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Sequential Engagement of FcεRI on Mast Cells and Basophil Histamine H₄ Receptor and FcεRI in Allergic Rhinitis

Yoshiki Shiraishi,* Yi Jia,* Joanne Domenico,* Anthony Joetham,* Hajime Karasuyama,[†] Katsuyuki Takeda,*¹ and Erwin W. Gelfand*,¹

Histamine H₄ receptor (H₄R)-deficient mice (H₄R^{-/-}), H₄R antagonist-treated wild-type (WT) mice, and WT mice depleted of basophils failed to develop early (EPR) or late phase (LPR) nasal responses following allergen sensitization and challenge. Basophil transfer from WT but not H₄R^{-/-} mice restored the EPR and LPR in H₄R^{-/-} mice. Following passive sensitization with OVA-specific IgE, FcεRI^{-/-} recipients of WT basophils plus OVA and histamine developed an EPR and LPR. OVA-IgE passively sensitized FcεRI^{-/-} recipients of H₄R^{-/-} basophils and OVA and histamine challenge failed to develop an EPR or LPR, and basophils were not detected in nasal tissue. In contrast, recipients of basophils from IL-13^{-/-} and IL-4^{-/-}/IL-13^{-/-} mice developed an EPR but not an LPR. These results demonstrate the development of allergic rhinitis proceeded in two distinct stages: histamine release from FcεRI-activated mast cells, followed by histamine-mediated recruitment of H₄R-expressing basophils to the nasal cavity and activation through FcεRI. *The Journal of Immunology*, 2013, 190: 000–000.

Allergic rhinitis (AR) is a common chronic disease, affecting >600 million people worldwide (1). The recurrent and chronic symptoms of allergic rhinitis, that is, sneezing, itching, rhinorrhea, and nasal congestion, impair the quality of life in these patients and contribute to a major socioeconomic burden. Similar to other allergic diseases, corticosteroids (topical) remain the most effective therapy in AR, despite major limitations (2). Only half of the patients gain adequate relief, whereas a large proportion suffers from persistent symptoms (3). New therapeutic targets in the treatment of AR are actively being investigated.

Histamine has long been recognized as an important mediator of allergic inflammation (4). To date, four subtypes of histamine receptors, H₁, H₂, H₃, and H₄, have been described (5, 6). Histamine H₁ receptor antagonists (inverse agonists) have often been used as first-line therapy in AR, but they are only modestly effective at best (7). The histamine H₄ receptor (H₄R) has been identified most recently (8–12) and shown to be involved in the recruitment and activation of cells involved in allergic inflammatory responses, including eosinophils, T cells, dendritic cells, basophils, mast cells, and sensory nerve cells (13–16). In allergic

rhinitis patients, the H₄R was upregulated in nasal tissue (17). As a result, the H₄R has been targeted in the treatment of allergic disease (18–22). For the most part, a primary focus in AR has been on mast cells. Mast cells share many features with basophils. Both cells express the high-affinity IgE receptor (FcεRI) and secrete effector molecules such as histamine, platelet-activating factor, cysteinyl leukotrienes, and Th2 cytokines following activation by Ag–IgE crosslinking of FcεRI (23). Both play key roles in the development of allergic inflammation. Basophils, unlike mast cells that reside in the tissue, circulate in the blood and migrate to sites of inflammation. Although little is known about the direct role of basophils in allergic disease, several studies have revealed some unique characteristics that may be important to the initiation phase of the allergic response (24, 25). Limited studies have implicated basophils in the development of AR. Basophils have been demonstrated in nasal washes of patients with allergic rhinitis (26–28).

We established a method to induce nasal responsiveness in mice without influencing the lower airways and demonstrated that increases in nasal cavity resistance (R_{NA}) were significantly correlated with decreases in respiratory frequency (RF), suggesting that RF is a valid, albeit indirect, indicator of nasal resistance or blockage in mice, which are obligate nasal breathers (29). In this experimental model of AR, we demonstrated that the combination of allergen, Ag-specific IgE, and FcεRI on mast cells resulted in the development of an early (EPR) or late phase (LPR) nasal response, with the latter also being dependent on IL-13 (29, 30). In this study, we investigated the role of basophils in the development of the EPR and LPR in this model of AR. The results identified a critical role for the H₄R on basophils in their recruitment to the nasal tissue.

Materials and Methods

Animals

Six-week-old female BALB/c mice were obtained from Harlan Laboratories (Indianapolis, IN) and H₄R-deficient (H₄R^{-/-}; BALB/c background) mice were initially provided by Dr. R. Thurmond (Johnson & Johnson Pharmaceutical Research and Development, San Diego, CA). FcεRI-deficient (FcεRI^{-/-}; BALB/cByJ background) mice were originally provided by Drs. D. Dombrowicz and J.P. Kinet (Harvard Medical School, Boston,

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Abbreviations used in this article: AR, allergic rhinitis; EPR, early phase nasal response; H₄R, histamine H₄ receptor; LPR, late phase nasal response; OVA/OVA, sensitized and challenged with OVA; OVA/saline, sensitized to OVA but challenged with saline; RF, respiratory frequency; R_{NA}, nasal cavity resistance; WT, wild-type.

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MA). IL-13-deficient (IL-13^{-/-}) and IL-4^{-/-}/IL-13^{-/-} BALB/c mice were initially provided by Dr. D. Umetsu (Harvard Medical School, Boston, MA). All animals were maintained and bred in the Animal Facility at National Jewish Health (Denver, CO). Mice were housed under specific pathogen-free conditions and maintained on an OVA-free diet. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of National Jewish Health.

OVA sensitization and nasal challenge

In this study, two models of AR were studied, one involving active sensitization and the other, passive sensitization. In the active sensitization approach, wild-type (WT) or H₄R^{-/-} mice were sensitized by i.p. injection of 20 µg OVA (Fisher Scientific, Pittsburgh, PA) emulsified in 2.25 mg aluminum hydroxide (Imject Alum; Thermo Scientific Pierce Protein Research Products, Rockford, IL) in a total volume of 100 µl on days 0 and 14. From days 28 to 33, up to six daily nasal challenges with OVA (10% [w/v] in saline [15 µl for each nostril]) or saline alone as control were administered without anesthesia.

In the passive sensitization model, FcεRI^{-/-} mice received OVA-specific IgE (2 µg/200 µl in HBSS, derived from OVA-specific IgE-secreting hybridomas) (31) or DNP-specific IgE (2 µg/200 µl in HBSS; Sigma-Aldrich) via the lateral tail vein on 2 consecutive days. Basophils from WT or H₄R^{-/-} mice were transferred 3 h after the last injection of IgE. Two hours after basophil transfer, a single nasal challenge was performed in the same manner as described in the active sensitization model. Some groups of mice received histamine (0.2, 2, or 20 nmol/mouse) intranasally together with OVA.

Nasal responsiveness

Nasal responsiveness was assessed as described monitoring RF and R_{NA} (29, 30, 32). In the active sensitization model, RF values were assessed beginning immediately after the fourth OVA challenge using restrained single-chamber whole-body plethysmography (Buxco, Troy, NY). After the fourth OVA challenge, RF values were determined at 4–7, 9–12, 14–17, 19–22, 24–27, and 29–32 min (EPR). Twenty-four hours after the sixth OVA challenge, RF and nasal cavity resistance (R_{NA}) were determined (LPR). Baseline RF values were determined before the first challenge.

In the passive sensitization model, baseline RF values were obtained 2 h after basophil transfer. Following a single OVA challenge, EPR was monitored just after a single OVA or saline challenge with/without 20 nmol/mouse histamine or vehicle (15 µl for each nostril) and LPR (RF and R_{NA}) was monitored 16 h after the EPR measurements. Measurements of R_{NA} were carried out after RF measurements in the LPR as described.

Nasal cavity lavage fluid

A blunt 19-gauge needle was inserted into the nasopharynx through the pharynx and the nasal cavity was lavaged with 1.0 ml ice-cold HBSS. Nasal cavity lavage fluids were then centrifuged at 480 × g at 4°C for 5 min, and the supernatants were collected and stored at –80°C until assayed.

Measurement of cytokine and histamine levels in nasal cavity lavage fluid

IL-4, IL-5, and IL-13 levels in the nasal cavity lavage fluid were collected after the determination of RF and nasal cavity resistance for LPR and stored at –80°C until assayed. The cytokine levels were assessed by ELISA (eBioscience, San Diego, CA) according to the manufacturer's directions. For the measurement of histamine levels in the active sensitization model, nasal cavity lavage fluid was collected 2 min after the fourth allergen challenge and stored at –80°C until assayed. Histamine levels were measured using an enzyme immunoassay kit (A05890; ALPCO Diagnostics, Salem, NH) as described by the manufacturer.

Depletion of circulating basophils following Ba103 Ab administration

To determine the effect of basophil depletion, a basophil-depleting Ab, Ba103 (50 µg/mouse), which recognizes CD200R3 (24, 25, 33, 34), or isotype control rat IgG (50 µg/mouse) was injected into mice via the lateral tail vein 1 d before initiation of the challenges. Peripheral blood samples were obtained 4 or 8 d following treatment. RBCs in samples were lysed with red blood cell lysing buffer (Sigma-Aldrich, St. Louis, MO). Following blockade of FcγR with anti-mouse CD16/CD32 Ab, cells were stained with anti-mouse FcεRIα conjugated with PE (MAR-1; eBioscience) and anti-mouse CD117 (c-Kit) conjugated with allophycocyanin (2B8; eBioscience). The labeled cells were analyzed by flow cytometry (Accuri C6; BD Biosciences).

H₄R antagonist treatment

The selective H₄R antagonist JNJ777120 was purchased from Sigma-Aldrich. JNJ777120 (1, 5, 10 mg/kg) or vehicle control (0.5% methylcellulose-water) were gavaged 2.5 h prior to each OVA challenge on 6 consecutive days from days 28 to 33.

Purification of basophils

To mobilize basophils in bone marrow, 10 µg mouse rIL-3 (BD Biosciences, San Jose, CA) and 5 µg anti-mouse IL-3 Ab (MP2-8F8; BD Biosciences) were mixed in 1.0 ml at room temperature for 1 min and the IL-3/anti-IL-3 Ab complex solution was injected into WT or H₄R^{-/-} mice via the lateral tail vein (35). Four days after injection of IL-3/anti-IL-3 Ab, bone marrow cells were collected from femurs and tibias. After FcγR blockade with anti-mouse CD16/CD32 Ab (2.4G2; BD Biosciences), cells were labeled with anti-mouse CD49b conjugated with PE (DX5; eBioscience), anti-mouse FcεRIα conjugated with FITC (MAR-1; eBioscience), and anti-mouse CD117 (c-Kit) conjugated with allophycocyanin (2B8; eBioscience). Labeled cells were sorted for CD117⁺, FcεRIα⁺, and CD49b⁺ double-positive populations using MoFlo XDP (Becton Coulter, Brea, CA). This provided a population of >95% basophils (Fig. 4Ad). Purity of isolated basophils (FcεRIα-FITC and CD49b-PE double-positive but CD117-allophycocyanin⁺ populations) was confirmed using flow cytometry.

H₄R expression on basophils

Expression of H₄R on purified bone marrow basophils was examined by flow cytometry using anti-human H₄R rabbit polyclonal Ab (MBL International, Woburn, MA). To identify the intracellular domain of the H₄R, basophils were prefixed with 2% formalin followed by permeabilization with 0.1% saponin. After blocking of Fcγ receptors with anti-mouse CD16/CD32, anti-human H₄R rabbit polyclonal Ab (MBL International) was used. These Abs recognize the first cytoplasmic domain of human H₄R, which is >90% homologous to the mouse domain. Purified rabbit IgG (Invitrogen, Carlsbad, CA) was used as an isotype control. Then, goat anti-rabbit IgG conjugated with Alexa Fluor 647 (Invitrogen) was added as the secondary Ab. The labeled cells were analyzed by flow cytometry (Accuri C6).

Histamine-induced basophil chemotaxis

Purified basophils from WT or H₄R^{-/-} mice were suspended to 1 × 10⁶ cells/ml in RPMI 1640 medium with 0.5% (w/v) BSA. Six hundred microliters of the same culture medium containing 0, 0.01, 0.1, 1, 10, or 100 µM histamine was placed in the 24-well plates and 100 µl suspended basophils was added onto the 5-µm pore size polycarbonate membrane Transwell insert (6.5-mm size; Costar, Cambridge, MA). The plates were incubated for 2 h at 37°C, 5% CO₂. Transwell inserts were removed gently and cells in the bottom chamber were resuspended in 100 µl RPMI 1640 medium with 0.5% BSA and counted.

In vitro cytokine production from basophils

Purified basophils from WT or H₄R^{-/-} mice were suspended in RPMI 1640 medium containing 10% FCS and transferred into 96-well plates (1 × 10⁵ cells in 100 µl/well). Basophils were incubated with histamine (1 µM) for 24 h. The dose of histamine was chosen based on maximum effects observed in chemotaxis assays. Basophils were also incubated with OVA-specific IgE (10 µg/ml) or DNP-specific IgE (10 µg/ml) followed by OVA (10 µg/ml) for 1 h. After incubation, supernatants were collected and stored at –80°C until IL-4 and IL-13 measurements by ELISA.

Adoptive transfer of purified basophils

In the active sensitization approach, basophils purified from H₄R^{-/-} or WT mice (0.6 × 10⁶ cells/300 µl in HBSS) or HBSS alone as control were injected i.v. 2 h prior to the fourth OVA challenge in actively sensitized H₄R^{-/-} or WT mice. The numbers of basophils used in adoptive transfer experiments were determined from the literature (36–39) and preliminary experiments. In the passive sensitization model, basophils purified from H₄R^{-/-}, IL-13^{-/-}, IL-4^{-/-}/IL-13^{-/-}, or WT mice (0.6 × 10⁶ cells/300 µl in HBSS) or HBSS alone were injected i.v. 2 h prior to a single OVA challenge in FcεRI^{-/-} recipient mice.

Adoptive transfer of OVA-specific IgE-pretreated basophils

Purified basophils from H₄R^{-/-} or WT mice (1 × 10⁶ cells/100 µl in HBSS) were incubated with OVA-specific IgE (500 µg/ml) or DNP-specific IgE (500 µg/ml) for 30 min at room temperature. After washing

with HBSS three times, basophils (0.6×10^6 cells in 300 μ l HBSS) or HBSS (vehicle control) were injected via the tail vein into naive Fc ϵ RI $^{-/-}$ mice. Two hours after basophil transfer, a single nasal challenge was performed in the same manner as described in the active sensitization model. Some groups of mice received histamine (20 nmol/mouse) intranasally together with OVA followed by the measurements of EPR and LPR as described in the passive sensitization model.

Detection of basophils in the nasal cavity lavage fluid

To identify basophils in the nasal cavity, isolated basophils that were purified from either WT or H $_4$ R $^{-/-}$ mice and labeled with CellVue Maroon (excitation maximum, 647 nm; emission maximum, 667 nm; Polysciences, Warrington, PA) were transferred into Fc ϵ RI $^{-/-}$ mice (0.6×10^6 cells/mouse) 3 h after passive sensitization with OVA-specific IgE or DNP-specific IgE and 2 h prior to a single OVA-vehicle, OVA-histamine, or histamine challenge. Two minutes after the challenge, the nasal cavity was lavaged with 1.0 ml HBSS containing 1% formalin and collected into 1.5-ml tubes. Timing for the in vivo analyses following basophil transfer was determined based on earlier studies (36–39). Cells were resuspended in 50 μ l 0.5% (w/v) BSA-PBS followed by cell counting and flow cytometry (Accuri C6).

Statistical analyses

Values for all measurements are expressed as means $\pm$ SEM. RF value changes were analyzed with repeated measures two-way ANOVA, followed by Bonferroni correction as a post hoc test. All other values were analyzed with one-way ANOVA, followed by Bonferroni correction. Statistical analyses were performed using GraphPad Prism 5.0d (GraphPad Software, La Jolla, CA). A p value <0.05 was considered statistically significant.

Results

H $_4$ R deficiency is associated with failure to develop an EPR and LPR

To identify the role of the H $_4$ R in the development of allergen-induced nasal responsiveness, we monitored RF and R $_{NA}$ in WT and H $_4$ R $^{-/-}$ mice. In the EPR, expressed as changes after the fourth OVA challenge, RF was significantly decreased in actively sensitized WT mice compared with saline-challenged mice (Fig. 1A). Alternatively, actively sensitized H $_4$ R $^{-/-}$ mice did not show a decrease in RF, similar to OVA-sensitized but saline-challenged (OVA/saline) WT or H $_4$ R $^{-/-}$ mice. In the LPR, actively sensitized WT mice demonstrated a significantly decreased RF whereas OVA-sensitized and -challenged (OVA/OVA) H $_4$ R $^{-/-}$ mice did not show a significant change in RF (Fig. 1B). In parallel, R $_{NA}$ in sensitized and OVA-challenged WT mice was significantly higher

than in saline-challenged WT mice, whereas R $_{NA}$ in sensitized and OVA-challenged H $_4$ R $^{-/-}$ mice was comparable to levels in sensitized and saline-challenged WT or H $_4$ R $^{-/-}$ mice (Fig. 1C). Analysis of cytokine levels in nasal cavity lavage fluid revealed that levels of IL-4, IL-5, and IL-13 were significantly increased in OVA/OVA WT mice, but not in H $_4$ R $^{-/-}$ mice (Fig. 1D).

Effect of H $_4$ R antagonist treatment on the development of AR in WT mice

To confirm the mechanism underlying the failure of H $_4$ R $^{-/-}$ mice to develop nasal responses to allergen sensitization and challenge, we determined whether treatment with a selective H $_4$ R antagonist could mimic these findings in WT mice. As shown in Fig. 2, H $_4$ R antagonist treatment of OVA-sensitized WT mice during the OVA challenge period prevented the development of both the EPR and LPR (Fig. 2A–C) in a dose-dependent manner. Cytokine analysis of nasal cavity lavage fluid showed that the increases in IL-4, IL-5, and IL-13 levels were also prevented by the inhibitor in a dose-dependent manner (Fig. 2D).

To establish whether levels of histamine, a ligand of the H $_4$ R, were increased in this model, nasal cavity lavage fluids were analyzed soon after the fourth OVA challenge. As shown in Fig. 3, histamine levels in nasal cavity lavage fluid were significantly increased 2 min following the fourth OVA challenge compared with the fourth saline challenge of OVA-sensitized WT or Fc ϵ RI $^{-/-}$ mice. In contrast, following the fourth OVA challenge of Fc ϵ RI $^{-/-}$ mice, which do not develop an EPR (29), nasal cavity histamine levels were extremely low. These data identify the importance of Fc ϵ RI-expressing resident mast cells as the major source of nasal cavity histamine (see above).

H $_4$ R expression and function on basophils

These results indicated a critical role for the H $_4$ R in the development of allergen-induced nasal responsiveness. In light of our previous demonstration that Fc ϵ RI was also essential in this setting (29), we focused on the role of basophils, circulating cells known to express high levels of Fc ϵ RI. We first demonstrated that H $_4$ R was indeed expressed on basophils from WT mice (Fig. 4B). To establish that the receptor was functional, chemotaxis assays were carried out using purified basophils from WT and H $_4$ R $^{-/-}$ mice. The numbers of basophils recovered from WT and H $_4$ R $^{-/-}$

FIGURE 1. EPR and LPR in OVA/OVA WT and H $_4$ R $^{-/-}$ mice. **(A)** EPR was monitored as changes in RF during a period of 32 min after the fourth OVA challenge. **(B)** LPR was detected as a change in RF 24 h after the sixth OVA challenge. **(C)** R $_{NA}$ in the LPR and **(D)** cytokine levels in nasal cavity lavage fluid. Shown are the means $\pm$ SEM; $n = 9$ –14 in each group. ** $p < 0.01$ compared with WT OVA/saline group, ## $p < 0.01$ compared with WT OVA/OVA group.

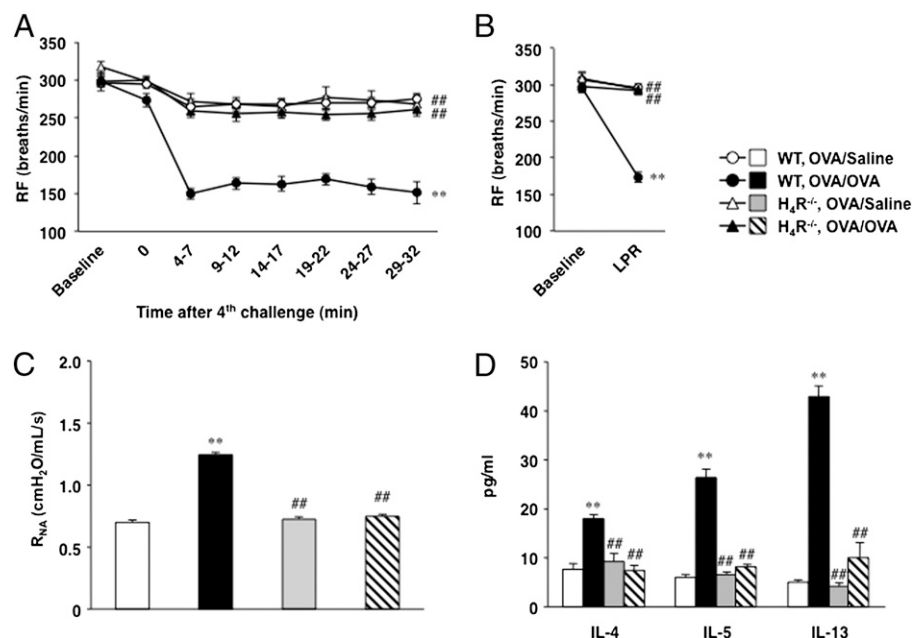
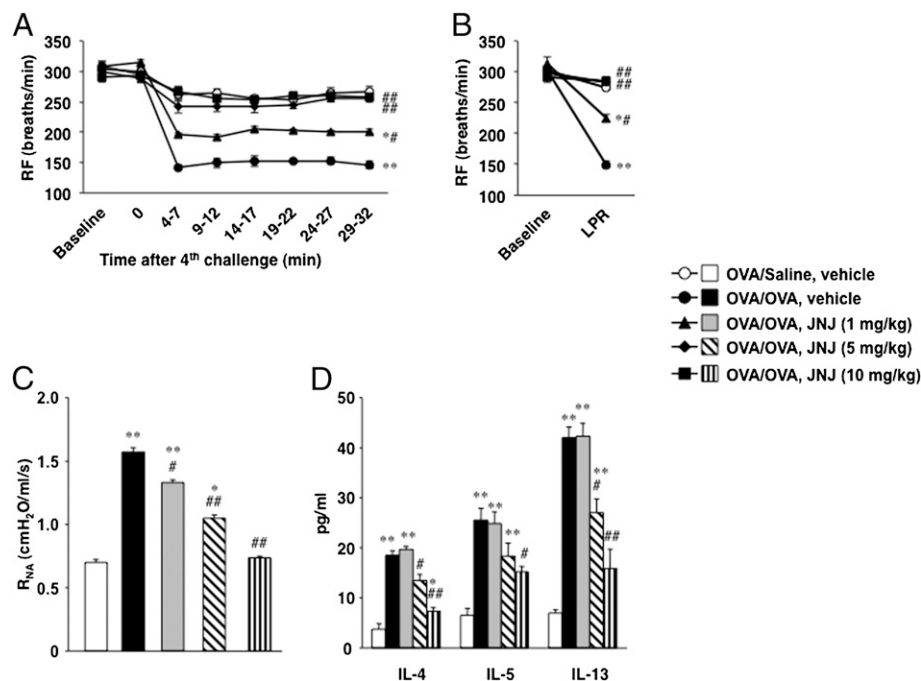


FIGURE 2. Effects of H_4R antagonist JNJ777120 treatment on OVA-induced nasal responses in the EPR and LPR of WT mice. The inhibitor (1, 5, or 10 mg/kg) was administered 2 h prior to each challenge. Changes in (A) EPR, (B) LPR, (C) R_{NA} , and (D) cytokine levels in nasal cavity lavage fluid. Shown are the means $\pm$ SEM; $n = 9-14$ in each group. * $p < 0.05$, ** $p < 0.01$ compared with OVA/saline vehicle group, # $p < 0.05$, ## $p < 0.01$ compared with OVA/OVA vehicle group.



mice were similar. Histamine induced WT basophil chemotaxis but not $H_4R^{-/-}$ basophils, confirming the importance of the H_4R in histamine-induced cell migration (Fig. 4C). Additionally, the capacity of basophils to produce cytokines was determined following stimulation with histamine or the combination of OVA and OVA-specific IgE. As shown in Fig. 4D, the combination of OVA and OVA-specific IgE triggered the production of IL-4 and IL-13 from purified basophils, whereas histamine alone failed to do so. The levels of IL-4 and IL-13 following OVA and OVA-specific IgE stimulation of $H_4R^{-/-}$ basophils were similar to levels from WT basophils (data not shown).

Basophil depletion reduces nasal responsiveness

To determine whether basophils are involved in the development of allergen-induced EPR and LPR, basophils from OVA-sensitized WT mice were depleted with Ba103 Ab prior to OVA challenge. Peripheral blood basophils were reduced by >80% up to 8 d following Ba103 Ab treatments (Fig. 5A). The number of mast cells in nasal tissue was not altered in Ba103-treated mice (data not shown), confirming the results of a previous study (24). Following basophil depletion, the development of both the EPR (Fig. 5B) and LPR (Fig. 5C, 5D) was inhibited, as were increases in Th2 cytokine levels in nasal cavity lavage fluid (Fig. 5E).

Adoptive transfer of basophils into actively sensitized $H_4R^{-/-}$ mice restores responsiveness

To establish the critical requirement for H_4R expression on basophils in the development of allergen-induced nasal responses, purified basophils from WT or $H_4R^{-/-}$ mice were transferred to sensitized and challenged $H_4R^{-/-}$ recipients prior to the fourth allergen challenge. Basophils from WT but not $H_4R^{-/-}$ mice restored the development of the EPR and LPR, decreases in R_{NA} , and increases in Th2 cytokine levels in nasal cavity lavage fluid (Fig. 6).

Adoptive transfer of basophils into passively sensitized $Fc\epsilon RI^{-/-}$ mice

Taken together, these data identified the role of the H_4R and basophils in the development of both the EPR and LPR. To more directly confirm the involvement of H_4R -expressing basophils in

these responses, $Fc\epsilon RI^{-/-}$ recipients were passively sensitized with IgE to bypass the need for active sensitization and mast cells. As $Fc\epsilon RI^{-/-}$ mice were not capable of releasing histamine into the nasal cavity (Fig. 3), histamine was administered intranasally together with allergen in certain groups. The mice were passively sensitized with OVA-specific IgE or DNP-specific IgE and were recipients of basophils from WT, $H_4R^{-/-}$, IL-13 $^{-/-}$, or IL-4 $^{-/-}$ /IL-13 $^{-/-}$ mice. As shown in Fig. 7A–C, only passively sensitized anti-OVA-IgE $Fc\epsilon RI^{-/-}$ recipients of WT basophils and challenged with OVA and intranasal histamine developed both an EPR and LPR. In the absence of histamine or passive sensitization with the irrelevant anti-DNP-IgE, no EPR or LPR developed. Recipients of $H_4R^{-/-}$ basophils failed to develop an EPR and LPR whereas recipients of IL-13 $^{-/-}$ (or IL-4 $^{-/-}$ /IL-13 $^{-/-}$, data not shown) basophils developed an EPR but not an LPR. In parallel, increases in nasal lavage fluid levels of IL-4 and IL-13 were only seen in the group that developed an EPR and LPR whereas recipients of IL-13 $^{-/-}$ basophils that developed an EPR but not LPR demonstrated increases in IL-4 levels (Fig. 7D). The response to histamine was dose-dependent: when mice were challenged with lower doses of histamine together with OVA, 2 nmol/mouse histamine induced modest nasal responses, but 0.2 nmol/mouse or lower doses did not elicit any nasal responses (data not shown).

To show the requirement for $Fc\epsilon RI$ on basophils in the development of nasal responses, purified basophils from WT or $H_4R^{-/-}$

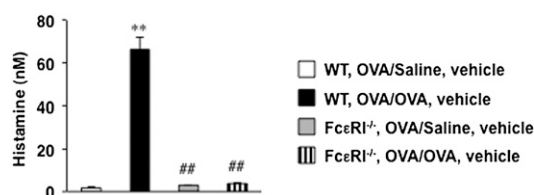


FIGURE 3. Histamine levels in nasal cavity lavage fluid 2 min following the fourth saline or fourth OVA challenge in OVA-sensitized WT or $Fc\epsilon RI^{-/-}$ mice. Shown are the means $\pm$ SEM; $n = 9-14$ in each group. ** $p < 0.01$ compared with OVA/saline vehicle group, ## $p < 0.01$ compared with OVA/OVA vehicle group.

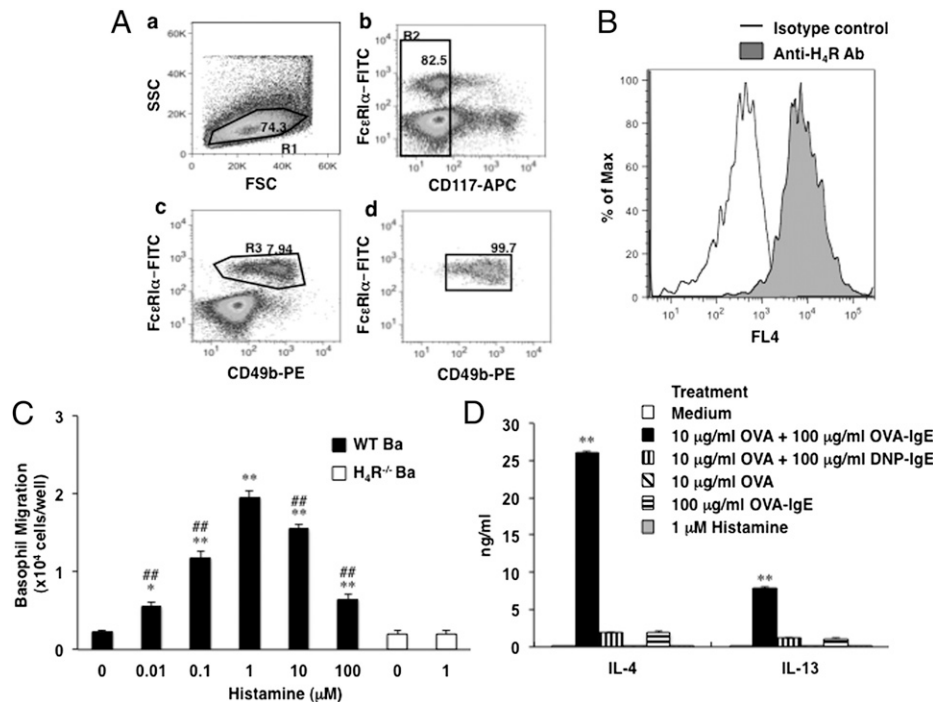


FIGURE 4. (A) Gating strategy for purification of basophils from bone marrow cells. Bone marrow cells from mice treated with IL-3 and anti-IL-3 Ab were stained with anti-mouse CD49b-PE, anti-mouse FcεRIα-FITC, and anti-mouse CD117 (c-Kit)-allophycocyanin after FcγR blockade with anti-mouse CD16/CD32 Ab. Basophils were purified by sorting (MoFlo XDP; Becton Coulter). (a) Bone marrow cells were gated on forward scatter and side scatter (region R1; 74.3% of total cells), and (b) region R1 was gated on CD117-allophycocyanin⁺ and FcεRIα-FITC⁺ cells (region 2), and then cells were collected in (c) FcεRIα-FITC and CD49b-PE double-positive populations (region R3). (d) After sorting of FcεRIα and CD49b double-positive but CD117⁺ populations, postsorting validation was performed (FcεRIα-FITC and CD49b-PE, 99.7% purity). (B) H₄R expression on WT basophils. CD117⁺ and FcεRIα and CD49b double-positive populations were stained with anti-H₄R rabbit Abs followed by goat anti-rabbit IgG Ab. (C) In vitro basophil chemotaxis assay to histamine. The chemotactic effects of 0, 0.01, 0.1, 1, 10, and 100 μM histamine on basophils from WT or H₄R^{-/-} mice were examined using 24-well plates and Transwell inserts and counting cells that migrated through to the bottom. Shown are the means ± SEM; *n* = 5 in each group. **p* < 0.05, ***p* < 0.01 compared with the 0 μM histamine and H₄R^{-/-} basophils, ###*p* < 0.01 compared with 1 μM histamine. (D) In vitro basophil cytokine production. Purified basophils (1 × 10⁵ cells/well) were incubated with or without 10 μg/ml OVA and/or 100 μg/ml OVA-specific or DNP-specific IgE for 24 h. Some groups of cells were incubated with 1 μM histamine for 24 h. Shown are the means ± SEM; *n* = 6 in each group. ***p* < 0.01 compared with medium group.

mice were cultured with OVA-specific IgE or DNP-specific IgE ex vivo prior to transfer into FcεRI^{-/-} mice. Among the different groups of recipients, only the group that received OVA-specific, IgE-pretreated WT basophils followed by OVA-histamine challenge developed an EPR and LPR (Fig. 8).

To establish that transferred basophils did indeed migrate into the target tissue, nasal cavity lavage fluid was collected 2 min after challenge following transfer of WT or H₄R^{-/-} basophils into the passively sensitized FcεRI^{-/-} mice. Basophil numbers in nasal lavage fluid only increased following transfer of WT basophils after the OVA-histamine challenge (Fig. 9B). Approximately 90% of the total cells were identified as basophils (Fig. 9A). WT basophils were also detected in the nasal cavity lavage fluid of OVA-specific or DNP-specific IgE passively sensitized mice that were exposed to histamine even without OVA (Fig. 9B), but they failed to develop any nasal responses. No basophils were detected in the absence of histamine or following transfer of H₄R^{-/-} basophils.

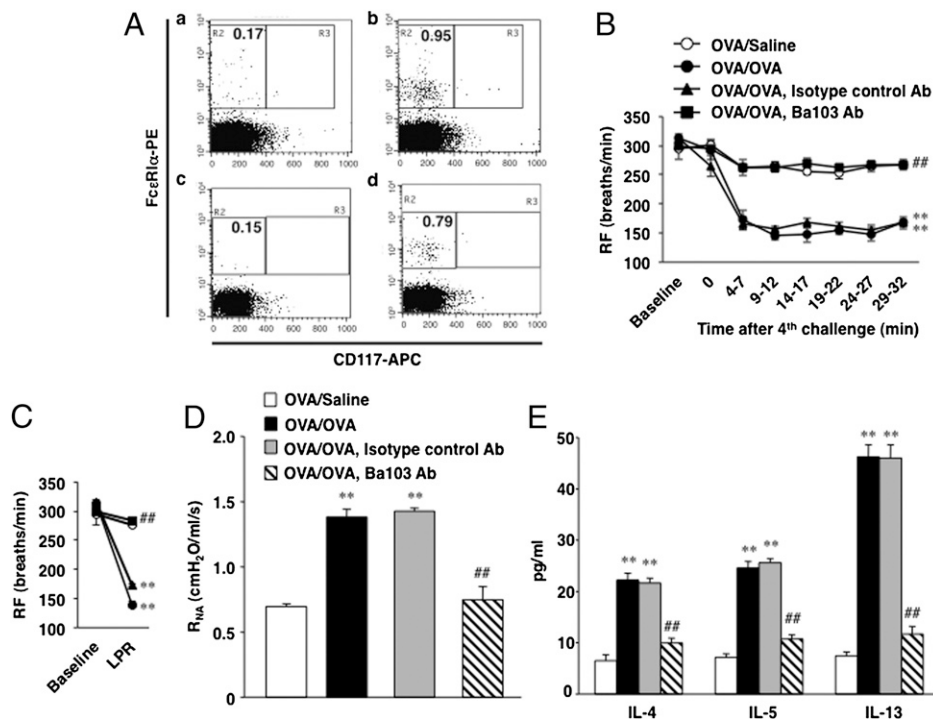
To identify which cell type in the nasal mucosa, a mast cell or basophil, was the initial source of histamine following activation through FcεRI, we assessed histamine levels in nasal cavity lavage fluid in OVA-specific IgE passively sensitized WT or FcεRI^{-/-} mice. Histamine levels in passively sensitized WT mice were significantly increased on OVA challenge, whereas passively sensitized FcεRI^{-/-} mice, even those that received WT basophils, failed to increase the levels of histamine in the nasal cavity following OVA challenge (Fig. 7E).

These data demonstrated that in the absence of mast cell activation (FcεRI^{-/-} hosts), basophils expressing both FcεRI and the H₄R were essential to the development of an EPR and LPR. Furthermore, in the absence of mast cell activation (FcεRI^{-/-} hosts), a source of histamine was also essential for the accumulation of basophils in the nasal cavity. Finally, to develop the LPR but not the EPR, the basophil release of IL-13 was also essential (Fig. 10).

Discussion

The symptoms of AR can be related to both the EPR and LPR with itching, sneezing, and rhinorrhea predominating in the EPR and congestion in the LPR. To better understand the underlying mechanisms, an experimental model of AR was developed in an attempt to mimic the human disease. A key to triggering nasal responsiveness without lower airway disease was intranasal administration of allergen without anesthesia and aspiration of allergen (29). In this approach, a distinct EPR and LPR could be elicited with changes in RF and R_{NA}, indicators of nasal obstruction, and the identification of individual pathways mediating these responses (29). RF was shown to correlate closely with R_{NA}, and a role for mast cells was demonstrated (30). In addition to the role of mast cells, in this study we identified an essential role for basophils and their expression of both the H₄R and FcεRI. In WT mice treated with an H₄R antagonist administered during allergen challenge, both the EPR and LPR were significantly reduced. To

FIGURE 5. (A) Effects of basophil depletion following Ba103 Ab administration. Ba103 Ab (50 μ g/mouse) was administered to WT mice and depletion was confirmed counting CD117⁺, Fc ϵ RI α ⁺ cells, (a) Ba103 Ab, (b) isotype control Ab on day 4 and on day 8, (c) Ba103 Ab, (d) isotype control Ab. (a and b) Peripheral blood basophils were depleted following Ba103 Ab treatment by 82–85% on day 4. (c and d) Peripheral blood basophils were depleted by Ba103 Ab by 81–86% on day 8. The effects of basophil depletion on OVA-induced nasal responses in WT mice are shown. Ba103 Ab or isotype control Ab was administered 1 d prior to initiation of 4 or 6 consecutive days of allergen challenge. (B) EPR, (C) LPR, (D) R_{NA} , and (E) cytokine levels in nasal cavity lavage fluid. Shown are the means $\pm$ SEM; $n = 6$ in each group. $^{##}p < 0.01$ compared with the OVA/OVA isotype control Ab-treated group, $^{**}p < 0.01$ compared with OVA/saline group.



confirm these findings and exclude concerns about the specificity and potential off-target effects of the H₄R antagonist, H₄R^{-/-} mice were also used. Allergen-sensitized and -challenged H₄R^{-/-} mice failed to develop both the EPR and LPR. These findings in receptor-deficient mice were confirmed in a different mouse model of AR where treatment with either an H₄R antagonist, JNJ7777120, or H₁R antagonist, ketotifen fumarate, prevented nasal symptoms such as sneezing and nasal rubbing, perhaps through blockade of the H₁R and H₄R on nasal sensory nerves (40). Because of the high doses of the H₁R antagonist used, the effects may reflect off-target activities (41). In addition to the effects on the EPR and LPR, antagonist treatment resulted in a significant reduction in Th2-type cytokine levels, IL-4, IL-5, or IL-13. Both IL-4 and IL-

13 are central to OVA-specific Ab production and we previously demonstrated the importance of IL-13 in development of the LPR (32). H₄R antagonism resulted in inhibition of T cell migration into the lung followed by decreased levels of Th2 cytokines in an experimental asthma model (42) and impairment of dendritic cell homing to lymph nodes in a model of atopic dermatitis (43). We previously demonstrated that Fc ϵ RI was also essential to the development of both the EPR and LPR (29). Fc ϵ RI is expressed primarily on mast cells and basophils, and activation through the receptor induces degranulation, histamine release, and cytokine secretion in both cell types (44). Given the requirement for expression of both receptors, the findings that the H₄R is effective in cell migration (5) and that mast cells are primarily tissue-resident

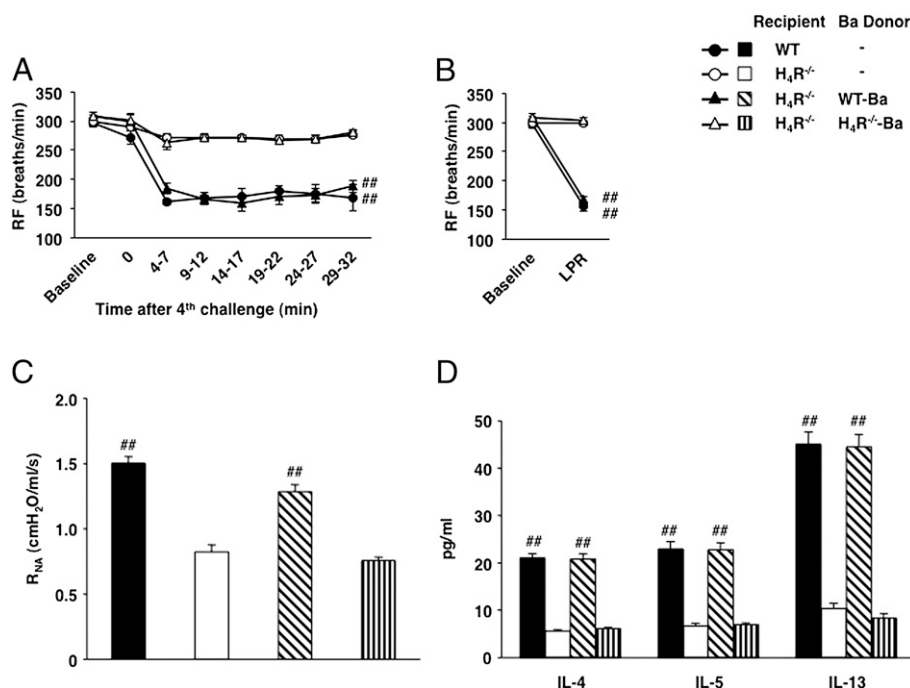
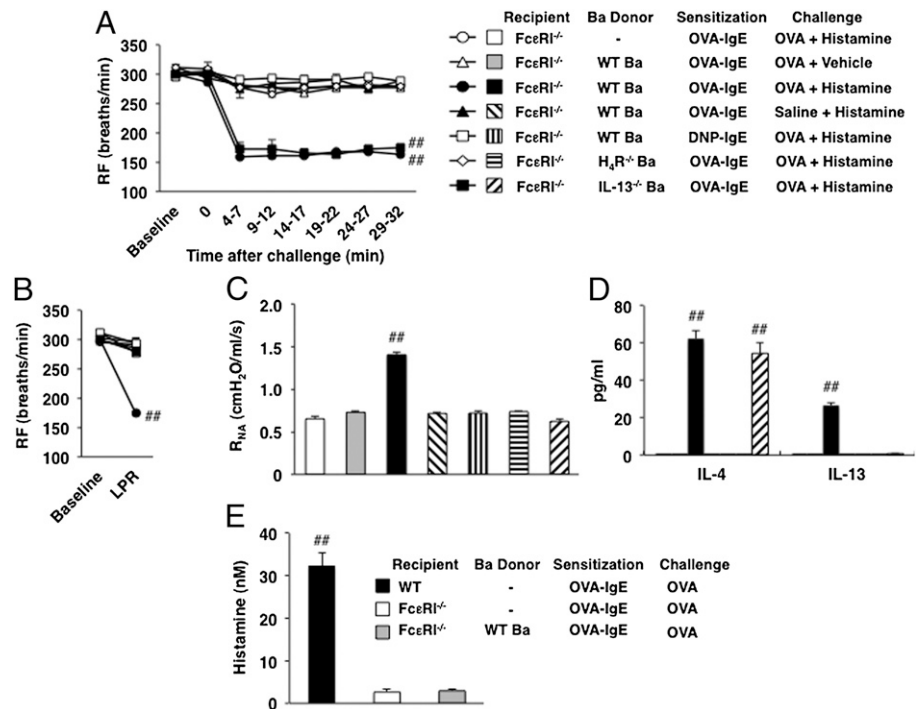


FIGURE 6. Transfer of WT but not H₄R^{-/-} basophils restores nasal responsiveness in sensitized and challenged H₄R^{-/-} recipients. Basophils isolated from WT or H₄R^{-/-} donors were transferred 2 h prior to the fourth OVA challenge. Changes in (A) EPR, (B) LPR, (C) R_{NA} , and (D) cytokine levels in nasal cavity lavage fluid. Shown are the means $\pm$ SEM; $n = 6-9$ in each group. $^{##}p < 0.01$ compared with H₄R^{-/-} recipients of H₄R^{-/-} basophils.

FIGURE 7. The combination of WT basophils, OVA-IgE passive sensitization, and OVA challenge with histamine restores nasal responsiveness in $Fc\epsilon R1^{-/-}$ recipients accompanied by increased basophil numbers in the nasal cavity. $Fc\epsilon R1^{-/-}$ recipient mice were passively sensitized with OVA-specific IgE or DNP-specific IgE followed by a single OVA or saline challenge with or without histamine. Basophils from WT, $H_4R^{-/-}$, or $IL-13^{-/-}$ donors were transferred into recipients 2 h prior to the single challenge. (A) EPR, (B) LPR (16 h after the single challenge), (C) R_{NA} , and (D) cytokine levels in nasal cavity lavage fluid. Nasal lavage fluid was collected 16 h after the single challenge. (E) Histamine levels in nasal cavity lavage fluid from OVA-specific IgE passively sensitized WT and OVA-specific IgE passively sensitized $Fc\epsilon R1^{-/-}$ mice with or without WT basophil transfer. Nasal cavity lavage fluid was collected 2 min after the single OVA challenge. Shown are the means $\pm$ SEM; $n = 6-9$ in each group. $^{##}p < 0.01$ compared with all other groups.



cells, we hypothesized that basophils, which circulate, fulfill these requirements in the development of AR. To directly examine their role in AR, basophils were depleted using the depleting Ab, Ba103. Previously, and in this study, Ba103 was shown to deplete basophils for up to 8 d in spleen, bone marrow, and peripheral blood, without altering mast cell numbers in peritoneal lavage fluid (24). We also confirmed that Ba103 depleted basophils but not mast cells. Following Ba103 treatment and depletion of $>80\%$ basophils in the peripheral blood, allergen-sensitized and -challenged mice failed to develop an EPR or LPR and prevented the increases in Th2 cytokine levels in nasal cavity lavage fluid. Following activation by OVA and OVA-specific IgE, basophils produced both IL-4 and IL-13, whereas histamine itself did not induce cytokine production.

We confirmed H_4R expression on basophils by flow cytometry analysis and increased histamine levels in nasal cavity lavage fluid. We then determined whether expression of this receptor was required for allergen-induced nasal responses. Basophils were isolated from WT or $H_4R^{-/-}$ mice and adoptively transferred into allergen sensitized $H_4R^{-/-}$ mice prior to the fourth allergen

challenge. $H_4R^{-/-}$ recipients that received WT but not $H_4R^{-/-}$ basophils developed decreases in RF in the EPR and LPR, increases in R_{NA} in the LPR, and increases in Th2 cytokine levels in the nasal cavity lavage fluid. These complementary approaches established a critical role for basophils in the development of AR, and the transfer experiments identified the importance of H_4R expression on these cells.

However, as mast cells can be activated through ligation of $Fc\epsilon R1$ as well as through H_4R in chemotaxis and calcium mobilization assays (15), their role in the development of AR could not be discounted. We previously suggested the involvement of mast cells triggered through $Fc\epsilon R1$ in AR (29, 30). To determine the role of basophils and exclude the activation of mast cells through $Fc\epsilon R1$, $Fc\epsilon R1^{-/-}$ mice were used as recipients of basophils isolated from WT or $H_4R^{-/-}$ mice. Additionally, to avoid systemic sensitization with OVA/aluminum hydroxide, recipients were passively sensitized with OVA-specific IgE prior to OVA challenge. Under these conditions only the transferred basophils but not host mast cells could be activated through the ligation of $Fc\epsilon R1$. With these restrictions, only $Fc\epsilon R1^{-/-}$ recipients that were

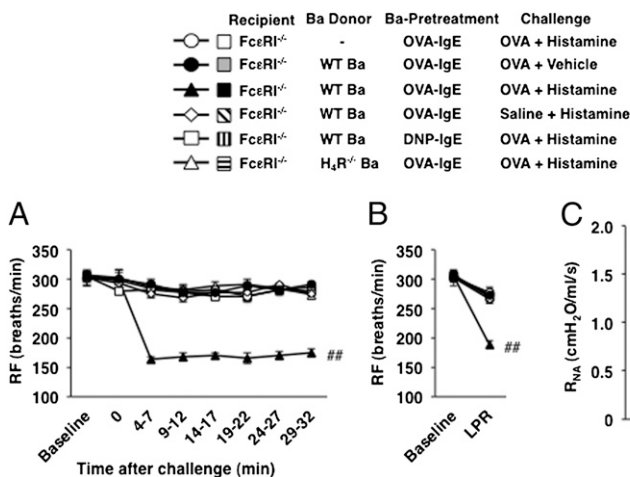


FIGURE 8. Transfer of OVA-specific IgE but not DNP-specific IgE-pretreated WT basophils restores nasal responsiveness in nonsensitized but OVA-histamine-challenged $Fc\epsilon R1^{-/-}$ recipient mice in both EPR and LPR. Isolated basophils from WT or $H_4R^{-/-}$ mice were transferred into nonsensitized $Fc\epsilon R1^{-/-}$ mice 2 h prior to a single challenge of OVA or saline with or without histamine (20 nmol/mouse). (A) EPR, (B) LPR (16 h after the EPR), and (C) R_{NA} . $^{##}p < 0.01$ compared with all other groups.

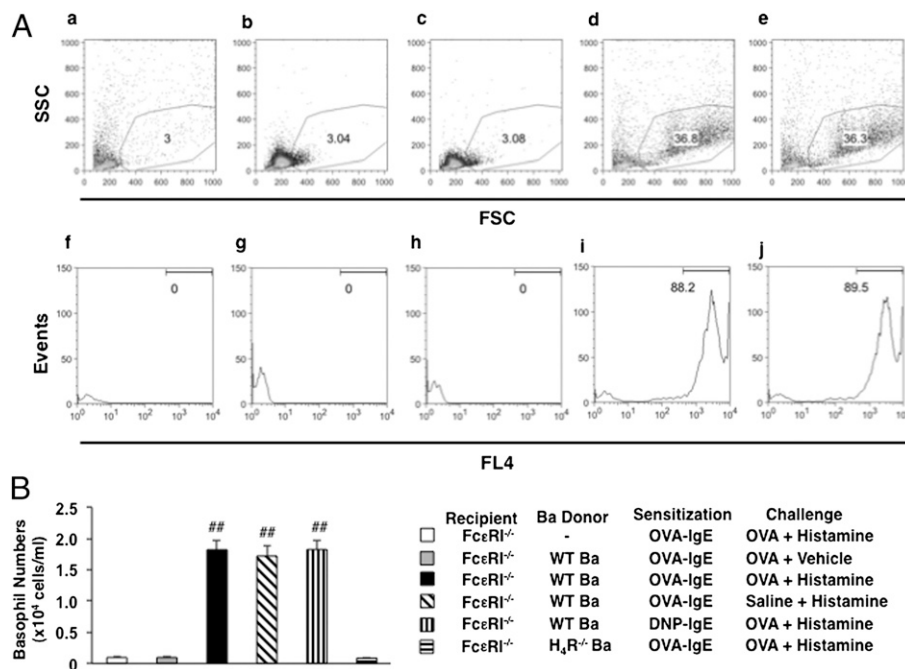


FIGURE 9. Detection of transferred basophils in nasal cavity lavage fluid. **(A)** Identification of transferred basophils in nasal cavity lavage fluid. Sorted basophils (CD117-allophycocyanin⁺ and FcεRIα-FITC and CD49b-PE double-positive populations) were labeled with CellVue Maroon and then transferred into passively sensitized FcεRI^{-/-} recipient mice. Nasal cavity lavage fluids were collected in 1 ml HBSS and suspended in 1% formalin 2 min after a single challenge. Labeled, live cells in nasal cavity lavage fluids were first gated on forward scatter/side scatter [dot plot, (a)–(e)]. Within the gated cells, numbers of labeled cells (transferred basophils) were identified (FL4). (a and f) Nasal cavity lavage fluid from FcεRI^{-/-} recipients treated with vehicle only following passive sensitization with OVA-specific IgE and OVA-histamine challenge. (b and g) FcεRI^{-/-} recipients of WT basophils following passive sensitization with OVA-specific IgE and OVA challenge. (c and h) FcεRI^{-/-} recipients of H₄R^{-/-} basophils following sensitization with OVA-specific IgE and OVA-histamine challenge. (d and i) FcεRI^{-/-} recipients of WT basophils following sensitization with OVA-specific IgE and OVA-histamine challenge. (e and j) FcεRI^{-/-} recipients of WT basophils following sensitization with OVA-specific IgE and challenge with histamine alone. **(B)** Basophil numbers in nasal lavage fluid, which was collected 2 min after a single OVA or saline challenge of passively sensitized FcεRI^{-/-} mice. Basophil numbers were increased in recipients of WT but not H₄R^{-/-} basophils and following histamine (20 nmol/mouse) challenge. Shown are the means $\pm$ SEM; $n = 6$ in each group. $^{##}p < 0.01$ compared with mice that did not receive basophils or that received H₄R^{-/-} basophils, or recipients that received OVA-vehicle challenge.

passively sensitized with OVA-specific IgE and received WT but not H₄R^{-/-} basophils developed the full spectrum of allergen-induced nasal responses following challenge with OVA and exogenous histamine. Furthermore, ex vivo pretreatment of basophils with OVA-specific IgE also restored responsiveness in OVA-histamine-challenged FcεRI^{-/-} recipients. These results indicated that FcεRI was essential to the activation of basophils in AR. Together with our earlier data on the role of mast cells in AR (29, 30), the requirement for exogenous histamine implied that FcεRI was also important in allergen-specific IgE mast cell activation for the release of histamine as the initiator of the basophil-driven response in AR. Although mast cells can be activated through IgE-FcγRIII ligation (45), exogenous histamine was essential to the development of the nasal responses. Thus, nasal mast cell-derived histamine initiated the cascade by directing the migration of basophils through the H₄R. In chemotaxis assays, only WT but not H₄R^{-/-} basophils responded to a histamine gradient. The response to histamine appeared to be restricted to chemotaxis, as addition of histamine to WT basophils did not result in degranulation or cytokine production. Confirming these findings, only WT but not H₄R^{-/-} basophils were demonstrated in the nasal cavity of OVA-IgE passively sensitized mice following OVA challenge together with histamine. Specificity of the response was shown because only OVA-specific IgE but not DNP-specific IgE triggered the EPR and LPR following WT basophil transfer and OVA-histamine administration in FcεRI^{-/-} mice. The administration of histamine in the challenge phase was essential to the accumulation of basophils in the nasal lavage fluid, but this was

not sufficient to elicit a nasal response. The development of nasal responses was also dependent on activation of the basophils through FcεRI in that the absence of either OVA challenge or

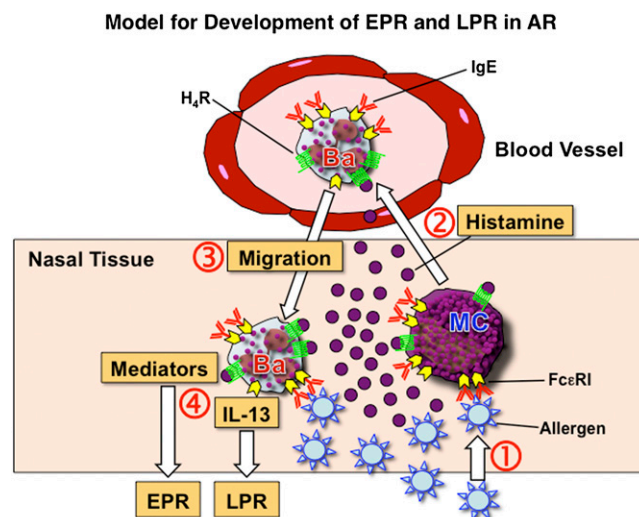


FIGURE 10. Model for development of EPR and LPR in AR. In step 1, activation of mast cells (MC) in the nasal tissue following crosslinking of FcεRI results in the release of histamine. In step 2, histamine binds to H₄R on basophils (Ba), leading to migration of basophils to the nasal tissue (step 3). In step 4, activation of basophils in the nasal tissue following cross-linking of FcεRI results in EPR mediator release and LPR cytokine production.

OVA-specific IgE passive sensitization, as EPR and LPR could not be detected. These data suggested that activated basophils were the source of the cytokines involved in AR, although we cannot rule out the contributions of other cell types. Basophils in the nose have been implicated in the pathogenesis of AR (46). Nasal allergen challenge in AR patients resulted in increased levels of FcεRI expression and IL-13 secretion by basophils (47). The number of basophils in the nasal mucosa of AR patients was shown to be increased significantly after segmental bronchial provocation, whereas the numbers of mast cells were decreased (27). In the experimental model of AR, we previously demonstrated that IL-13 played an essential role in the development of an LPR (32). As shown in the present study, both IL-13^{-/-} and IL-4^{-/-}/IL-13^{-/-} basophils were capable of inducing an EPR but not an LPR in OVA-IgE, passively sensitized FcεRI^{-/-} recipients following challenge with OVA and histamine.

Taken together, these findings identify the important role for both the H₄R and FcεRI on basophils in AR and a sequential or biphasic cascade of events beginning with mast cell activation (through FcεRI) in the nasal tissue, the release of histamine followed by the recruitment of basophils to the nasal tissue (through the H₄R), and subsequent activation (through FcεRI) of basophils in the nasal mucosa in an allergen-specific manner (Fig. 10). This mast cell/histamine/H₄R/basophil/FcεRI axis provides new opportunities for therapeutic intervention, overcoming the limitations of current treatment with H₁R antagonists (inverse agonists) and intranasal corticosteroids.

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Disclosures

The authors have no financial conflicts of interest.

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