β2-Adrenoceptor signaling in airway epithelial cells promotes eosinophilic inflammation, mucous metaplasia, and airway contractility

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The mostly widely used bronchodilators in asthma therapy are β2-adrenoceptor (β2AR) agonists, but their chronic use causes paradoxical adverse effects. We have previously determined that β2AR activation is required for expression of the asthma phenotype in mice, but the cell types involved are unknown. We now demonstrate that β2AR signaling in the airway epithelium is sufficient to mediate key features of the asthmatic responses to IL-13 in murine models. Our data show that inhibition of β2AR signaling with an aerosolized antagonist attenuates airway hyperresponsiveness (AHR), eosinophilic inflammation, and mucous production responses to IL-13, whereas treatment with an aerosolized agonist worsens these phenotypes, suggesting that β2AR signaling on resident lung cells modulates the asthma phenotype. Labeling with a fluorescent β2AR ligand shows the receptors are highly expressed in airway epithelium. In β2AR−/− mice, transgenic expression of β2ARs only in airway epithelium is sufficient to rescue IL-13–induced AHR, inflammation, and mucus production, and transgenic overexpression in WT mice exacerbates these phenotypes. Knockout of β2-arrestin-2 (βarr-2−/−) attenuates the asthma phenotype as in β2AR−/− mice. In contrast to eosinophilic inflammation, neutrophilic inflammation was not promoted by β2AR signaling. Together, these results suggest β2ARs on airway epithelial cells promote the asthma phenotype and that the proinflammatory pathway downstream of the β2AR involves βarr-2. These results identify β2AR signaling in the airway epithelium as a master regulator of key features of asthma.

Significance

Activation of β2-adrenoceptors (β2-ARs) on airway smooth muscle cells produces airway relaxation, and β2AR agonists are the most widely used bronchodilators for treating asthma. Paradoxically, murine models show β2AR activation is also required for expression of cardinal features of the asthma phenotype, including airway hyperresponsiveness (AHR), inflammation, and mucous metaplasia. However β2ARs are expressed on all the cell types implicated in the pathogenesis and maintenance of asthma, and which cell type(s) control these asthmatic effects is unknown. Here we show activation of β2AR signaling solely on airway epithelium is sufficient to restore/promote the cardinal features of asthma, including inflammation, mucous metaplasia, and AHR. These studies support the role of the airway epithelium as a master regulator of key features of asthma.


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**Results**

**Topical Administration of β2AR Ligands Affects Allergic Lung Inflammation.**

As an initial exploration of whether the β2AR signals resident or recruited cells to promote allergic inflammation, we exposed mouse lung parenchymal cells directly to IL-13, a key cytokine in allergic asthma (17–20). Intratracheal administration of IL-13 to WT mice induced a marked increase 24 h later in airway epithelial mucus and lung lavage eosinophils, both of which were attenuated in β2AR−/− mice (Fig. 2A and B). This suggested that IL-13 acting directly on lung parenchymal cells requires β2AR signaling within resident cells because of the short interval between IL-13 instillation and phenotypic assessment. Supporting a lung parenchymal site of β2AR action, Rag1−/− mice, which lack all anti-specific lymphocytes that contribute to the asthma phenotype (21, 22), exhibited mucous metaplasia and lung eosinophilia comparable to WT mice in response to IL-13 instillation (Fig. S1), although this does not rule out a role for innate lymphoid cells or other leukocytes (23).

To further test local interactions between IL-13 and β2AR signaling within the lungs, we exposed mice to aerosolized β2AR ligands at concentrations not likely to have systemic effects. In previous time-course studies in OVA models, the β2AR antagonist nadolol (250 ppm) when administered in the diet for 7 d partially suppressed and when administered for 28 d strongly suppressed mucous metaplasia (7). To more precisely define the time required for maximal effects of nadolol, a time-course study was performed by assessing mice every 3 d for 28 d. The results showed a plateau of effect on lung eosinophilia by day 7 and on mucous metaplasia after 22 d (Fig. S2A). Based on these results, mice in the current study were exposed to aerosolized nadolol for 21 d. In previous dose–response studies, nadolol in the diet at 5 ppm did not reduce the eosinophilia and only mildly reduced mucous metaplasia, while at 25 ppm there was a significant reduction of the eosinophilia and an almost complete suppression of the mucous metaplasia (10). We measured the plasma concentration of nadolol given in the diet at 5 ppm as 0.65 ng/mL (± 0.1 SEM, n = 4) and at 25 ppm as 3.1 ng/mL (± 0.4 SEM, n = 3). When 1.5 mg of nadolol was aerosolized twice daily for 21 d, the plasma level was only 0.25 ng/mL (± 0.1 SEM, n = 5). Despite the subtherapeutic plasma nadolol level (less than half the blood levels produced by the 5-ppm oral dose), mucous metaplasia and lung eosinophilia in response to IL-13 were reduced nearly as much by aerosolized nadolol as by genetic deletion of β2AR (Fig. 2C). Thus, nadolol appeared to have powerful effects acting locally within the lungs. Further supporting a lung parenchymal rather than circulating leukocyte site of action, Rag1−/− mice exposed to aerosolized nadolol exhibited attenuated eosinophilia and metaplasia in response to IL-13 comparable to that in WT mice (Fig. S1).

We also tested the local activity of the high-efficacy β2AR agonist formoterol given by aerosol. In previous time-course studies, we found that restoration of β2AR signaling in phenyl ethanolamine N-methyltransferase (PNMT) null mice, which lack epinephrine, by twice daily i.p. injection of formoterol required 12 d to reach a maximal effect on eosinophilia and 19 d to reach a maximal effect on mucous metaplasia (9). We obtained similar results in time-course studies restoring β2AR signaling by withdrawing nadolol (250 ppm) from the diet of WT mice in an OVA model (Fig. S2B). Based on these results, mice in the current study were exposed to aerosolized formoterol for 21 d. We used the clinical dose of 20 μg nebulized twice daily to maximize pulmonary effects and minimize systemic effects (24); drug deposition in the lungs scales with animal size, allowing the assumption of comparable delivery between humans and mice (25, 26). This regimen augmented lung eosinophilia and mucous metaplasia in response to IL-13 (Fig. 2D), further supporting a site of action for β2AR signaling within the lungs and demonstrating that an exogenous β2AR agonist can cause a gain-in-
To explore PNAS PLUS mice carrying the E9165 βTg-Epith- mice did not impede IL-13 +6) as intense as the labeling mice (Fig. 4) mice (white bars) mice administered or not administered 3 µg IL-13 intratracheally. All analyses were performed using two-way β0.0001 compared with WT mice challenged with IL-13). Published online October 9, 2017

We next explored the sig- mics resulted in increased epi- 2.7%, SEM, Tg-Epith- βmice was sufficient for recovery of IL-13 expression in WT mice carrying the Scgb1a1-HA-β2AR transgenic allele (β2AR/−/ + Tg-Epith-β2) and was augmented in β2AR WT mice carrying the Scgb1a1-β2AR allele (WT + Tg-Epith-β2) compared with WT mice (Fig. 4A). Transgenic expression of β2AR in airway epithelial secretory cells of β2AR−/− mice was sufficient for recovery of IL-13-induced mucous metaplasia and lung eosinophilia (Fig. 4B), while overexpression in WT + Tg-Epith-β2 mice resulted in increased epithelial mucin content, (Fig. 4B), epithelial height, and lung eosinophilia compared with WT mice (Fig. 4B). These results indicate that β2AR signaling in airway secretory cells alone is sufficient for the promotion of two cardinal features of the asthma phenotype and that increased β2AR signaling can cause a gain-in-function similar to that observed with the exogenous agonist formetol (Fig. 2D).

We then tested whether β2AR signaling in airway secretory cells alone is sufficient to promote AHR, a third cardinal feature of asthma. Intratracheal IL-13 caused AHR in methacholine (MCh)-treated WT mice (Fig. 4C), which was markedly reduced in β2AR−/− mice, as we have previously observed (8). Transgenic expression of β2AR in airway epithelial secretory cells was sufficient for recovery of IL-13-induced AHR in β2AR−/− + Tg- Epith-β2 mice, although overexpression in WT + Tg-Epith-β2 mice did not cause significantly increased AHR (Fig. 4C). Aerosolized isoproterenol, a βAR agonist, functionally antagonized IL-13−/− induced AHR in WT mice but did not further reduce the attenuated AHR in β2AR−/− mice (Fig. 4D). Isoproterenol treatment of β2AR−/− + Tg-Epith-β2 mice did not impede IL-13−/− induced AHR (Fig. 4D), suggesting that β2ARs on airway smooth muscle cells are necessary for mediating bronchodilation.

βarr-2 Gene Deletion and Pharmacologic Inhibition of MAP Kinases Also Attenuate Allergic Inflammation. We next explored the signaling pathway downstream of the β2AR that promotes allergic function above the endogenous agonist in promoting the asthma phenotype.

Airway Epithelial Cells Express β2ARs. To explore β2AR distribution within the lungs, we labeled mouse peripheral lung sections with boron dipyrromethene (BODIPY-TMR-CGP-12177), a fluorescent β2AR ligand (27). This revealed fluorescence in airway epithelial cells and blood vessels (arrowheads and arrows, respectively, in Fig. 3A). Pretreatment of tissue sections with bromoacetyl alpenolol menthane (BAAM), an irreversible β2AR antagonist, resulted in diminished fluorescence in airway epithelial cells but not blood vessels (Fig. 3B), indicating that fluorescence associated with airway epithelium, but not blood vessels, was due to specific β2AR binding. Blood vessels were visible even in the absence of BODIPY-TMR-CGP-12177 (Fig. 3C), indicating autofluorescence that is probably due to elastin fibers that appear black in the histochemically stained section (Fig. 3D). Central airways containing smooth muscle were also examined to compare receptor density in epithelium with a cell type known to have high β2AR density (25). As determined by photometry, labeling of epithelial cells by BODIPY-TMR-CGP-12177 was 77.6% (± 2.7% SEM, n = 6) as intense as the labeling of smooth muscle cells (Fig. 3E–G).

Transgenic Expression of the β2AR in Airway Secretory Cells of β2AR−/− Mice Restores a Full Asthma Phenotype. As a test of the site of β2AR signaling in promoting allergic inflammation, we evaluated whether restoration of receptors only in airway epithelial cells of β2AR−/− mice could rescue the asthma phenotype in response to IL-13. We generated transgenic (Tg-Epith-β2) mice expressing hemagglutinin-tagged human β2AR under control of the secretoglobulin Scgb1a1 promoter (Fig. S3), which is efficient and selective for airway epithelial secretory (club) cells (28, 29). Expression of β2AR was confirmed using anti-hemagglutinin antibody (Fig. S3D). BODIPY-TMR-CGP-12177 labeling was restored in the airway epithelium of β2AR−/− mice carrying the Scgb1a1-HA-β2AR transgenic allele (β2AR−/− + Tg-Epith-β2) and was augmented in β2AR WT mice carrying the Scgb1a1-β2AR allele (WT + Tg-Epith-β2) compared with WT mice (Fig. 4A).
inflammation. Previous studies of βarr-2−/− mice indicate that these mice mimic β2AR−/− mice in models of OVA immunization and challenge, acting in both hematopoietic and nonhematopoietic cells (15, 30). We tested whether βarr-2−/− mice also attenuate responses to acute topical exposure to IL-13 to highlight the function of βarr-2 in epithelial cells. Reductions in IL-13-induced mucous metaplasia and lung eosinophilia in βarr-2−/− mice (Fig. 4E) were similar to those in β2AR−/− mice (Fig. 2B), supporting the possibility that these function in the same pathway in epithelial cells.

Under certain conditions MAP kinases have been found to function downstream of βarr-2 (31, 32), so we tested whether intratracheal instillation of U0126, an inhibitor of MEK1/2 (33), before the instillation of IL-13 reduced mucous metaplasia and eosinophilia (Fig. S4A), similar to the genetic loss of β2ARs (Fig. 2B) or βarr-2 (Fig. 4E). The addition of U0126 also produced a reduction in mucin 5AC (MUC5AC) transcripts in cultured human airway epithelial cells exposed to both epinephrine and IL-13 (Fig. S4B). Together these results suggest that MAP kinases function downstream of β2AR and βarr-2 in airway epithelial cells to promote allergic airway inflammation (34, 35).

Epinephrine-Mediated β2AR Signaling in Isolated Human Bronchial Epithelial Cells. To further isolate the direct effects of β2AR activation on epithelial cells, we analyzed normal human bronchial epithelial (NHBE) cells in vitro. We confirmed that NHBE cells required epinephrine in addition to IL-13 to increase expression of MUC5AC transcripts (Fig. 5A and ref. 36), a molecular correlate of mucous metaplasia (37). Besides the effects on mucous metaplasia, our studies in mice suggested that airway epithelial β2AR signaling promotes allergic inflammation because leukocyte recruitment to the lungs varied with changes in epithelial β2AR signaling (Figs. 2 and 4B). To test this, we measured secretion from NHBE cells of the epithelial-derived chemokines chemokine (C-C motif) ligand 24 (CCL24), chemokine (C-X-C motif) ligand 1 (CXCL1), and chemokine (C-C motif) ligand 2 (CCL2) that participate in allergic lung inflammation (38–42) and chemokine (C-C motif) ligand 20 (CCL20) that participates in neutrophilic inflammation (43). NHBE cells required β2AR signaling to increase secretion of CCL24, CXCL1, and CCL2, but not CCL20, in response to IL-13 (Fig. 5B). These results in primary human cells confirm that epinephrine acts directly on airway epithelial cells to promote proinflammatory activities of IL-13. As expected for a β2AR-mediated effect, in the presence of epinephrine, nadolol inhibited MUC5AC expression and the increased secretion of CCL24, CXCL1, and CCL2, but not CCL20 (Fig. 5). Since at circulating levels norepinephrine activates all adrenoreceptors except β2AR, this confirms the receptor specificity of epinephrine in mediating these effects.

β2AR Signaling Does Not Promote Neutrophilic Inflammation. We next addressed whether β2AR signaling is nonspecifically proinflammatory or is selective for allergic inflammation. Previous experimental models utilized sensitization and then challenge by OVA (7–9). Here the effects of genetic deletion of β2AR on a variety of innate stimuli of eosinophilic and neutrophilic inflammation were compared. Crude extrinsic stimuli (lysates of Aspergillus species protease or nontypeable Haemophilus influenzae, NTHi), chemically well-defined extrinsic stimuli [chitin or the Toll-like receptor agonists Pam2CSK4 and ODN M362 (PAM2/ODN)], and intrinsic stimuli (IL-13 or IL-17A) were introduced into the lungs of WT and β2AR−/− mice (Fig. 6). As expected, the loss of β2AR signaling resulted in the attenuation of lung eosinophilia in response to all three eosinophilic stimuli (Fig. 6A). However, lack of β2AR signaling caused no loss or even a slight augmentation of lung neutrophilia in response to the neutrophilic stimuli (Fig. 6B).

We have previously shown that the promotion of eosinophilic inflammation by the β2AR is ligand dependent rather than constitutive (9). To examine the effects of endogenous epinephrine on neutrophilic inflammation, mice in which epinephrine was depleted genetically by deletion of Pnmt, which is responsible for the final step in epinephrine synthesis, or pharmacologically by exposure to reserpine, as we have described previously (9), were exposed to PAM2/ODN. In contrast to our previous results with eosinophilic inflammation (9), neither genetic (Fig. 6C) nor pharmacologic (Fig. 6D)
depletion of epinephrine caused a reduction in neutrophilic lung inflammation.

To determine the cell types involved, PAM2/ODN was used to identify the involvement of specific receptors in β2AR−/+ + Tg-Epith-β2 mice with transgenic expression of the receptor only in airway epithelial cells (Fig. S5). The transgenic expression of the β2AR solely on airway epithelial cells was sufficient to restore neutrophilic inflammation equal to that of the WT mice.

Discussion

Together these studies provide several lines of evidence that airway epithelial cells are the principle cell type through which β2AR signaling promotes the asthma phenotype. First, the effect of topical administration of IL-13 to the lung was sensitive to expression of β2AR (Figs. 2 A and B and 4B), and topical administration of a hydrophilic β-blocker (Fig. 2C) or β2AR agonist (Fig. 2D) together with IL-13 modulated the asthma phenotype, suggesting that the site of interaction between IL-13 and β2AR signaling is in resident lung cells. Second, β2ARs are highly expressed in airway epithelial cells in addition to smooth muscle cells, as shown by fluorescent ligand labeling (Fig. 3). Third, transgenic expression of β2AR only in airway epithelial cells rescues the loss of function shown by β2AR−/− mice and induces a gain of function in WT mice (Fig. 4B). Fourth, we show that β2AR signaling is responsible for the increased expression of MUC5AC and secretion of Th2 cytokines by IL-13 in isolated human airway epithelial cells (Fig. 5).

The airway epithelium is known to be an important site of IL-13 action (19, 44, 45), and our in vitro studies suggest that IL-13 and β2AR signaling interact cell-autonomously in airway epithelium (Fig. 5 and Fig. S4B). In their location at the interface of the lungs with the outside environment, airway epithelial cells are best positioned to integrate signals from inhaled air (e.g., pathogen-associated molecular patterns), the immune system (e.g., IL-13), and the nervous system (e.g., epinephrine). They respond to these signals both directly through the increased production of mucins and indirectly by signaling to leukocytes (46–49). Our studies also show that β2AR signaling on the airway epithelium can modulate airway smooth muscle function (Fig. 4C).
and ref. 19), reinforcing an analogy between the airway epithelium and the vascular endothelium (50, 51) that might extend to all cells lining hollow organs. These cells have traditionally often been called “barrier” cells because evidence shows that in disease it is often this barrier function that is disrupted (52, 53). However, in the last 30 y the vascular endothelium has emerged from this view of serving primarily a barrier function to being arguably the most important cell type in coordinating and maintaining vascular smooth muscle cell tone (54, 55). However, unlike the production of nitric oxide by the vascular endothelium, no relaxing or contracting factor released by airway epithelium has yet been widely recognized (50). This may be because epithelial regulation of the airway smooth muscle could be regulated by raising cytokine levels rather than by paracrine signaling as shown for CCL2, CCL24, and CXCL1 (Fig. 5B). However, it should be noted that the ability to coordinate an integrated response is common to all cells lining hollow organs.

Our studies also provide support for the hypotheses that explain the epidemiologic observation of a loss of asthma control and increase in asthma mortality with the regular use of AR ligands that activate adenylyl cyclase to induce relaxation (Fig. 4<sup>A</sup>) activation as the potential pathway mediating the proinflammatory responses. Last, our results show the β<sub>2</sub>AR signaling on airway epithelium is selective for promoting cosinophilic inflammation, as β<sub>2</sub>AR signaling has little effect on models of neutrophilic inflammation. These results reinforce the emerging role of the airway epithelium as a master-controller cell type capable of modulating the functions of other cells, as has been shown for the vascular endothelium.

Materials and Methods

**Supplies.** Chemicals and reagents were obtained from Sigma-Aldrich unless otherwise stated.

**Animals.** Six- to twelve-week-old mice were housed under specific pathogen-free conditions and were handled in accordance with the University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee, which also approved all the experiments conducted in these studies. All mice were on an FVB/N background except for jarr-2<sup>−/−</sup> mice, which were on a C57BL/6 background and used C57BL/6 controls. β<sub>2</sub>AR and jarr-2<sup>−/−</sup> mice have been described previously (8, 15). To generate epithelial-specific transgenic mice (Fig. 53), the β<sub>2</sub>AR gene was cloned into a plasmid encoding the mouse Scgb1a1 promoter and a hemagglutinin tag (28, 29). The β<sub>2</sub>AR sequence in the cloned construct was confirmed to be identical to contig NW_001838953, and the construct was injected into FVB/N blastocysts by the MD Anderson Genetically Engineered Mouse Facility. Scgb1a1-HA-β<sub>2</sub>AR transgenic offspring were identified by PCR using the primers CTTGCGGTTTGTGCGTGCATC and GAAGCCAGCGAGTGAAGGAAAG. These mice were crossed to β<sub>2</sub>AR<sup>−/−</sup> mice identified using the primers TCTCATGCTGCAATGCAAT and TTGTGCTCTTTCA AGCAGAACTT to produce offspring (50).

**Drug Administration.** Recombinant mouse IL-13 (3 μg in 25 μL 0.9% NaCl in water; PeproTech or BioLegend) was instilled into the tracheas of anesthetized (pentobarbital 6.5 mg) mice using a gel-loading pipette under direct visualization. Mice were killed 24 h later for histological and lung lavage fluid analyses as described (7). U0126 (15 mg/g mouse weight in 2% DMSO in 0.9% NaCl in water; Promega) was instilled intratracheally 2 h before IL-13 instillation and every 8 h until mice were killed. For aerosol delivery of drugs (nadolol, formoterol, PAM2/ODN), an AerOmist CA-209 jet nebulizer (Cis-USS) was driven by 10 L/min of room air supplemented with 5% CO<sub>2</sub> to promote deep ventilation. The nebulizer was connected by polyethylene tubing to a 10-L polyethylene chamber containing mice, which was closed except for efflux polyethylene tubing vented to a low-resistance filter. Solutions of nadolol (1.5 mg) or formoterol (20 μg) in 0.9% NaCl in water (6 mL) were delivered twice daily for 21 d. PAM2/ODN, 4 μM and 1 μM,
look-up-table (LUT) was applied to some fluorescent mice (NA 1.0) objective were performed using one-way ANOVA and Dunnett D0.0001 compared with WT mice challenged with eosinophilic or E9169 For mucin quantification, lungs were inflated with 4% zoom; green HeNe laser, 643-nm excitation/590-nm emission; and were μ = μ = μ = < published online October 9, 2017

Expression Assay (Life Technologies). Relative MUC5AC expression was calculated using the corresponding equation. Mucin content of airway epithelium is expressed as mucin volume density, signifying the measured volume of mucin overlying a unit area of epithelial basal lamina as described (7, 8, 68).

For examination of βAR distribution, lungs were inflated and fixed with 10% phosphate-buffered formalin and then were embedded in paraffin. Deparaffined sections were incubated in the dark at 20 °C for 60 min with water containing the fluorescent ligand b-lysin (BODIPY-TMR-CGP-12177; Molecular Probes) (100 nM in water), or with the irreversible antagonist BAAM (1 μM in 0.0001% ethanol in water) followed by BODIPY-TMR-CGP-12177 for 60 min, or with water alone for 120 min (27, 69, 70). The lung slices were observed with a Radiance 2100 confocal microscope (; Bio-Rad), 20× (NA 0.75) or 40× (NA 1.0) objective and 4x zoom; green HeNe laser, 643-nm excitation/590-nm emission; and were optimized to compare the fluorescence intensity between slices. For display purposes, a “smart” look-up-table (LUT) was applied to some fluorescent ligand images using ImageJ. This LUT assigns redyellow hues to all values greater than 103 in an eight-bit image (0–225). For identification of lung tissues to compare with BODIPY-TMR-CGP labeling, sections were stained with Verhoeff’s elastic stain and picrosiris red and were examined by brightfield microscopy.

Lung Physiology. Mice were anesthetized, tracheotomized, and connected to a computer-controlled ventilator (flexiVent; SCIREQ) (9). To induce bronchoconstriction, acetyl-β-methylcholine chloride (methacholine, MCh) in increasing doses from 10 to 100 μg/mL in 0.9% NaCl in water was aerosolized using the built-in nebulizer. Following each MCh dose, the Newtonian resistance (Raw) was recorded at 1-min intervals for 4 min and then averaged, with the largest value obtained referred to as “peak Raw” (9). To induce bronchoconstriction, the β-AR agonist isoproterenol (1 μg/mL in 0.9% NaCl in water) was aerosolized 2 min before MCh.

Cell Culture. NHBE cells (Lonza) were seeded into Transwell-Clear culture inserts (24.5-mm diameter, 0.45-μm pore; Corning) and grown in a 1:1 mixture of Bronchial Epithelial Basal Medium (Lonza) and Dulbecco’s Modified Eagle’s Medium (Life Technologies) supplemented with 30 μg/mL bovine pituitary extract, 0.5 μg/mL BSA, 0.5 μg/mL epinephrine, 50 μg/mL gentamycin, 50 ng/mL amphotericin B, 0.5 ng/mL human EGF, 0.5 μg/mL hydrocortisone, 5 μg/mL insulin, 7 ng/mL triiodothyronine, 10 ng/mL transferrin, and 0.1 ng/mL retinoic acid. When the cells reached confluence, the apical medium was removed to establish an air–liquid interface, and the basolateral medium was substituted with medium with or without epinephrine (3 μM), IL-3 (20 ng/mL), nadolol (10 μM), and U0126 (3 μM) and was replaced every 48 h for 14 d. For quantitative PCR, RNA was extracted from cells that had grown at the air–liquid interface for 4 d using TRIzol reagent (ThermoFisher Scientific) and was reverse transcribed using SuperScript III (Life Technologies). MUC5AC and 18S mRNA were quantified by real-time quantitative PCR using the Taqman Gene Expression Assay (Life Technologies). Relative MUC5AC expression was calculated using the ΔΔCT method. For chemokine measurement, basolateral medium was withdrawn from NHBE cells that had grown at the air–liquid interface for 5 d. Samples were flash frozen, and chemokines

respectively, dissolved in water was aerosolized once daily for 20 min to unrestrained mice.

Bronchoalveolar Lavage. The lungs of the killed mice were lavaged twice with 1 mL 0.9% NaCl in water through a tracheal catheter, total cells were counted with a hemacytometer, and the differential count was obtained by cyto-centrifugation of 300 μL of bronchoalveolar lavage (BAL) fluid at 450 × g for 5 min, followed by Wright–Giemsa staining as described (8).

Eosinophilic Stimuli. Fresh Aspergillus fumigatus was passed several times through an EmulsiFlex homogenizer (Avestin Inc.) to create a homogenized lysate, which was then diluted to 2.5 mg/mL. Neutrophilic Stimuli. NTHI lysate was prepared as described by Evans et al. (65) and was administered as previously described (66). Briefly, 6 mL of the lysate at a concentration of 2.5 mg/mL was delivered to mice by an Aeromist CA-

Fig. 6. Effect of βAR signaling on innate eosinophilic or neutrophilic inflammation. (A) BAL eosinophil counts obtained from WT and βAR−/− mice after exposure to extrinsic crude (15 mg A. fumigatus), extrinsic defined (100 μg chitin), and intrinsic (2 μg IL-13) eosinophilic stimuli (n = 5–9 mice per group). (B) BAL neutrophil counts obtained from WT and βAR−/− mice after exposure to extrinsic crude (15 mg NTHI), extrinsic defined (PAM2/ODN at daily doses of 30.5 μg and 48.3 μg, respectively), and intrinsic (1 μg IL-17A) neutrophilic stimuli (n = 5–9 mice per group). (C) WT or WT mice (D) previously treated with a loading dose of 5 mg/kg reserpine, followed by a maintenance dose of 0.3 mg/kg for the next 5 d (n = 4 mice per group). Error bars show SEM; *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 compared with WT mice challenged with eosinophilic or neutrophilic crude stimuli. Analyses in A and B were performed using two-way ANOVA and Tukey’s multiple comparisons post-hoc test. Analyses in C and D were performed using one-way ANOVA and Dunnett’s multiple comparisons post-hoc test.

βAR−/− mice were treated with the largest value obtained referred to as “peak Raw” (9). To induce bronchoconstriction, the β-AR agonist isoproterenol (1 μg/mL in 0.9% NaCl in water) was aerosolized 2 min before MCh.
Blood from treated mice was recovered in EDTA (1.8 mg/mL) and centrifuged to retrieve the plasma supernatant. Plasma samples were diluted in water and analyzed in the MD Anderson Pharmaceutical Development Center using LC/MS (Acquity UPLC and Xevo TQ-S; Waters). For the chromatography, a C8 reversed-phase column (Kinetex; Phenomenex) was eluted using gradients of acetonitrile and 20 mM ammonium acetate in 0.1% formic acid. Nadolol reference standards were used to set a linear range of detection from 0.5 to 1,000 ng/mL.

**Statistical Analysis.** Statistical analysis of multiple groups was performed using one- or two-way ANOVA followed by Dunnett’s or Tukey’s multiple comparison tests (GraphPad Prism version 6 for Mac OS X; GraphPad Software). Data are presented as mean ± SEM. P < 0.05 was considered statistically significant.

**Data-Sharing Plan.** The authors will make the data and materials available upon request. Please note that some of the genetically altered mice lines used in the experiments are no longer available, but the transgenic constructs to recreate the mice will be shared.

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