

β_2 -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 β_2 -adrenoreceptor (β_2 AR) agonists, but their chronic use causes paradoxical adverse effects. We have previously determined that β_2 AR activation is required for expression of the asthma phenotype in mice, but the cell types involved are unknown. We now demonstrate that β_2 AR 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 β_2 AR signaling with an aerosolized antagonist attenuates airway hyperresponsiveness (AHR), eosinophilic inflammation, and mucus-production responses to IL-13, whereas treatment with an aerosolized agonist worsens these phenotypes, suggesting that β_2 AR signaling on resident lung cells modulates the asthma phenotype. Labeling with a fluorescent β_2 AR ligand shows the receptors are highly expressed in airway epithelium. In β_2 AR^{-/-} mice, transgenic expression of β_2 ARs 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 β -arrestin-2 (β arr-2^{-/-}) attenuates the asthma phenotype as in β_2 AR^{-/-} mice. In contrast to eosinophilic inflammation, neutrophilic inflammation was not promoted by β_2 AR signaling. Together, these results suggest β_2 ARs on airway epithelial cells promote the asthma phenotype and that the proinflammatory pathway downstream of the β_2 AR involves β arr-2. These results identify β_2 AR signaling in the airway epithelium as capable of controlling integrated responses to IL-13 and affecting the function of other cell types such as airway smooth muscle cells.

asthma | β_2 -adrenoceptor | airway epithelium | mucous metaplasia | airway hyperresponsiveness

The β_2 -adrenoreceptor (β_2 AR) agonists are the most widely used and effective bronchodilators in asthma, but their chronic use may cause a loss of asthma control and an increase in asthma mortality (1–6). We have shown that a possible explanation for the paradoxical adverse chronic effects of β_2 AR agonists is due to β_2 AR signaling being required for full expression of the asthma phenotype in mice (7–9). Using both pharmacological and genetic approaches in antigen-driven murine asthma models, we showed that inhibition of β_2 AR signaling by administration of certain β_2 AR-blockers or by genetic deletion of the β_2 AR resulted in a marked reduction of three cardinal features of asthma: airway hyperresponsiveness (AHR), inflammation, and mucous metaplasia (7–10). Furthermore, removal of the endogenous ligand for the β_2 AR, epinephrine, again using both pharmacological and genetic approaches, also produced an attenuation of the asthma phenotype (9). These findings were translated to pilot clinical trials that demonstrated chronic treatment with the β -blocker nadolol produced a dose-dependent reduction in AHR (11, 12), although another β -blocker showed no improvement in a different

subset of asthmatics (13). Previous murine studies were performed using global deletion of the β_2 AR or of the enzyme responsible for epinephrine synthesis and systemic administration of β_2 AR antagonists or reserpine (to deplete epinephrine) (8, 9). Therefore, the cell types that were critical in mediating the antigen-driven features of the asthma phenotype remained unknown.

The aim of the present study was to identify the principal cell type and the signaling pathway by which activation of the β_2 AR promotes the asthma phenotype (Fig. 1). Our previous studies suggested the airway epithelium might be a key site of β_2 AR signaling because inhibition of β_2 AR signaling had a greater

Significance

Activation of β_2 -adrenoreceptors (β_2 ARs) on airway smooth muscle cells produces airway relaxation, and β_2 AR agonists are the most widely used bronchodilators for treating asthma. Paradoxically, murine models show β_2 AR activation is also required for expression of cardinal features of the asthma phenotype, including airway hyperresponsiveness (AHR), inflammation, and mucous metaplasia. However β_2 ARs 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 β_2 AR 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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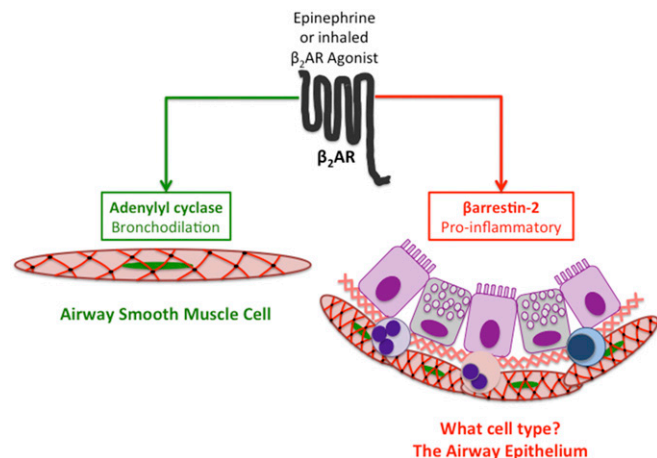


Fig. 1. Schematic of potential β_2 AR signaling in models of asthma. The β_2 AR can activate at least two distinct pathways: the canonical Gs-cAMP-mediated pathway and a β arr-2-mediated pathway. The canonical pathway is thought to be responsible for the potent bronchodilator effects of β_2 AR agonist by activating β_2 ARs on airway smooth muscle cells. However, the β arr-2-mediated pathway has been implicated as proinflammatory in asthma models (15, 16), but the cell type(s) mediating these effects is unknown. The aim of the present study is to test the hypothesis that the airway epithelium may be a main site of action for the proinflammatory effects of β_2 AR agonists.

effect on reducing mucous metaplasia than on inflammation in mouse ovalbumin (OVA) models of asthma. These results suggested that epithelial effector responses are upstream of leukocyte recruitment (7, 8, 10). In this study we used genetic and pharmacological approaches to show an essential role of the airway epithelium in the mediation of the asthma phenotype produced by intratracheal administration of IL-13. Our results demonstrate that both expressing the β_2 AR solely on the airway epithelium in mice with systemic deletion of the β_2 AR can restore the cardinal features produced by IL-13, and that aerosolized delivery of a hydrophilic β_2 AR blocker to WT mice is sufficient to attenuate the response to IL-13 to the same extent as the attenuation of the asthma phenotype produced by systemic β_2 AR blocker administration in antigen-driven murine models.

Additionally, the β_2 AR has been shown to be able to activate at least two signaling pathways: the canonical *Gs*-cAMP pathway and a β -arrestin-2 (β arr-2)-dependent pathway (14). Activation of the β_2 AR- β arr-2 pathway has been implicated in mediating the cardinal features of asthma in murine models (Fig. 1) (15, 16) and may lead to activation of MAP kinases. In the current study we present evidence that epithelial signaling via β_2 AR- β arr-2 mediates development of the IL-13-induced asthma phenotype. Previous studies using global deletion of β arr-2 implicate this pathway as mediating the asthma phenotype in murine antigen-driven models (15). Our results show that selective deletion of β arr-2 in airway epithelium is sufficient to attenuate the IL-13-induced asthma phenotype response.

Last, we show that β_2 AR signaling from the airway epithelium does not affect inflammation in neutrophilic models. These data suggest the proinflammatory β_2 AR signaling from the airway epithelium selectively modulates eosinophilic inflammation. Taken together, our results suggest β_2 AR signaling via a β arr-2 pathway solely on the airway epithelium is sufficient to modulate key features of the asthma phenotype produced by intratracheal administration of IL-13. These results further contribute to the increasing role of the cells that line hollow organs, such as the vascular endothelium, in sensing and coordinating integrated responses to hormones, antigens, or other stimuli.

Results

Topical Administration of β_2 AR Ligands Affects Allergic Lung Inflammation.

As an initial exploration of whether the β_2 AR 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 mucin and lung lavage eosinophils, both of which were attenuated in β_2 AR^{−/−} mice (Fig. 2 *A* and *B*). This suggested that IL-13 acting directly on lung parenchymal cells requires β_2 AR signaling within resident cells because of the short interval between IL-13 instillation and phenotypic assessment. Supporting a lung parenchymal site of β_2 AR 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 β_2 AR signaling within the lungs, we exposed mice to aerosolized β_2 AR ligands at concentrations not likely to have systemic effects. In previous time-course studies in OVA models, the β_2 AR 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. S24). 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 ($\pm$ 0.1 SEM, $n = 4$) and at 25 ppm as 3.1 ng/mL ($\pm$ 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 ($\pm$ 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 β_2 AR (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 β_2 AR agonist formoterol given by aerosol. In previous time-course studies, we found that restoration of β_2 AR signaling in phenylethanolamine *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 β_2 AR 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 μ g 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 β_2 AR signaling within the lungs and demonstrating that an exogenous β_2 AR agonist can cause a gain-in-

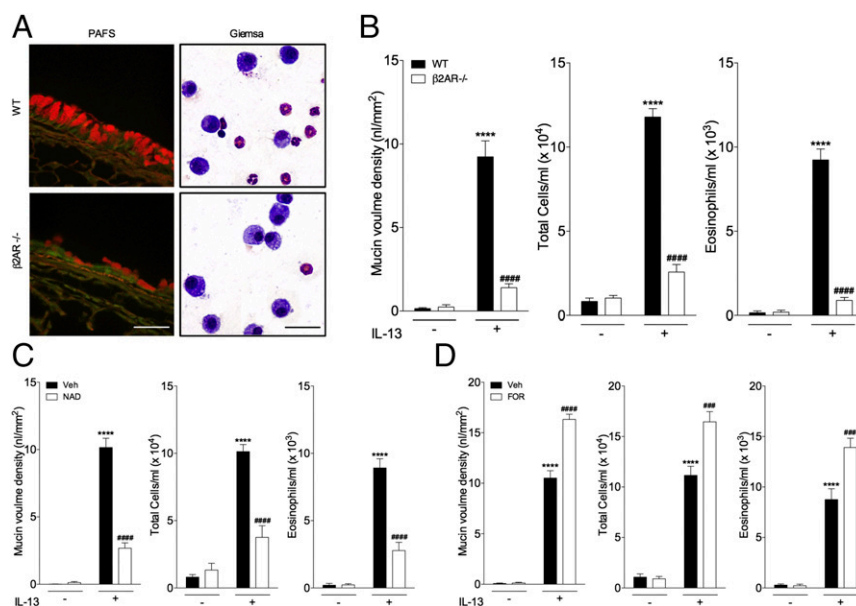


Fig. 2. Interaction of IL-13 and β_2 AR signaling in the lungs of mice. (A) WT and β_2 AR $^{-/-}$ mice were administered 3 μ g IL-13 intratracheally, and 24 h later lungs were harvested and stained with PAFS for mucin (Left) or were lavaged and stained with Wright-Giemsa staining for inflammatory cells (Right). (Scale bars: Left, 50 μ m; Right, 100 μ m.) (B) Epithelial mucin content (Left), lung lavage total cell counts (Center), and lung lavage eosinophil counts (Right) from WT (black bars) and β_2 AR $^{-/-}$ (white bars) mice administered or not administered 3 μ g IL-13 intratracheally. (C) Mucin content and lavage cell counts from WT mice treated for 21 d with aerosolized vehicle alone (Veh, black bars) or with 1.5 mg nadolol twice daily (white bars) and administered or not administered 3 μ g IL-13 intratracheally. (D) Mucin content and lavage cell counts from WT mice treated for 21 d with aerosolized vehicle alone (black bars) or with 20 μ g formoterol twice daily (white bars) and administered or not administered 3 μ g IL-13 intratracheally ($n = 6-8$; error bars show SEM; **** $P < 0.0001$ compared with the vehicle-treated control group; ### $P < 0.001$ and #### $P < 0.0001$ compared with WT mice challenged with IL-13). All analyses were performed using two-way ANOVA and Tukey's multiple comparisons post-hoc test.

function above the endogenous agonist in promoting the asthma phenotype.

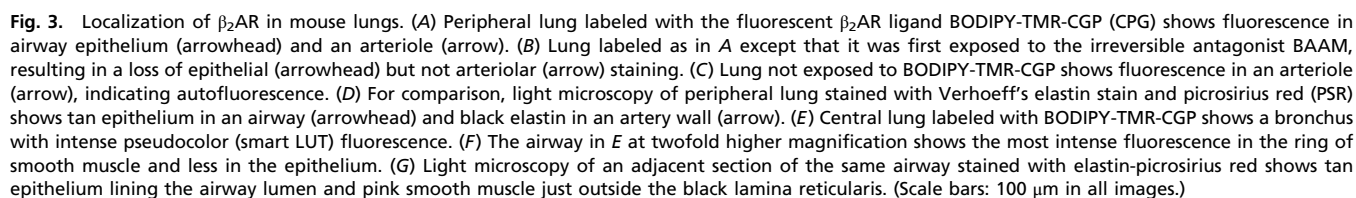
Airway Epithelial Cells Express β_2 ARs. To explore β_2 AR distribution within the lungs, we labeled mouse peripheral lung sections with boron dipyrromethene (BODIPY-TMR-CGP-12177), a fluorescent β_2 AR ligand (27). This revealed fluorescence in airway epithelial cells and blood vessels (arrowheads and arrows, respectively, in Fig. 3A). Pretreatment of tissue sections with bromoacetyl alprenolol menthane (BAAM), an irreversible β_2 AR 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 β_2 AR 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 β_2 AR density (25). As determined by photometry, labeling of epithelial cells by BODIPY-TMR-CGP-12177 was 77.6% ($\pm 2.7\%$ SEM, $n = 6$) as intense as the labeling of smooth muscle cells (Fig. 3E–G).

Transgenic Expression of the β_2 AR in Airway Secretory Cells of β_2 AR $^{-/-}$ Mice Restores a Full Asthma Phenotype. As a test of the site of β_2 AR signaling in promoting allergic inflammation, we evaluated whether restoration of receptors only in airway epithelial cells of β_2 AR $^{-/-}$ mice could rescue the asthma phenotype in response to IL-13. We generated transgenic (Tg-Epith- β_2) mice expressing hemagglutinin-tagged human β_2 AR under control of the secretoglobulin Scgb1a1 promoter (Fig. S3), which is efficient and selective for airway epithelial secretory (club) cells (28, 29). Expression of β_2 AR was confirmed using anti-hemagglutinin antibody (Fig. S3D). BODIPY-TMR-CGP-12177 labeling was

restored in the airway epithelium of β_2 AR $^{-/-}$ mice carrying the Scgb1a1-HA- β_2 AR transgenic allele (β_2 AR $^{-/-}$ + Tg-Epith- β_2) and was augmented in β_2 AR WT mice carrying the Scgb1a1- β_2 AR allele (WT + Tg-Epith- β_2) compared with WT mice (Fig. 4A). Transgenic expression of β_2 AR in airway epithelial secretory cells of β_2 AR $^{-/-}$ mice was sufficient for recovery of IL-13-induced mucous metaplasia and lung eosinophilia (Fig. 4B), while overexpression in WT + Tg-Epith- β_2 mice resulted in increased epithelial mucin content, (Fig. 4B), epithelial height, and lung eosinophilia compared with WT mice (Fig. 4B). These results indicate that β_2 AR signaling in airway secretory cells alone is sufficient for the promotion of two cardinal features of the asthma phenotype and that increased β_2 AR signaling can cause a gain-in-function similar to that observed with the exogenous agonist formoterol (Fig. 2D).

We then tested whether β_2 AR 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 β_2 AR $^{-/-}$ mice, as we have previously observed (8). Transgenic expression of β_2 AR in airway epithelial secretory cells was sufficient for recovery of IL-13-induced AHR in β_2 AR $^{-/-}$ + Tg-Epith- β_2 mice, although overexpression in WT + Tg-Epith- β_2 mice did not cause significantly increased AHR (Fig. 4C). Aerosolized isoproterenol, a β AR agonist, functionally antagonized IL-13-induced AHR in WT mice but did not further reduce the attenuated AHR in β_2 AR $^{-/-}$ mice (Fig. 4D). Isoproterenol treatment of β_2 AR $^{-/-}$ + Tg-Epith- β_2 mice did not impede IL-13-induced AHR (Fig. 4D), suggesting that β_2 ARs on airway smooth muscle cells are necessary for mediating bronchodilation.

β arr-2 Gene Deletion and Pharmacologic Inhibition of MAP Kinases Also Attenuate Allergic Inflammation. We next explored the signaling pathway downstream of the β_2 AR that promotes allergic



inflammation. Previous studies of $\beta\text{arr-2}^{-/-}$ mice indicate that these mice mimic $\beta_2\text{AR}^{-/-}$ mice in models of OVA immunization and challenge, acting in both hematopoietic and nonhematopoietic cells (15, 30). We tested whether $\beta\text{arr-2}^{-/-}$ mice also attenuate responses to acute topical exposure to IL-13 to highlight the function of $\beta\text{arr-2}$ in epithelial cells. Reductions in IL-13-induced mucous metaplasia and lung eosinophilia in $\beta\text{arr-2}^{-/-}$ mice (Fig. 4E) were similar to those in $\beta_2\text{AR}^{-/-}$ mice (Fig. 2B), supporting the possibility that these function in the same pathway in epithelial cells.

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 β_2 AR-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 adrenoceptors except β_2 AR, this confirms the receptor specificity of epinephrine in mediating these effects.

We have previously shown that the promotion of eosinophilic inflammation by the β_2 AR 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)

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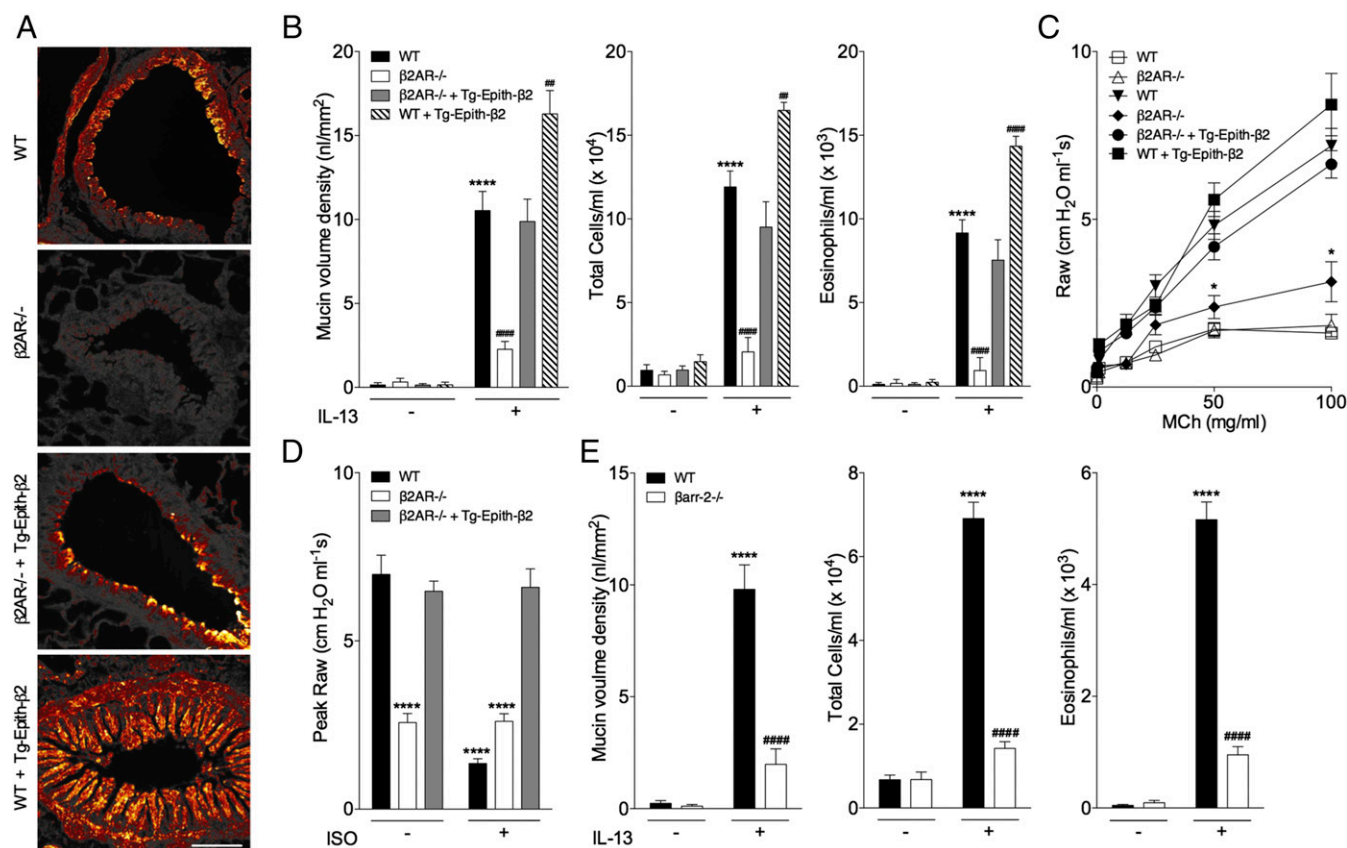


Fig. 4. Effect of airway epithelial β_2AR and β arr-2 signaling on the asthma phenotype. (A) BODIPY-TMR-CGP labeling of lung from WT mice shows fluorescence in airway epithelium; lung from $\beta_2AR^{-/-}$ mice shows no labeling; lung from *Scgb1a1*-HA- β_2AR transgenic mice crossed to $\beta_2AR^{-/-}$ mice ($\beta_2AR^{-/-}$ + Tg-Epith- β_2) shows fluorescence in epithelium comparable to that in WT mice; and lung from *Scgb1a1*-HA- β_2AR mice on a WT background (WT + Tg-Epith- β_2) shows more intense fluorescence than in WT mice. (Scale bar: 50 μ m.) (Please note that the long structure on the left of the WT image at the top of A is an adjacent airway that has been damaged/folded during the section cutting process). (B) Epithelial mucin content (Left), lung lavage total cell counts (Center), and lung lavage eosinophil counts (Right) from mice of the genotypes in A administered or not administered 3 μ g IL-13 intratracheally (n = 5–7 mice in each group; error bars show SEM; **** P < 0.0001 compared with the vehicle-treated control group; ### P < 0.01 and #### P < 0.0001 compared with WT mice administered IL-13). (C) Newtonian airway resistance (Raw, recorded at 1-min intervals for 4 min and then averaged) in response to increasing doses of aerosolized MCh (10–100 mg/mL) in mice administered (filled symbols) or not administered (open symbols) 3 μ g IL-13 intratracheally 24 h earlier (n = 5–7 mice in each group; * P < 0.05 compared with WT mice administered IL-13). (D) Peak Raw in response to aerosolized MCh of mice administered 3 μ g IL-13 intratracheally 24 h earlier and treated or not treated with 1 mg/mL isoproterenol (ISO) to induce bronchodilation (n = 5–7 mice in each group; **** P < 0.0001 compared with WT mice not treated with isoproterenol). (E) Epithelial mucin content (Left), lung lavage total cell counts (Center), and lung lavage eosinophil counts (Right) from WT (black bars) and β arr-2 $^{-/-}$ (white bars) mice administered or not administered 3 μ g IL-13 intratracheally (n = 5–6 mice in each group; error bars show SEM; **** P < 0.0001, compared with the vehicle-treated control group; ##### P < 0.0001, compared with WT challenged with IL-13). All analyses were performed using two-way ANOVA and Tukey's multiple comparisons post-hoc test.

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 $\beta_2AR^{-/-}$ + Tg-Epith- β_2 mice with transgenic expression of the receptor only in the airway epithelial secretory cells (Fig. S5). The transgenic expression of the β_2AR solely on airway epithelial cells was sufficient to restore neutrophilic inflammation equaled 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 β_2AR signaling promotes the asthma phenotype. First, the effect of topical administration of IL-13 to the lung was sensitive to expression of β_2AR (Figs. 2A and B and 4B), and topical administration of a hydrophilic β -blocker (Fig. 2C) or β_2AR agonist (Fig. 2D) together with IL-13 modulated the asthma phenotype, suggesting that the site of interaction between IL-13 and β_2AR signaling is in resident lung cells. Second, β_2AR s 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 β_2AR only in airway epithelial cells rescues the loss of function shown by $\beta_2AR^{-/-}$ mice and induces a gain of function in WT mice (Fig. 4B). Fourth, we show that β_2AR 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 β_2AR 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 β_2AR signaling on the airway epithelium can modulate airway smooth muscle function (Fig. 4C

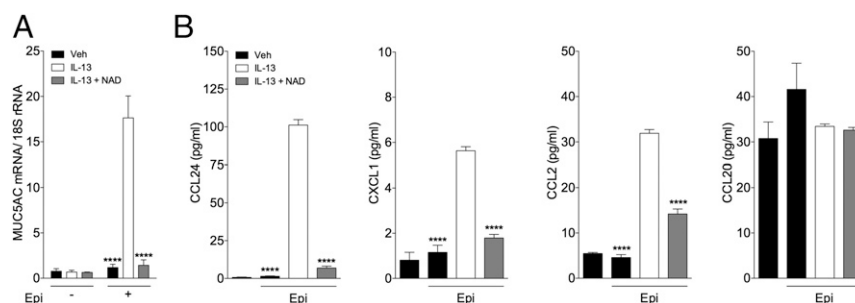


Fig. 5. β_2 AR signaling and effect on MUC5AC and chemokines in NHBE cells. (A) Fold change in MUC5AC transcript levels in NHBE cells grown in medium supplemented or not supplemented with 3 μ M epinephrine (EPI) and containing vehicle alone (Veh, black bars), 20 ng/mL IL-13 (white bars), or 20 ng/mL IL-13 and 10 μ M nadolol (gray bars); ($n = 3$ –4 donors; **** $P < 0.0001$ compared with cells in medium treated with IL-13 and epinephrine). (B) Cytokines from the basolateral medium of NHBE cells grown in the presence of 3 μ M epinephrine or not and containing vehicle alone (black bars), 20 ng/mL IL-13 (white bars), or 20 ng/mL IL-13 and 10 μ M nadolol (NAD) (gray bars) ($n = 3$ –4 donors; **** $P < 0.0001$ compared with cells treated with IL-13 and epinephrine). All analyses were performed using two-way ANOVA and Tukey's multiple comparisons post-hoc test.

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 β_2 AR agonists (1–3, 5). The augmentation of airway mucous metaplasia, lung eosinophilia, and AHR we observed when the agonist formoterol was administered chronically (Fig. 2D) or the β_2 AR was transgenically overexpressed in airway epithelium (Fig. 4A–D) supports a mechanistic explanation for the observation that chronic activation of β_2 AR to a level higher than that induced by endogenous epinephrine makes the asthma phenotype more severe. This is consistent with clinical findings in controlled trials of increased cough and sputum (4) and increased sputum eosinophils (4, 56, 57) with regular use of β_2 AR agonists. It is also conceptually consistent with our previous loss-of-function studies in mice and humans (7–9, 11, 12, 58) and is supported by previous gain-of-function studies by us and others in mice (9, 59–62). Our studies of the signaling pathway downstream of β_2 ARs in asthma promotion suggest that, whereas β_2 ARs in airway smooth muscle cells signal most prominently through G_s and adenylyl cyclase to induce relaxation (Fig. 4D), β_2 ARs in epithelial cells signal most prominently through β arr-2 and MEK1/2 to promote mucous metaplasia and inflammation (Fig. 4B and Fig. S44). Biased β_2 AR ligands that activate adenylyl cyclase signaling and inhibit β arr-2 signaling could retain the desirable bronchodilatory effect without incurring the undesirable mucus hypersecretory and inflammatory effects (60). Our results are also consistent with the beneficial effects of long-acting β -agonists, without adverse effects, in chronic obstructive pulmonary disease, a disease of neutrophilic inflammation (63).

Our studies identify the airway epithelium as the primary cell type and suggest β_2 AR signaling via β arr-2 (and possibly MEK1/

2) activation as the potential pathway mediating the proinflammatory responses. Last, our results show the β_2 AR signaling on airway epithelium is selective for promoting eosinophilic inflammation, as β_2 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 β arr-2 $^{-/-}$ mice, which were on a C57BL/6 background and used C57BL/6 controls. β_2 AR and β arr-2 $^{-/-}$ mice have been described previously (8, 15). To generate epithelial-specific transgenic mice (Fig. S3), the β_2 AR gene was cloned into a plasmid encoding the mouse *Scgb1a1* promoter and a hemagglutinin tag (28, 29). The β_2 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_2 AR transgenic offspring were identified by PCR using the primers CGTGCTGGTATTGTGCTGCTC ATC and GCAGCCAGCAGAGGGTGAAAG. These mice were crossed to β_2 AR $^{-/-}$ mice identified using the primers TCCATCATGGCTGATGCAAT and TTGTGCTCTTCA AGCAGAACTT to produce offspring expressing β_2 AR only in epithelial secretory cells. For Western blots to identify lines expressing the transgene, lungs were lysed in SDS sample buffer, and proteins were separated by SDS/PAGE and transferred to a PVDF membrane (Millipore) as described (59). Membranes were blocked in 3% BSA (1 h, 20 °C), incubated overnight with horseradish peroxidase-conjugated anti-HA antibody (1:1,000, 4 °C), washed in saline, and visualized using chemiluminescent substrate (SuperSignal West Pico; Thermo Fisher Scientific).

Drug Administration. Recombinant mouse IL-13 (3 μ g in 25 μ L 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-US) was driven by 10 L/min of room air supplemented with 5% CO₂ 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 μ g) in 0.9% NaCl in water (6 mL) were delivered twice daily for 21 d. PAM2/ODN, 4 μ M and 1 μ M,

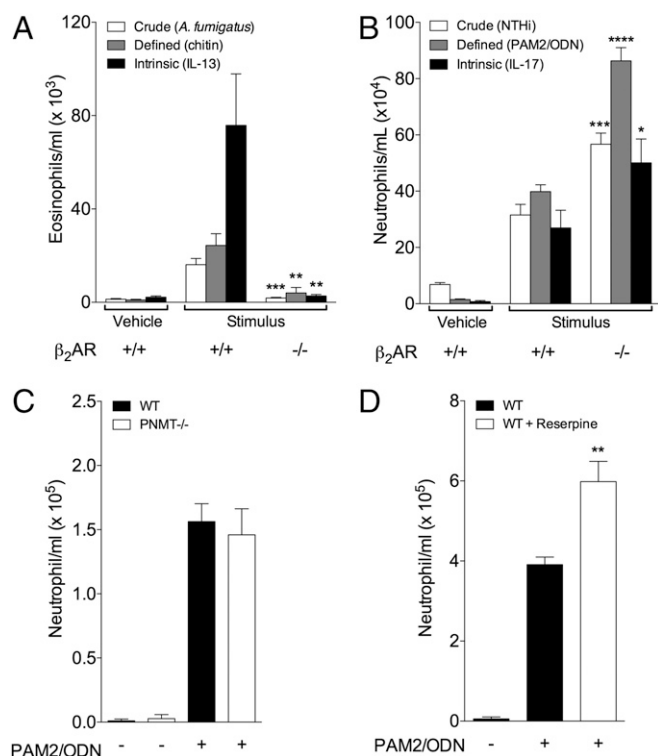


Fig. 6. Effect of β_2 AR signaling on innate eosinophilic or neutrophilic inflammation. (A) BAL eosinophil counts obtained from WT and β_2 AR^{-/-} mice after exposure to extrinsic crude (15 mg *A. fumigatus*), extrinsic defined (100 μ g chitin), and intrinsic (2 μ g IL-13) eosinophilic stimuli ($n = 5-9$ mice per group). (B) BAL neutrophil counts obtained from WT and β_2 AR^{-/-} mice after exposure to extrinsic crude (15 mg NTHi), extrinsic defined (PAM2/ODN at daily doses of 30.5 μ g and 48.3 μ g, respectively), and intrinsic (1 μ g IL-17A) neutrophilic stimuli ($n = 5-9$ mice per group). (C and D) BAL neutrophil counts 48 h after exposure to PAM2/ODN in *Pmmt*^{-/-} mice ($n = 4$ mice per group) (C) 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.

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 μ L of bronchoalveolar lavage (BAL) fluid at 450 $\times$ 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. *Aspergillus oryzae* lysate was generously provided by David B. Corry, Immunology, Allergy, and Rheumatology Section, Baylor College of Medicine, Houston. The first group of mice was administered 6 mL of this lysate by aerosol and was killed 48 h later for BAL analysis. The next group was given 100 μ g of suspended chitin, prepared as described by Reese et al. (64). It was delivered in 25 μ L of PBS intratracheally once daily for two administrations. These mice were killed for BAL analysis 24 h after the second treatment. The final group was given 2 μ g of IL-13 (PeproTech) in 50 μ L of PBS intratracheally; these mice were killed 48 h later for BAL analysis.

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-

209 jet nebulizer. PAM2/ODN (InvivoGen) was used as the defined stimulus. PAM2/ODN was used in place of NTHi to identify the involvement of specific receptors. PAM2/ODN were reconstituted and delivered once by jet nebulization to mice as previously described (67). The final group was given 1 μ g murine IL-17A (PeproTech) in 25 μ L of PBS, delivered intratracheally. All animals were killed for BAL analysis 48 h after stimulus administration.

Gain-of-Function Studies. Before PAM2/ODN administration, 20 μ g of formoterol fumarate was delivered in 6 mL of saline (pH 5) by jet nebulization, administered twice per day for 21 d (Fig. S5) to mice as described above. Mice were killed 48 h later for BAL analysis.

Mechanism Studies. Chitin and PAM2/ODN were prepared and delivered as described above. U0126 (Sigma) was prepared in a 2% DMSO solution to a concentration of 5 mg/mL, and 250 μ g was delivered intratracheally to WT C57 mice every 8 h, starting 2 h before the initial experimental administration. Mice were killed 48 h later for BAL analysis.

Histochemistry. For mucin quantification, lungs were inflated with 4% paraformaldehyde in PBS (20 °C) through a tracheal cannula and then were removed and further fixed overnight (4 °C) before paraffin embedding. Deparaffined sections were stained with periodic acid fluorescent Schiff's (PAFS) reagent, and images were acquired and analyzed as described below by investigators blinded to mouse genotype and treatment. The intracellular 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 β_2 AR 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 boron dipyrromethene (BODIPY-TMR-CGP-12177; Molecular Probes) (100 nM in water), or with the irreversible antagonist BAAM (1 μ M 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 $\times$ (NA 0.75) or 40 $\times$ (NA 1.0) objective and 4 $\times$ 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 red/yellow 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 picrosirius 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 mg/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 bronchorelaxation, the β -AR agonist isoproterenol (1 mg/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- μ m 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 μ g/mL bovine pituitary extract, 0.5 μ g/mL BSA, 0.5 μ g/mL epinephrine, 50 μ g/mL gentamycin, 50 ng/mL amphotericin B, 0.5 ng/mL human EGF, 0.5 μ g/mL hydrocortisone, 5 μ g/mL insulin, 7 ng/mL triiodothyronine, 10 μ g/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 μ M), IL-13 (20 ng/mL), nadolol (10 μ M), and U0126 (3 μ M) 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 14 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 $\Delta\Delta$ CT 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

were measured by indirect ELISA (Aushon Biosystems). All experiments were repeated with cells from three donors.

Plasma Nadolol Measurement. 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 multi-

comparison tests (GraphPad Prism version 6 for Mac OS X; GraphPad Software). Data are presented as mean $\pm$ 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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- National Asthma Education and Prevention Program (2007) Expert panel report 3: Guidelines for the diagnosis and management of asthma. *J Allergy Clin Immunol* 120 (Suppl 5):S94–S138, and erratum (2008) 121:1330.
- Chowdhury BA, Dal Pan G (2010) The FDA and safe use of long-acting beta-agonists in the treatment of asthma. *N Engl J Med* 362:1169–1171.
- Salpeter SR, Wall AJ, Buckley NS (2010) Long-acting beta-agonists with and without inhaled corticosteroids and catastrophic asthma events. *Am J Med* 123:322–8.e2.
- Sears MR, et al. (1990) Regular inhaled beta-agonist treatment in bronchial asthma. *Lancet* 336:1391–1396.
- Taylor DR (2009) The beta-agonist saga and its clinical relevance: On and on it goes. *Am J Respir Crit Care Med* 179:976–978.
- Wright JM, et al. (2004) Adverse effects of short-acting beta-agonists: Potential impact when anti-inflammatory therapy is inadequate. *Respirology* 9:215–221.
- Nguyen LP, et al. (2008) Chronic exposure to beta-blockers attenuates inflammation and mucin content in a murine asthma model. *Am J Respir Cell Mol Biol* 38:256–262.
- Nguyen LP, et al. (2009) Beta2-adrenoceptor signaling is required for the development of an asthma phenotype in a murine model. *Proc Natl Acad Sci USA* 106:2435–2440.
- Thanawala VJ, et al. (2013) β 2-Adrenoceptor agonists are required for development of the asthma phenotype in a murine model. *Am J Respir Cell Mol Biol* 48:220–229.
- Nguyen LP, et al. (2012) Complementary anti-inflammatory effects of a β -blocker and a corticosteroid in an asthma model. *Naunyn Schmiedeberg's Arch Pharmacol* 385:203–210.
- Hanania NA, et al. (2008) The safety and effects of the beta-blocker, nadolol, in mild asthma: An open-label pilot study. *Pulm Pharmacol Ther* 21:134–141.
- Hanania NA, et al. (2010) Response to salbutamol in patients with mild asthma treated with nadolol. *Eur Respir J* 36:963–965.
- Short PM, Williamson PA, Anderson WJ, Lipworth BJ (2013) Randomized placebo-controlled trial to evaluate chronic dosing effects of propranolol in asthma. *Am J Respir Crit Care Med* 187:1308–1314.
- Reiter E, Ahn S, Shukla AK, Lefkowitz RJ (2012) Molecular mechanism of β -arrestin-biased agonism at seven-transmembrane receptors. *Annu Rev Pharmacol Toxicol* 52:179–197.
- Walker JK, et al. (2003) Beta-arrestin-2 regulates the development of allergic asthma. *J Clin Invest* 112:566–574.
- Thanawala VJ, et al. (2015) β -Blockers have differential effects on the murine asthma phenotype. *Br J Pharmacol* 172:4833–4846.
- Grünig G, et al. (1998) Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282:2261–2263.
- Wills-Karp M, et al. (1998) Interleukin-13: Central mediator of allergic asthma. *Science* 282:2258–2261.
- Kuperman DA, et al. (2002) Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med* 8:885–889.
- Zhen G, et al. (2007) IL-13 and epidermal growth factor receptor have critical but distinct roles in epithelial cell mucin production. *Am J Respir Cell Mol Biol* 36:244–253.
- Cohn L, Elias JA, Chupp GL (2004) Asthma: Mechanisms of disease persistence and progression. *Annu Rev Immunol* 22:789–815.
- Paul WE, Zhu J (2010) How are T(H)2-type immune responses initiated and amplified? *Nat Rev Immunol* 10:225–235.
- Walker JA, Barlow JL, McKenzie AN (2013) Innate lymphoid cells—How did we miss them? *Nat Rev Immunol* 13:75–87.
- Gross NJ, Donohue JF (2010) Nebulized formoterol: A review of clinical efficacy and safety in COPD. *Int J Chron Obstruct Pulmon Dis* 5:223–232.
- Bide RW, Armour SJ, Yee E (2000) Allometric respiration/body mass data for animals to be used for estimates of inhalation toxicity to young adult humans. *J Appl Toxicol* 20:273–290.
- Patton JS, et al. (2010) The particle has landed—Characterizing the fate of inhaled pharmaceuticals. *J Aerosol Med Pulm Drug Deliv* 23(Suppl 2):S71–S87.
- Daly CJ, McGrath JC (2011) Previously unsuspected widespread cellular and tissue distribution of β -adrenoceptors and its relevance to drug action. *Trends Pharmacol Sci* 32:219–226.
- Moghaddam SJ, et al. (2008) Haemophilus influenzae lysate induces aspects of the chronic obstructive pulmonary disease phenotype. *Am J Respir Cell Mol Biol* 38:629–638.
- Stripp BR, et al. (1992) cis-acting elements that confer lung epithelial cell expression of the CC10 gene. *J Biol Chem* 267:14703–14712.
- Hollingsworth JW, et al. (2010) Both hematopoietic-derived and non-hematopoietic-derived beta-arrestin-2 regulates murine allergic airway disease. *Am J Respir Cell Mol Biol* 43:269–275.
- Shenoy SK, et al. (2006) beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. *J Biol Chem* 281:1261–1273.
- Shenoy SK, Lefkowitz RJ (2011) β -Arrestin-mediated receptor trafficking and signal transduction. *Trends Pharmacol Sci* 32:521–533.
- Favata MF, et al. (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* 273:18623–18632.
- Duan W, Chan JH, Wong CH, Leung BP, Wong WS (2004) Anti-inflammatory effects of mitogen-activated protein kinase kinase inhibitor U0126 in an asthma mouse model. *J Immunol* 172:7053–7059.
- Lee PJ, et al. (2006) ERK1/2 mitogen-activated protein kinase selectively mediates IL-13-induced lung inflammation and remodeling in vivo. *J Clin Invest* 116:163–173.
- Al-Sawalha N, et al. (2015) Epinephrine activation of the β 2-adrenoceptor is required for IL-13-induced mucin production in human bronchial epithelial cells. *PLoS One* 10:e0132559.
- Fahy JV, Dickey BF (2010) Airway mucus function and dysfunction. *N Engl J Med* 363:2233–2247.
- Coleman JM, et al. (2012) Epithelial eotaxin-2 and eotaxin-3 expression: Relation to asthma severity, luminal eosinophilia and age at onset. *Thorax* 67:1061–1066.
- Fitzpatrick AM, Higgins M, Holguin F, Brown LA, Teague WG; National Institutes of Health/National Heart, Lung, and Blood Institute's Severe Asthma Research Program (2010) The molecular phenotype of severe asthma in children. *J Allergy Clin Immunol* 125:851–857.e18.
- Holgate ST, et al. (1997) Release of RANTES, MIP-1 alpha, and MCP-1 into asthmatic airways following endobronchial allergen challenge. *Am J Respir Crit Care Med* 156:1377–1383.
- Komiyama A, et al. (2003) Concerted expression of eotaxin-1, eotaxin-2, and eotaxin-3 in human bronchial epithelial cells. *Cell Immunol* 225:91–100.
- Meyer-Hoffert U, et al. (2003) Th2- and to a lesser extent Th1-type cytokines upregulate the production of both CXC (IL-8 and gro-alpha) and CC (RANTES, eotaxin, eotaxin-2, MCP-3 and MCP-4) chemokines in human airway epithelial cells. *Int Arch Allergy Immunol* 131:264–271.
- Kao CY, et al. (2005) Up-regulation of CC chemokine ligand 20 expression in human airway epithelium by IL-17 through a JAK-independent but MEK/NF-kappaB-dependent signaling pathway. *J Immunol* 175:6676–6685.
- Kuperman DA, et al. (2005) IL-4 receptor signaling in Clara cells is required for allergen-induced mucus production. *J Immunol* 175:3746–3752.
- Whittaker L, et al. (2002) Interleukin-13 mediates a fundamental pathway for airway epithelial mucus induced by CD4 T cells and interleukin-9. *Am J Respir Cell Mol Biol* 27:593–602.
- Fahy JV, Locksley RM (2011) The airway epithelium as a regulator of Th2 responses in asthma. *Am J Respir Crit Care Med* 184:390–392.
- Holgate ST, Roberts G, Arshad HS, Howarth PH, Davies DE (2009) The role of the airway epithelium and its interaction with environmental factors in asthma pathogenesis. *Proc Am Thorac Soc* 6:655–659.
- Lambrecht BN, Hammad H (2012) The airway epithelium in asthma. *Nat Med* 18:684–692.
- Lloyd CM, Saglani S (2010) Asthma and allergy: The emerging epithelium. *Nat Med* 16:273–274.
- Vanhoutte PM (2013) Airway epithelium-derived relaxing factor: Myth, reality, or naivety? *Am J Physiol Cell Physiol* 304:C813–C820.
- Wu T, et al. (2017) Identification of BP1FA1/SPLUNC1 as an epithelium-derived smooth muscle relaxing factor. *Nat Commun* 8:14118.
- Carlier FM, Sibille Y, Pilette C (2016) The epithelial barrier and immunoglobulin A system in allergy. *Clin Exp Allergy* 46:1372–1388.
- Steelant B, et al. (2016) Restoring airway epithelial barrier dysfunction: A new therapeutic challenge in allergic airway disease. *Rhinology* 54:195–205.
- Vanhoutte PM, Shimokawa H, Feletou M, Tang EH (2017) Endothelial dysfunction and vascular disease - a 30th anniversary update. *Acta Physiol (Oxf)* 219:22–96.
- Vanhoutte PM (2016) Endothelial lessons. *Curr Vasc Pharmacol* 14:175–180.
- Aldridge RE, et al. (2000) Effects of terbutaline and budesonide on sputum cells and bronchial hyperresponsiveness in asthma. *Am J Respir Crit Care Med* 161:1459–1464.
- Aldridge RE, et al. (2002) Eosinophils and eosinophilic cationic protein in induced sputum and blood: Effects of budesonide and terbutaline treatment. *Ann Allergy Asthma Immunol* 89:492–497.

58. Callaerts-Vegh Z, et al. (2004) Effects of acute and chronic administration of beta-adrenoceptor ligands on airway function in a murine model of asthma. *Proc Natl Acad Sci USA* 101:4948–4953.
59. Lin R, et al. (2012) Chronic treatment in vivo with β -adrenoceptor agonists induces dysfunction of airway $\beta(2)$ -adrenoceptors and exacerbates lung inflammation in mice. *Br J Pharmacol* 165:2365–2377.
60. Walker JK, Penn RB, Hanania NA, Dickey BF, Bond RA (2011) New perspectives regarding $\beta(2)$ -adrenoceptor ligands in the treatment of asthma. *Br J Pharmacol* 163: 18–28.
61. Riesenfeld EP, et al. (2010) Inhaled salmeterol and/or fluticasone alters structure/function in a murine model of allergic airways disease. *Respir Res* 11:22.
62. Kamachi A, et al. (2001) Enhancement of goblet cell hyperplasia and airway hyperresponsiveness by salbutamol in a rat model of atopic asthma. *Thorax* 56: 19–24.
63. Kew KM, Mavergames C, Walters JA (2013) Long-acting beta2-agonists for chronic obstructive pulmonary disease. *Cochrane Database Syst Rev*, CD010177.
64. Reese TA, et al. (2007) Chitin induces accumulation in tissue of innate immune cells associated with allergy. *Nature* 447:92–96.
65. Evans CM, Kim K, Tuvim MJ, Dickey BF (2009) Mucus hypersecretion in asthma: Causes and effects. *Curr Opin Pulm Med* 15:4–11.
66. Clement CG, et al. (2008) Stimulation of lung innate immunity protects against lethal pneumococcal pneumonia in mice. *Am J Respir Crit Care Med* 177:1322–1330.
67. Duggan JM, et al. (2011) Synergistic interactions of TLR2/6 and TLR9 induce a high level of resistance to lung infection in mice. *J Immunol* 186:5916–5926.
68. Piccotti L, Dickey BF, Evans CM (2012) Assessment of intracellular mucin content in vivo. *Methods Mol Biol* 842:279–295.
69. Briones AM, et al. (2005) Direct demonstration of beta1- and evidence against beta2- and beta3-adrenoceptors, in smooth muscle cells of rat small mesenteric arteries. *Br J Pharmacol* 146:679–691.
70. Baker JG, Hall IP, Hill SJ (2003) Pharmacology and direct visualisation of BODIPY-TMR-CGP: A long-acting fluorescent beta2-adrenoceptor agonist. *Br J Pharmacol* 139:232–242.

Supporting Information

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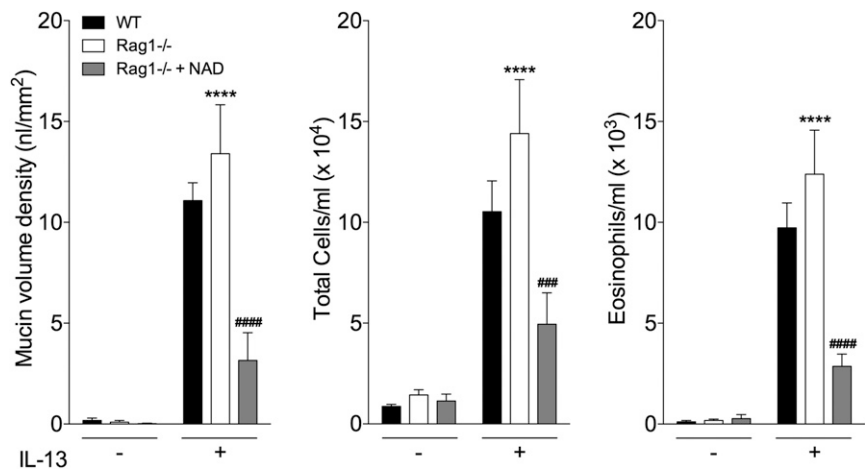


Fig. S1. Direct effect of exogenous IL-13 on lung parenchymal cells in the absence of lymphocytes. WT mice (black bars), Rag1^{-/-} mice (white bars), or Rag1^{-/-} mice pretreated twice daily for 21 d with 1.5 mg aerosolized nadolol (gray bars) were administered or not administered 3 μ g IL-13 intratracheally. After 24 h, epithelial mucin content (*Left*), lung lavage total cell counts (*Center*), and lung lavage eosinophil counts (*Right*) were measured ($n = 6$; error bars show SEM; **** $P < 0.0001$ compared with the vehicle-treated Rag1^{-/-} group; ### $P < 0.001$ and #### $P < 0.0001$ compared with Rag1^{-/-} mice challenged with IL-13). All analyses were performed using two-way ANOVA and Tukey's multiple comparisons post-hoc test.

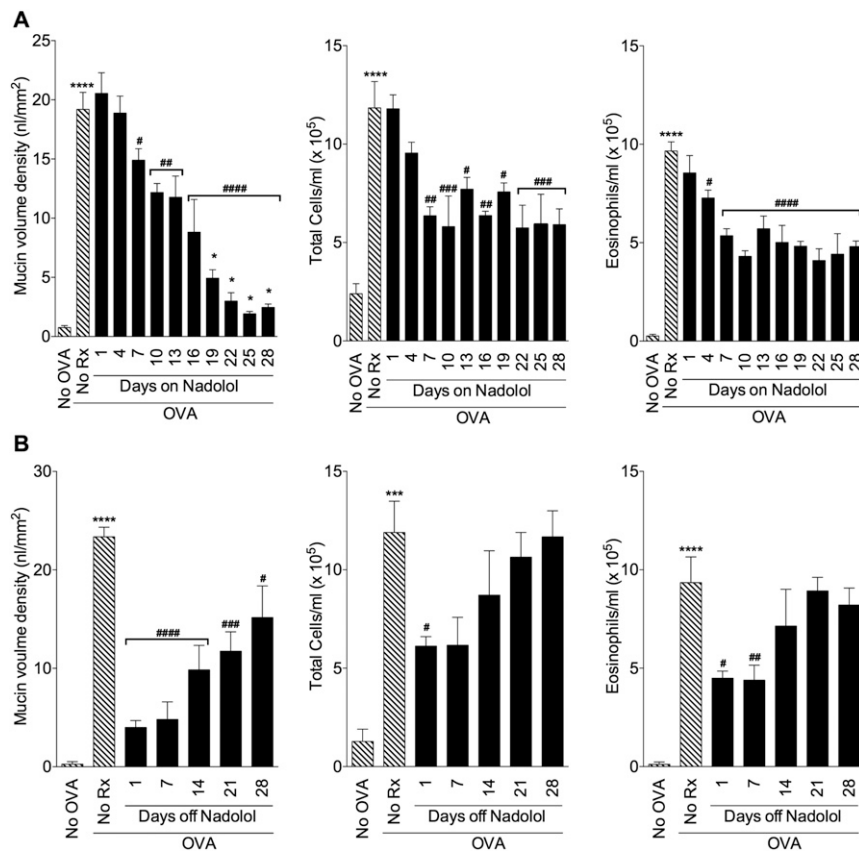


Fig. S2. Time courses of the effect from the onset and loss of nadolol. (A) Mice were immunized with OVA and alum as described (7–9) and then were placed on a diet containing 250 ppm nadolol for the number of days indicated (black bars) or were not treated with nadolol (No Rx). (B) Mice were immunized to OVA and then were placed on a diet containing 250 ppm nadolol for 28 d (black) or were not treated with nadolol (No Rx). Nadolol was then withdrawn from the diet for the number of days indicated. All mice except the control group (No OVA) were challenged daily for 5 d with intranasal OVA for the indicated number of days on (A) or off (B) nadolol and then were killed and analyzed. Epithelial mucin content (Left), lung lavage total cell counts (Center), and lung lavage eosinophil counts (Right) were measured ($n = 4–10$; error bars show SEM; * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ compared with no OVA; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, and #### $P < 0.0001$ compared with no nadolol). All analyses were performed using one-way ANOVA and Dunnett's multiple comparisons post-hoc test.

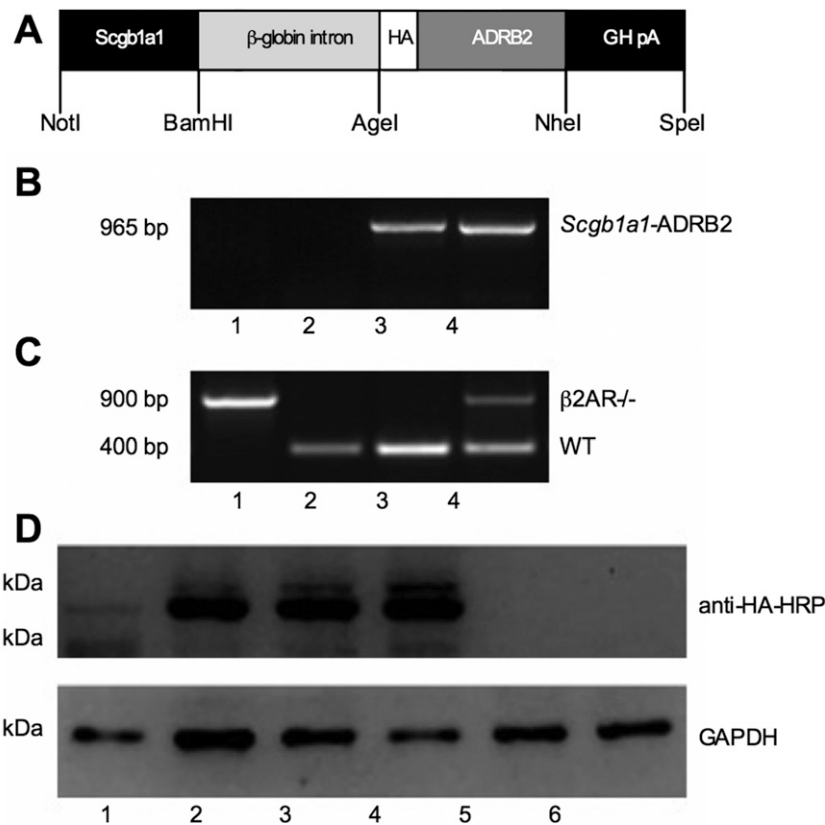


Fig. S3. Generation of transgenic mice expressing β_2 AR in airway epithelial secretory cells. (A) Diagram of the expression construct showing the mouse *Scgb1a1* promoter, a rabbit β -globin intron, a hemagglutinin tag, the β_2 AR coding region, and the cattle growth hormone polyadenylation signal, together with restriction sites. (B) Genomic DNA from F2 mouse tails was amplified using PCR with primers corresponding to the coding sequence of the β_2 AR. WT (lane 1) and β_2 AR^{-/-} (lane 2) controls fail to provide a template, whereas DNA of the predicted size was amplified from two mouse lines that incorporated the *Scgb1a1*-HA- β_2 AR transgene (lanes 3 and 4). (C) *Scgb1a1*-HA- β_2 AR mice were crossed to β_2 AR^{-/-} mice, yielding offspring that expressed β_2 AR in airway epithelial secretory cells on a homozygous β_2 AR^{-/-} background (lane 1), a homozygous WT background (lanes 2 and 3), or a heterozygous background (lane 4). (D) Expression of HA- β_2 AR protein (~47 kDa) in lung homogenates using anti-HA antibody in a Western blot in three of four mouse lines that incorporated the transgene (lanes 1–4) compared with WT (lane 5) and β_2 AR^{-/-} (lane 6) mice. GAPDH was used for loading controls.

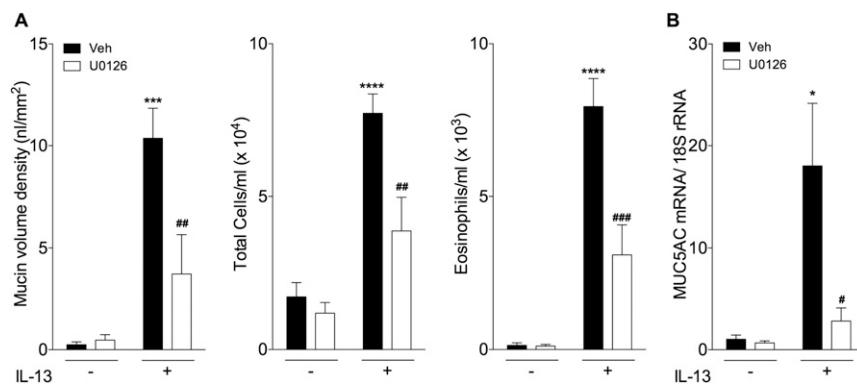


Fig. S4. Effect of the MEK1/2 inhibitor U0126 on mucin content and inflammation. (A) Mucin content (Left), lavage total cell counts (Center), and eosinophil counts (Right) from WT mice treated with vehicle alone (Veh, black bars) or the MEK1/2 inhibitor 250 μ g U0126 (white bars) and administered or not administered 3 μ g IL-13 intratracheally ($n = 5$ –6 mice in each group, error bars show SEM; *** $P < 0.001$, **** $P < 0.0001$ compared with vehicle-treated control group; ## $P < 0.01$, ### $P < 0.001$ compared with WT mice challenged with IL-13). (B) Fold change in MUC5AC transcript levels in NHBE cells grown in medium supplemented with 3 μ M epinephrine, with or without 20 ng/mL IL-13, and with vehicle alone (black bars) or with 3 μ M U0126 (white bars) ($n = 5$ –6 mice in each group or three donors for NHBE cells; error bars show SEM; * $P < 0.05$ compared with the vehicle-treated control group; # $P < 0.05$ compared with WT mice challenged with IL-13). All analyses were performed using two-way ANOVA and Tukey's multiple comparisons post-hoc test.

