells per millimeter of basement membrane (BM). Peribronchial inflammation was assessed in saline-challenged (saline FcεRI^{+/+}; *n* = 8) and OVA-challenged (OVA FcεRI^{+/+}; *n* = 8) FcεRI^{+/+} mice and in saline-challenged (saline FcεRI^{-/-}; *n* = 6) and OVA-challenged (OVA FcεRI^{-/-}; *n* = 8) FcεRI^{-/-} mice. Data were obtained from two independent experiments; the mean ± SEM are given. *, *p* < 0.05 compared with all other groups.

measured levels of IL-13 in lung homogenates. Indeed, OVA-challenged wild-type mice exhibited increased levels of IL-13 in lung homogenates 48 h after the last challenge compared with levels in saline-challenged control mice (Fig. 4). However, OVA-challenged FcεRI^{-/-} mice showed no elevation in the levels of IL-13 in their lung homogenates (Fig. 5). Similarly, lung homogenates of OVA-challenged mast cell-deficient mice exhibited significantly (*p* < 0.05) lower IL-13 levels compared with those of OVA-challenged mast cell-sufficient mice (Fig. 5).

IL-13 mediates the development of AHR after 10-day OVA challenge

To elucidate the role of IL-13 in the development of AHR after 10-day OVA challenge, we monitored tracheal smooth muscle responses to EFS in IL-13^{-/-} and IL-13^{+/+} mice challenged with OVA as well as after treatment with an inhibitor of IL-13 (sIL-13Rα2). OVA-challenged IL-13^{-/-} mice showed no differences in EFS compared with saline-challenged IL-13^{-/-} and IL-13^{+/+} mice, and they were significantly different than the responses in OVA-challenged IL-13^{+/+} mice (Fig. 6). In parallel, OVA-challenged IL-13^{+/+} mice treated with sIL-13Rα2 during the challenge period normalized their EFS responses to levels seen in saline-challenged IL-13^{+/+} mice or IL-13^{-/-} animals (Fig. 6). The OVA-challenged IL-13^{-/-} and IL-13^{+/+} mice treated with sIL-13Rα2 also showed no PAS-positive cells. Importantly, in these experiments, serum Ab levels were not statistically different in OVA-challenged IL-13^{+/+} vs IL-13^{-/-} mice or in sIL-13Rα2- vs human IgG-treated mice (Table I).

Transfer of BMMC from wild-type to FcεRI-deficient mice restores their ability to develop AHR

In rodents, FcεRI is expressed only on mast cells and basophils (39). We therefore investigated whether the inability of high affinity IgE receptor-deficient (FcεRI^{-/-} IL-13^{+/+}) and IL-13-deficient mice (FcεRI^{+/+} IL-13^{-/-}) to develop AHR could be overcome by adoptive transfer of BMMC from wild-type (FcεRI^{+/+} IL-13^{+/+}) animals. Bone marrow cells from FcεRI^{+/+} IL-13^{+/+}, FcεRI^{-/-} IL-13^{+/+}, and FcεRI^{+/+} IL-13^{-/-} mice were cultured

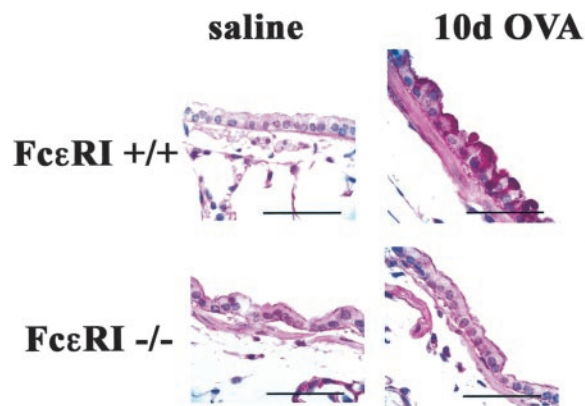


FIGURE 3. Role of FcεRI in goblet cell hyperplasia after allergen exposure. Goblet cell hyperplasia was detected using PAS staining 48 h after the last airway challenge in either 10-day saline-exposed (saline) or OVA-exposed (10 day OVA) wild-type (FcεRI^{+/+}) or FcεRI-deficient (FcεRI^{-/-}) mice. Bar = 50 μm.

under conditions described in *Materials and Methods*. After 4 wk, 98% of the cells in culture were considered to be mast cells, because they expressed c-Kit (CD117) and FcεRI (in the case of FcεRI^{+/+} donors) on their surfaces (data not shown).

Mast cells were injected i.v. into wild-type, FcεRI^{-/-}, and IL-13^{-/-} recipients 1 day before starting the 10-day OVA challenge protocol. None of the recipients exposed to saline challenge alone showed any response to EFS (Fig. 7). The positive response to EFS was restored in FcεRI^{-/-} IL-13^{+/+} mice that received BMMC from FcεRI^{+/+} IL-13^{+/+} or FcεRI^{+/+} IL-13^{-/-} mice before 10-day OVA challenge. Administration of sIL-13Rα2 to the FcεRI^{-/-} IL-13^{+/+} recipients prevented the reconstitution of EFS responsiveness after transfer of BMMC from FcεRI^{+/+} IL-13^{+/+} (mean ± SEM; ES₅₀, 4.13 ± 0.33 Hz; *n* = 8 from two independent experiments) and FcεRI^{+/+} IL-13^{-/-} donors (4.37 ± 0.27 Hz; *n* = 7 from two independent experiments) and OVA exposure. In contrast, FcεRI^{-/-} IL-13^{+/+} mice receiving autologous BMMC from FcεRI^{-/-} IL-13^{+/+} mice and 10-day OVA challenge showed

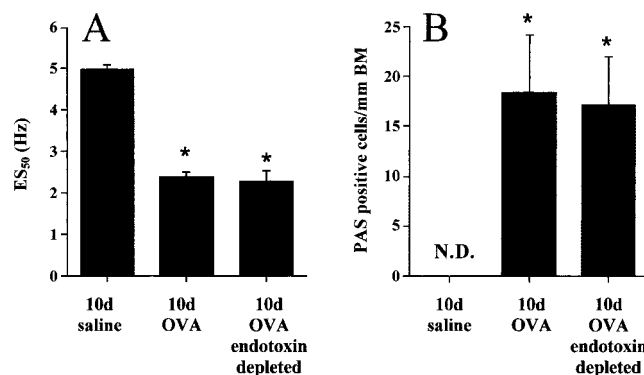
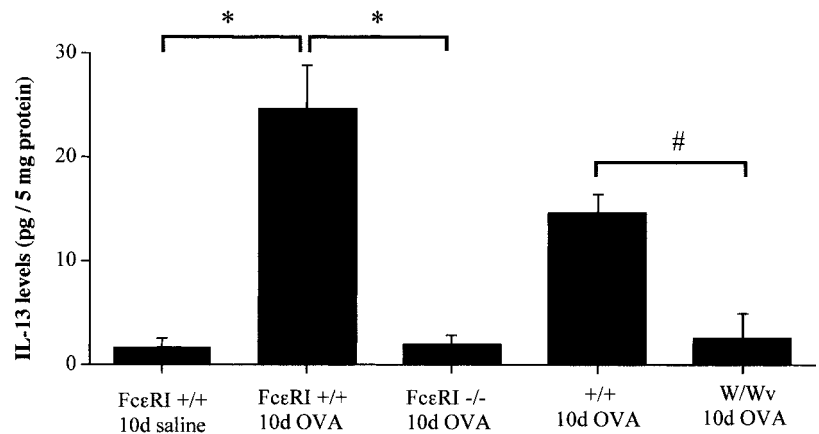


FIGURE 4. Endotoxin contamination does not affect airway responsiveness and goblet cell hyperplasia after allergen exposure. To assess the effect of endotoxin contamination on the development of increased airway responsiveness, BALB/c mice were exposed to 10-day saline (10 day saline; *n* = 8 from two independent experiments), 10-day OVA (10 day OVA; *n* = 8 from two independent experiments), or 10-day OVA that had been depleted of endotoxin (10 day OVA endotoxin-depleted). Forty-eight hours after the last challenge, airway responsiveness was assessed by EFS (A), and lung tissue was obtained and stained with PAS for quantification of goblet cells (B). N.D., not detected. The mean ± SEM are given. *, *p* < 0.01 compared with 10-day saline.

FIGURE 5. Levels of IL-13 in lung homogenates. Levels of IL-13 and total protein content were assessed in lung homogenates 48 h after the last challenge from either saline-challenged (FcεRI^{+/+} 10 day saline; *n* = 8) or OVA-challenged (FcεRI^{+/+} 10 day OVA; *n* = 8) wild-type mice, OVA-challenged FcεRI^{-/-} mice (FcεRI^{-/-} 10 day OVA; *n* = 7), and OVA-challenged mast cell-deficient mice (W/W^v 10 day OVA; *n* = 8) as well as their respective OVA-challenged wild-type controls (+/+ 10 day OVA; *n* = 8). The mean ± SEM from two independent experiments are given. *, *p* < 0.01; #, *p* < 0.05.



no response to EFS. This confirms the requirement for FcεRI-sufficient mast cells in the development of a positive response to EFS.

The results were similar when BMMC transfer was performed 20 wk before starting the 10-day OVA exposure. Again, FcεRI^{-/-} IL-13^{+/+} mice receiving BMMC from FcεRI^{+/+} IL-13^{+/+} mice before 10-day OVA exposure showed a significantly (*p* = 0.012) lower ES₅₀ (mean ± SEM; 3.1 ± 0.3 Hz; *n* = 9 from two independent experiments) compared with FcεRI^{-/-} IL-13^{+/+} mice receiving autologous BMMC from FcεRI^{-/-} IL-13^{+/+} mice before 10-day OVA exposure (4.9 ± 0.2 Hz; *n* = 6 from two independent experiments).

Transfer of BMMC from wild-type to IL-13-deficient mice does not restore their ability to develop AHR

In addition to the need for FcεRI-sufficient mast cells in the development of smooth muscle hyper-responsiveness to EFS after 10-day OVA challenge, a role for IL-13 was also identified. Mast cells are a potential source of IL-13 (40). However, in the adoptive transfer experiments described in Fig. 7, FcεRI^{+/+} IL-13^{-/-} recipients of FcεRI^{+/+} IL-13^{+/+} BMMC failed to develop EFS responses after 10-day OVA challenge. This occurred despite the fact that these cells, in the same numbers, were capable of reconstituting the EFS response in FcεRI^{-/-} IL-13^{+/+} mice (Fig. 7).

Mast cells can be detected in tracheal tissue of FcεRI^{-/-} mice

To assess whether mast cells can be detected in tracheal tissue from FcεRI^{-/-} mice, tracheal sections from 10-day OVA-challenged FcεRI^{-/-} mice and 10-day OVA-challenged FcεRI^{-/-} after transfer of BMMCs were stained with toluidine blue. In both groups mast cells were detected in the subepithelial region as well as in close proximity to smooth muscle (Fig. 8). To further assess whether transferred BMMCs migrate to tracheal tissue, green-fluorescent BMMCs from UBI-GFP mice were transferred to C57BL/6 recipients 1 day before initiating the airway challenges. After 10-day exposure to either saline or OVA, transferred green-fluorescent cells could be detected in the tracheal tissue 48 h after the last challenge (Fig. 8). In saline-exposed mice we detected 1 ± 1 GFP⁺ mast cells per section, whereas in OVA-exposed mice, we detected 4 ± 1.5 GFP⁺ mast cells (*n* = 4 in each group).

Discussion

Among the many features of allergic asthma is an increase in levels of serum IgE, and in epidemiologic studies a relationship between IgE serum levels and severity of AHR has been established (41). In the present study we examined the role of the high affinity

IgE receptor (FcεRI) and by implication IgE in the development of allergic airway disease, using mice deficient in FcεRI due to disruption of the FcεRIα gene. These mice are essentially normal in all aspects of their development and maturation, have functional mast cells (22, 23), which can be found in tracheal tissue. Additionally, FcεRI^{-/-} mice are able to mount an immune response to allergen exposure, as shown by elevated levels of total IgE and allergen-specific IgE and IgG1. In these respects FcεRI-deficient mice are similar to wild-type mice. In contrast, in other aspects of allergic airway disease, allergen-exposed FcεRI^{-/-} mice failed to develop increased airway reactivity, goblet cell hyperplasia, and airway inflammation. This phenotype in FcεRI^{-/-} mice is unmasked only when they are exposed to allergen, in this case via the airways, in the absence of adjuvant. This contrasts with their response to systemic sensitization (together with adjuvant), followed by airway challenge, where FcεRI^{-/-} mice develop AHR and airway inflammation similar to those exhibited by their sensitized and challenged wild-type littermates (42). Furthermore, in the 10-day OVA challenge model the heightened airway reactivity they develop is detected as a change in the response of tracheal smooth muscle to EFS, a consequence of altered muscarinic receptor 2 function and increased acetylcholine release (18). The allergen exposure protocols have been associated with differences in the approaches to detect altered airway function. After the 10-day aerosolized allergen exposure, these mice do not develop MCh-induced AHR as seen in systemically sensitized mice (42).

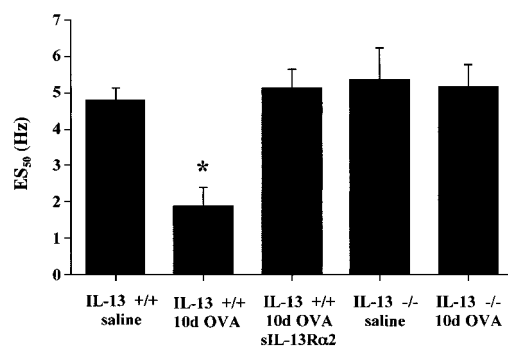
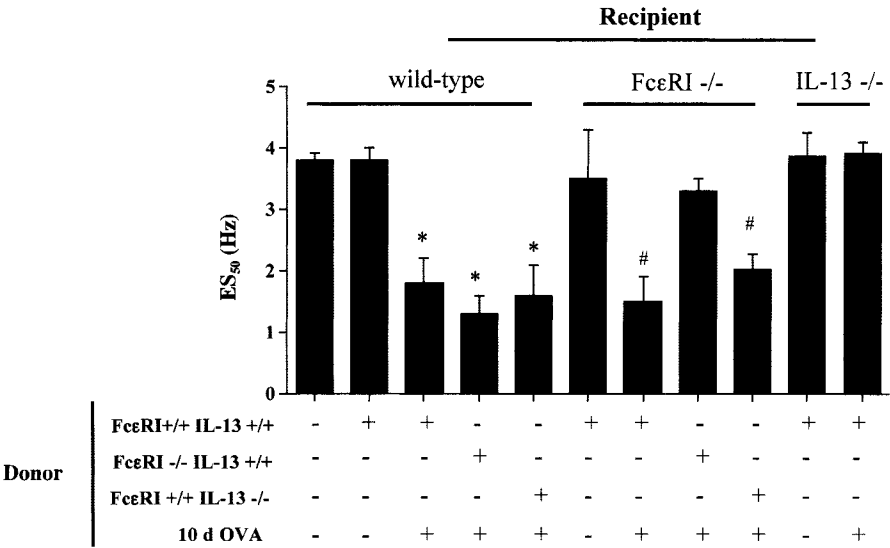


FIGURE 6. IL-13 mediates the development of increased airway responsiveness after 10-day OVA exposure. Wild-type mice treated with control Ab (IL-13^{+/+}, 10 day OVA; *n* = 6) or with sIL-13Rα2 (IL-13^{+/+}, 10 day OVA; *n* = 10) as well as IL-13-deficient mice (IL-13^{-/-} 10 day OVA; *n* = 8) were exposed to 10-day OVA challenge. As a control, IL-13-deficient mice were exposed to 10-day saline (IL-13^{-/-} saline OVA; *n* = 6). The mean ± SEM from two independent experiments are given. *, *p* < 0.01 compared with all other groups.

FIGURE 7. Transfer of wild-type BM-MCs to FcεRI^{-/-} mice reconstitutes their ability to develop AHR. BMMCs derived from wild-type mice (FcεRI^{+/+} IL-13^{+/+}), FcεRI-deficient mice (FcεRI^{-/-} IL-13^{+/+}), or IL-13-deficient mice (FcεRI^{+/+} IL-13^{-/-}) were transferred to wild-type (wild-type), FcεRI-deficient (FcεRI^{-/-}), or IL-13-deficient (IL-13^{-/-}) recipients, which were then challenged with OVA (10 day OVA). Each condition contained eight mice, and the mean ± SEM from two independent experiments are given. *, *p* < 0.01 compared with saline-challenged (10 day OVA-) wild-type recipients; #, *p* < 0.01 compared with saline-challenged (OVA-) and FcεRI^{-/-} IL-13^{+/+} transferred FcεRI^{-/-} recipients.



In rodents, FcεRI has been found to be expressed only on mast cells and basophils (39). Indeed, in support of the findings in FcεRI^{-/-} mice, mast cell-deficient mice also failed to develop increased airway reactivity after 10-day OVA exposure. This finding is somewhat in contrast to previous studies using mast cell-deficient mice, which suggested that the induction of airway inflammation and AHR does not require the presence of mast cells

(13, 15). Similarly, it has been shown that the presence of IgE is not mandatory for the development of allergic airway disease (43, 44). In most of these studies animals were sensitized systemically by injection of the allergen in combination with an adjuvant and then challenged via the airways, which leads to marked inflammatory changes in the lung, high serum levels of allergen-specific Abs, increased airway reactivity including sensitivity to inhaled MCh, and prominent goblet cell hyperplasia. In the present study mice were exposed to a less potent exposure protocol using aerosolized allergen alone. This resulted in little airway inflammation and less goblet cell hyperplasia than seen after systemic sensitization.

Under these conditions without adjuvant, mast cells appear to play an essential role in the development of allergic airway disease, as mast cell-deficient mice fail to develop increased airway reactivity after aerosolized allergen exposure. Similarly, mice lacking the FcεRI fail to develop AHR after this exposure protocol. These findings share some features with recent reports using different systemic allergen exposure protocols (16, 45). In those reports, where mast cells and FcεRI were found to be critical for the development of AHR and airway inflammation, the exposure protocol deviated from those using repeated systemic sensitization and adjuvant, conditions under which mast cells and IgE were shown not to be important (15, 43). Williams et al. (16) found that mast cell-deficient mice exhibited weaker airway responsiveness and lung tissue eosinophil inflammation after a systemic sensitization protocol, but without adjuvant. In a different study, Mayr et al. (45) described the inability of FcεRI-deficient mice to develop allergic airway inflammation and AHR to MCh after systemic sensitization consisting of a single OVA injection (with adjuvant) and consecutive intranasal challenges.

IgE can bind either to FcεRI or to the low affinity IgE receptor (FcεRII, CD23). Whereas cross-linking of FcεRI with IgE and allergen has been associated with mast cell activation, ligation of FcεRII can be either activating (carrying an immunoreceptor tyrosine-based activation motif) or inhibitory (carrying an immunoreceptor tyrosine-based inhibitory motif). In allergic airway disease, FcεRII has been shown to exhibit negative regulatory effects (46). In the present study cross-linking of FcεRI seemed to be activating in the development of increased airway reactivity and goblet cell hyperplasia, as FcεRI^{-/-} mice failed to develop increased smooth muscle responsiveness after allergen exposure. Interestingly, transfer of FcεRI^{+/+} BMMC to FcεRI^{-/-} animals 14 days before the assessment of airway function fully reconstituted

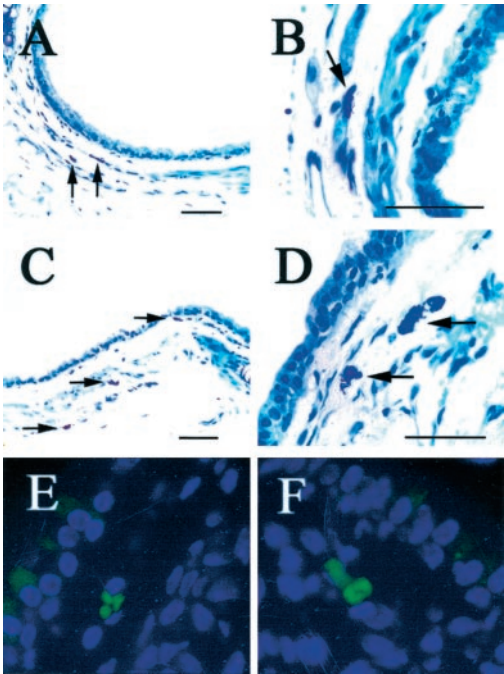


FIGURE 8. Transferred BMMCs can be found in tracheal tissue. Sections of tracheal tissue from 10-day OVA-challenged FcεRI^{-/-} (A and B) and from 10-day OVA-challenged FcεRI^{-/-} after transfer of BMMCs (C and D) were stained with toluidine blue. Mast cells (arrows) were detected in the subepithelial region as well as in close proximity to smooth muscle (bar = 50 μm). After transfer, BMMCs derived from UBI-GFP mice were detected in tracheal tissue of 10-day saline-challenged recipient C57BL/6 mice (E) as well as 10-day OVA-challenged recipient C57BL/6 mice (F) 48 h after the last challenge. In saline- as well as OVA-challenged mice, fluorescent signals from GFP positive cells (green) were detected in tracheal tissue. Blue staining are nuclei stained with DAPI. Note the weak background fluorescence in epithelial cells. Magnification, ×100.

their ability to develop increased airway reactivity, suggesting that in this model the expression of FcεRI on the mast cells is important and that mast cells alone can reconstitute this response. Previously, transfer experiments with BMMCs have been performed mainly to reconstitute mast cells in mast cell-deficient mice (16, 47–49). In that approach the analysis was conducted 10 wk or more after mast cell transfer. In the present study we assessed the response to 10-day OVA challenge at 2 and 20 wk after transfer of FcεRI^{+/+} BMMC into FcεRI^{-/-} mice. In both cases the responses were similar. After transfer of green fluorescent BMMCs derived from GFP-overexpressing mice, we were able to detect transferred cells in the tracheal tissue 14 days post-transfer, suggesting that this time period is sufficient for migration of transferred cells to the trachea. Transfer of FcεRI^{+/+} cells reconstituted the ability of FcεRI^{-/-} mice to develop increased airway reactivity independent of the time point after the transfer was made. In other disease models, mast cell reconstitution in mast cell-deficient mice normalized the ability to mount a mast cell-dependent response 2 days (50) and 9 days (51) after the transfer. In the present study we used BMMCs cultured in a mixture of IL-3 and Kit ligand for 4 wk. Under these conditions, >98% of the cells expressed FcεRI as well as c-Kit on their surface and previous studies have suggested that the overwhelming majority of cells grown under these culture conditions are mast cells (52, 53) as well as our own electron microscopy studies. A very small percentage have features of basophils (52, 53), so it cannot be completely excluded that basophils, which may also express FcεRI and c-Kit on their surface, may contribute to the reconstitution of the response. The finding that FcεRI^{+/+} mast cells alone can restore responsiveness in FcεRI^{-/-} mice does not differentiate between the activated mast cells having a direct role in the EFS response and their having an indirect effect through the release of mediators that trigger other cells necessary for the EFS response.

Activation of lung mast cells through FcεRI leads to the release of a variety of mediators via degranulation. Activation also triggers cytokine transcription and protein synthesis. A significant finding was that lung homogenates from allergen-exposed FcεRI^{-/-} mice had significantly lower levels of IL-13 than those from allergen-exposed wild-type controls. Altered airway function and goblet cell hyperplasia have been closely linked to IL-13 expression in the lung (35–38, 54), and IL-13 may directly act on epithelial cells to induce goblet cell hyperplasia and airway hyper-reactivity (55). As a pleiotropic cytokine, IL-13 is secreted by activated Th2 cells and exhibits immunoregulatory activities that partially overlap with those of IL-4 (56). IL-13 also facilitates the preferential recruitment of eosinophils (and T cells) to the airway tissues (57) and airway mucus secretion, which can exacerbate airway responsiveness (54, 58). Although not necessary for, or even capable of, inducing Th2 development, IL-13 plays a regulatory role in Th2 cell activation (24). Administration of IL-13 or overexpression of IL-13 in the airways induced airway eosinophilia, mucus production, and AHR to varying degrees (35, 36, 54).

In further attempts to link mast cells, FcεRI, and IL-13 in the present model, we chose two approaches: using IL-13 gene-targeted mice (24, 37) and the other treatment of mice during the challenge period with the decoy receptor sIL-13Rα2 (35, 36, 38). Both IL-13^{-/-} mice and sIL-13Rα2-treated mice had levels of serum Abs similar to those of the wild-type mice, confirming that in mice, IL-13 does not regulate serum Ab production (37, 38). Furthermore, IL-13^{-/-} mice have fully functional mast cells that signal through FcεRI, degranulate, express similar amounts of cytokines (other than IL-13) and release of lipid mediators and after activation with allergen-specific IgE and allergen (data not shown). Both approaches revealed that the presence of IL-13 was necessary

for development of the heightened response to EFS and goblet cell hyperplasia after the 10-day OVA challenge.

In addition to T cells, mast cells produce and secrete IL-13 (40). In rodents it has been shown that, depending on their phenotype, mast cells express IL-13 mRNA either constitutively or only after FcεRI-mediated activation (59). All of our results in the 10-day OVA challenge model are consistent with the idea that mast cells are an important source of IL-13. Mast cells may produce IL-13 after stimulation with endotoxin (33, 34). However, in the present model endotoxin contamination of OVA seemed to be irrelevant to the development of increased airway reactivity and goblet cell hyperplasia, as OVA depleted of endotoxin, induced similar degrees of airway reactivity and goblet cell hyperplasia. Interestingly, IL-13^{-/-} mice failed to develop AHR even after adoptive transfer of IL-13^{+/+} mast cells, suggesting that the reconstitution solely of mast cells capable of producing IL-13 is not enough to change airway reactivity. This observation, together with the demonstration that transfer of BMMC from FcεRI^{+/+} IL-13^{-/-} mice to FcεRI^{-/-} IL-13^{+/+} recipients restores their ability to develop AHR, indicates that activation of mast cells through FcεRI is directing IL-13 production in the lung, a critical element in the development of altered airway function. However, under these conditions, mast cells may not be the predominant source of the IL-13 needed for this response. This does not diminish the need for IL-13, as reconstitution of airway responsiveness after mast cell transfer (even from IL-13^{-/-} mice) was still critically dependent on IL-13; treatment with IL-13Rα2 after the mast cell transfer completely inhibited the reconstitution of increased airway responsiveness. We may speculate either that the IL-13 derived from the numbers of transferred mast cells (IL-13^{+/+} into IL-13^{-/-}) was insufficient to restore AHR or that other cell types are a major source of IL-13 when stimulated by factors released from activated mast cells. There are a number of other cell types that may serve as a source of IL-13 after activation by a factor(s) released from IgE-FcεRI-activated mast cells, including T cells, smooth muscle cells (60), basophils, and NKT cells (61, 62). Studies assessing these potential sources are underway.

In summary, we have demonstrated essential roles for FcεRI, mast cells, and IL-13 in the development of allergic airway disease after allergen exposure exclusively via the lungs in the absence of adjuvant. FcεRI on mast cells mediates these effects by regulating the production of IL-13. As aeroallergen exposure via the lungs in the absence of adjuvant is a major factor in the etiology of asthma, this IgE-mast cell-IL-13 pathway may play a significant contributory role in the development, maintenance, and exacerbation of the disease.

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