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Leukotriene E_4 elicits respiratory epithelial cell mucin release through the G-protein–coupled receptor, GPR99

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Cysteinyl leukotrienes (cysLTs), leukotriene C_4 (LTC_4), LTD_4, and LTE_4 are proinflammatory lipid mediators with pathobiologic function in asthma. LTE_4, the stable cysLT, is a weak agonist for the type 1 and type 2 cysLT receptors (CysLTRs), which constrict airway smooth muscle, but elicits airflow obstruction and pulmonary inflammation in patients with asthma. We recently identified GPR99 as a high-affinity receptor for LTE_4 that mediates cutaneous vascular permeability. Here we demonstrate that a single intranasal exposure to extract from the respiratory pathogen *Alternaria alternata* elicits profound epithelial cell (EpC) mucin release and submucosal swelling in the nasal mucosa of mice that depends on cysLTs, as it is absent in mice deficient in the terminal enzyme for cysLT biosynthesis, LTC_4 synthase (LTC_4S). These mucosal changes are associated with mast cell (MC) activation and absent in MC-deficient mice, suggesting a role for MCs in control of EpC function. Of the three CysLTRs, only GPR99-deficient mice are fully protected from EpC mucin release and swelling elicited by *Alternaria* or by intranasal LTE_4. GPR99 expression is detected on lung and nasal EpCs, which release mucin to doses of LTE_4 one log lower than that required to elicit submucosal swelling. Finally, mice deficient in MCs, LTC_4S, or GPR99 have reduced baseline numbers of goblet cells, indicating an additional function in regulating EpC homeostasis. These results demonstrate a novel role for GPR99 among CysLTRs in control of respiratory EpC function and suggest that inhibition of LTE_4 and of GPR99 may have therapeutic benefits in asthma.

cysteinyl leukotrienes | mast cells | mucosal immunology | lung | epithelial cell

Cysteinyl leukotrienes (cysLTs), leukotriene C_4 (LTC_4), LTD_4, and LTE_4 are lipid mediators detected during asthma exacerbations triggered by allergen (1), aspirin (2, 3), and respiratory viruses (4). The cysLTs elicit vascular permeability, inflammation, and bronchoconstriction through three G-protein–coupled receptors. The type 1 cysLT receptor (CysLTR), $CysLT_1R$, is the high-affinity receptor for LTD_4 and the dominant CysLTR mediating airway smooth muscle constriction (5–8). The type 2 CysLTR, $CysLT_2R$, has prominent effects on the vascular endothelium (9–12) and also elicits bronchial constriction (13, 14). LTE_4, the stable cysLT (15–18), is a weak agonist for $CysLT_1R$ and $CysLT_2R$ in transfected cells (5, 19), but elicits airflow obstruction in patients with asthma (20–22). Moreover, LTE_4 has comparable activity to LTC_4 and LTD_4 in eliciting a wheal and flare response in human skin (23), and LTE_4 elicits cutaneous vascular permeability in mice lacking both $CysLT_1R$ and $CysLT_2R$, suggesting the existence of a high-affinity receptor for LTE_4, which was recently identified as GPR99 (24, 25). However, the mechanism by which LTE_4 induces lung pathobiology and the role of GPR99 remain poorly understood.

The cysLTs are derived from arachidonate through the serial enzymatic actions of 5-lipoxygenase and leukotriene C_4 synthase (LTC_4S). LTC_4, the terminal product of intracellular biosynthesis, is generated in activated leukocytes, exported extracellularly, and rapidly metabolized to LTD_4 and then to LTE_4. cysLT generation is elicited in the effector phase of allergic inflammation by the IgE-dependent cross-linking of FcεR1 on mast cells (MCs). However, cysLT generation can also be elicited by some allergen extracts in an IgE-independent fashion (26, 27), and recent reports demonstrate a role for cysLTs in shaping primary type 2 immune responses in the lung (28, 29). Thus, we sought to better understand cysLT function in the innate response to *Alternaria alternata*, a ubiquitous airborne fungus that contains potent aeroallergens. Here we demonstrate that a single intranasal (i.n.) exposure to *Alternaria* extract elicits profound epithelial cell (EpC) mucin release and submucosal swelling in the nasal mucosa of mice, which depends on MC activation, cysLT generation, and the CysLTR GPR99. Furthermore, GPR99, expressed on respiratory EpCs, mediates mucin release in response to low doses of i.n. LTE_4 injection and regulates baseline numbers of goblet cells, demonstrating a novel role for GPR99 among CysLTRs in control of respiratory EpCs.

Results

cysLT-Dependent Submucosal Swelling and Mucin Release. To determine cysLT function in innate immune responses in the airway, we evaluated mucosal changes in the nose of WT and LTC_4S-deficient (*Ltc4s*$^{-/-}$) mice 1 h after a single i.n. application of *Alternaria*. In the nasal septum (Fig. 1*A*), the highly vascular submucosa (Fig. 1*B*, submucosal area marked with punctated lines) responded to 30 μg *Alternaria* with marked swelling in WT

Significance

Leukotriene E_4 (LTE_4), a lipid mediator detected in asthma exacerbations triggered by allergen, viruses, and aspirin, elicits airflow obstruction and lung inflammation in asthmatics. GPR99 is the recently identified high-affinity receptor for LTE_4 and is resistant to blockade by commercially available cysteinyl leukotriene (cysLT) receptor antagonists. Here we find that GPR99 is expressed in respiratory epithelial cells and mediates mucin release and submucosal swelling in response to LTE_4 or to cysLTs elicited by *Alternaria*, a common airborne fungus. Furthermore, among cysLT receptors, only GPR99 regulates baseline numbers of mucin-containing goblet cells. These studies demonstrate a unique role for GPR99 in epithelial cell homeostasis and activation and indicate that epithelial cells may be a dominant site of LTE_4 action in the lung.

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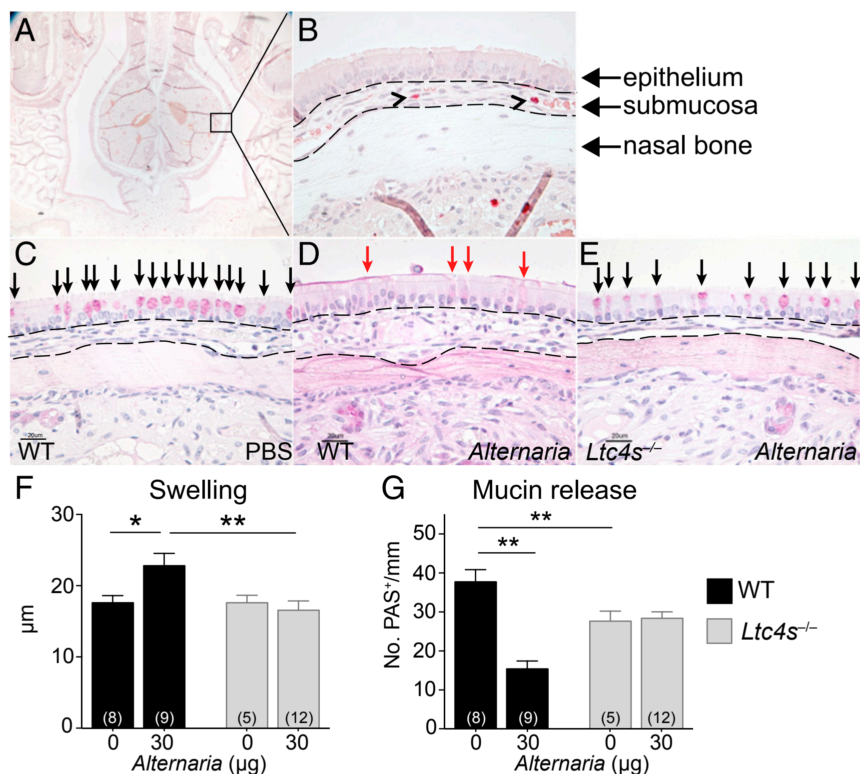

Fig. 1. cysLT-dependent submucosal swelling and mucin release elicited by *Alternaria*. (*A*) Representative histological coronal section through the incisors of the mouse skull of a PBS-challenged mouse with CAE stain (50× magnification). (*B*) A 630× magnification of *Inset* in *A*, rotated 90°. Black arrowheads point to MCs. (*C*–*E*) PAS staining of nasal septum 1 h after i.n. administration of (*C*) PBS or (*D* and *E*) 30 μg of *Alternaria* (magnification: 630×). (Scale bar, 20 μm.) Black arrows indicate mucin-containing GCs. Red arrows indicate GCs with partial extrusion of mucin. (*F* and *G*) WT (black bars) and *Ltc4s*$^{-/-}$ (gray bars) mice. (*F*) Submucosal swelling, indicated by the difference in the submucosal area between PBS (0 μg) and *Alternaria*-treated (30 μg) mice. (*G*) Mucin release, indicated by the difference in the total number of PAS$^+$ cells detected between PBS and *Alternaria*-treated mice. Results are means ± SEM pooled from four independent experiments with the number of mice per group indicated on each bar in parentheses. *$P < 0.05$, **$P < 0.01$.

mice, compared with PBS-treated controls (Fig. 1 *C* and *D*). Submucosal swelling was due to both vasodilatation and interstitial edema. There was no detectable cellular infiltration. The nasal epithelium of naive or PBS-challenged mice contains a large number of mucin granule-containing Periodic acid Schiff (PAS)-reactive goblet cells (GCs) (Fig. 1*C*, black arrows). *Alternaria* challenge led to extrusion of mucin from EpC granules with loss of PAS reactivity (Fig. 1*D*). Both submucosal edema and EpC mucin release were absent in *Alternaria*-treated *Ltc4s*$^{-/-}$ mice (Fig. 1 *E*–*G*), indicating a critical role for cysLTs in the response of respiratory tissue. Baseline numbers of PAS-reactive GCs were reduced in *Ltc4s*$^{-/-}$ mice compared with WT mice (Fig. 1*G*).

Innate Activation of MCs Is Required for *Alternaria*-Elicited Mucosal Responses. The submucosa of the nasal septum and nasal cavity floor contained MCs, and *Alternaria* challenge elicited release of their chloroacetate esterase (CAE)-reactive granules (Fig. S1 *A*–*C*). Degranulation, defined by the presence of more than five extracellular granules per cell, was elicited in 35 ± 2% of MCs in *Alternaria*-challenged WT mice, compared with 8 ± 1% in PBS-challenged WT mice (Fig. 2*A*). MC activation was confirmed by the detection of prostaglandin D_2 (PGD_2) in the nasal lavage (NL) of *Alternaria*-treated WT mice, which was absent in *Alternaria*-treated MC-deficient (Mcpt5/DTA) controls (Fig. 2*B*). To establish that *Alternaria* activated MCs in an innate fashion, we assessed *Fcer1g*$^{-/-}$ mice. The percentage of degranulating MCs (26 ± 4%) and the levels of PGD_2 in the NL of *Alternaria*-treated *Fcer1g*$^{-/-}$ mice were comparable to those of WT mice (Fig. 2 *A* and *B* and Fig. S1 *D*–*F*), demonstrating that *Alternaria* activates MCs independently of signaling through FcεR1 or activating FcγRs.

Alternaria also elicited robust cysLT generation from WT bone marrow-derived mast cells (BMMCs) in a dose-dependent fashion with a peak response at 100 μg/mL *Alternaria* (Fig. 2*C*). There was no cytotoxicity at these doses. As expected, no cysLTs were detected from *Alternaria*-treated *Ltc4s*$^{-/-}$ BMMCs, whereas *Fcer1g*$^{-/-}$ BMMCs had no attenuation in *Alternaria*-elicited cysLT production (Fig. 2*C*). Finally, we found a robust dose-dependent release of β-hexosaminidase from *Alternaria*-stimulated BMMCs and from human CD34$^+$ cell-derived MCs (Fig. 2*D*), indicating that *Alternaria* induces MC activation across species. Innate MC activation was essential for the epithelial and vascular changes in the *Alternaria*-challenged mucosa as both submucosal edema (Fig. 2*E*) and mucin release (Fig. 2*F*) were absent in *Alternaria*-treated Mcpt5/DTA mice and present in *Alternaria*-treated *Fcer1g*$^{-/-}$ mice. Mcpt5/DTA mice had reduced baseline numbers of mucin-containing goblet cells, similar to *Ltc4s*$^{-/-}$ mice.

GPR99 Controls *Alternaria*-Elicited EpC Mucin Release and Baseline EpC Composition. We next addressed which CysLTR might regulate these epithelial and submucosal changes. *Alternaria*-elicited submucosal swelling was absent in mice deficient in the type 1 CysLTR, $CysLT_1R$ (*Cysltr1*$^{-/-}$), but intact in $CysLT_2R$-deficient (*Cysltr2*$^{-/-}$) mice, compared with WT controls (Fig. 3*A*). EpC mucin release was partially attenuated in *Cysltr1*$^{-/-}$ mice and intact in *Cysltr2*$^{-/-}$ mice (Fig. 3*B*). Notably, both nasal submucosal swelling and mucin release were completely abrogated in *Alternaria*-treated *Gpr99*$^{-/-}$ mice (Fig. 3 *C* and *D*). MC degranulation in the submucosa and PGD_2 levels in the NL of *Alternaria*-treated *Cysltr1*$^{-/-}$ and *Gpr99*$^{-/-}$ mice were comparable

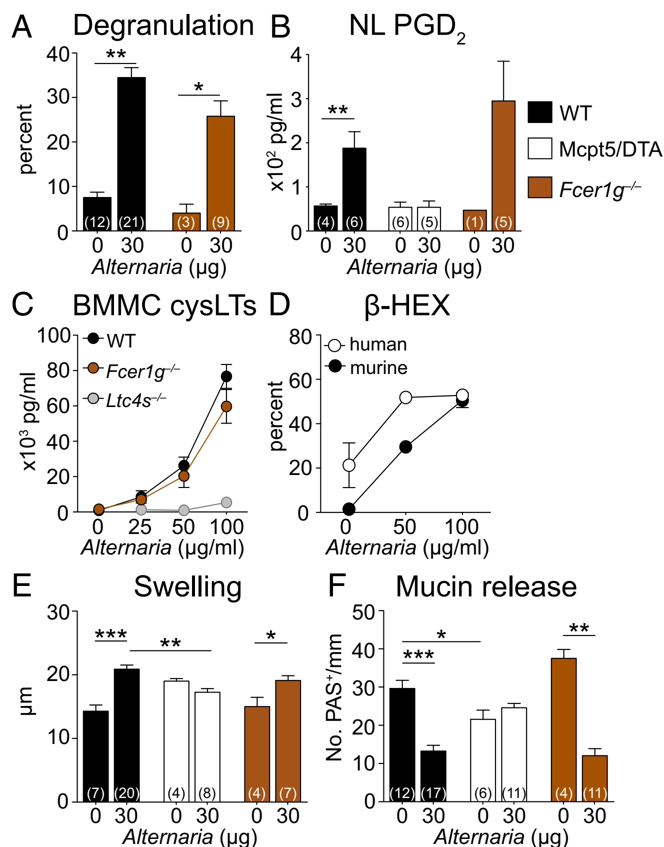

Fig. 2. The innate activation of MCs is required for *Alternaria*-elicited mucosal responses. (*A*) Percentage of degranulating MCs in the nasal submucosa 1 h after i.n. administration of PBS (0 μg) or *Alternaria* (30 μg) to WT (black bars) and *Fcer1g*$^{-/-}$ (brown bars) mice. (*B*) ELISA quantification of PGD_2 in the NL 1 h after i.n. administration of PBS or *Alternaria* to WT, *Fcer1g*$^{-/-}$, and Mcpt5/DTA (white bars) mice. (*C*) BMMCs from WT, *Fcer1g*$^{-/-}$, or *Ltc4s*$^{-/-}$ mice were harvested after 5–8 wk of culture and stimulated with *Alternaria*. The concentrations of cysLTs in the supernatants were measured by enzyme immunoassay at 30 min. Results are means ± SEM pooled from three independent experiments with cultures obtained from 10 WT mice, 3 *Ltc4s*$^{-/-}$ mice, and 3 *Fcer1g*$^{-/-}$ mice. (*D*) β-Hexosaminidase release into the supernatant at 30 min as a percentage of total. Results are means ± SEM pooled from three independent BMMC cultures and three independent human $CD34^+$ cell-derived MC cultures. (*E*) Submucosal swelling and (*F*) mucin release in WT, Mcpt5/DTA, and *Fcer1g*$^{-/-}$ mice 1 h after a single dose of PBS or *Alternaria*. Results are means ± SEM pooled from three (*A*) or five (*B*, *E*, and *F*) independent experiments with the number of mice per group indicated on each bar in parentheses. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

to *Alternaria*-treated WT mice (Fig. S2), indicating no defects in MC activation.

Baseline numbers of PAS-reactive GCs in PBS-treated *Gpr99*$^{-/-}$ mice were reduced by 29% compared with PBS-treated WT mice (Fig. 3*D*). Although there was no reduction in baseline GC numbers in *Fcer1g*$^{-/-}$ or *Cysltr1*$^{-/-}$ and *Cysltr2*$^{-/-}$ mice (Figs. 2*F* and 3*B*), baseline GC numbers were decreased by 34% in *Ltc4s*$^{-/-}$ mice (Fig. 1*G*) and by 27% in Mcpt5/DTA mice (Fig. 2*F*), compared with WT controls. These results demonstrate a constitutive effect of MCs, cysLTs, and GPR99 on EpC composition.

GPR99 Is Expressed in Respiratory EpCs and Mediates LTE_4-Elicited Submucosal Swelling and Mucin Release. Quantitative RT-PCR showed expression of all three CysLTRs in the nasal mucosa of naive WT mice (Fig. 4*A* and Fig. S3). Additionally, *Gpr99*$^{-/-}$ mice expressed *Escherichia coli* β-galactosidase (Fig. 4*A*), inserted in the targeted deletion of the *Gpr99* gene, confirming GPR99 expression in the nasal mucosa. Strong X-gal staining in naive *Gpr99*$^{-/-}$ nasal mucosa localized to respiratory EpCs and was not detected in WT mice (Fig. 4*B* and Fig. S4). X-gal staining also confirmed GPR99 expression in bronchial EpCs (Fig. 4*C* and Fig. S4). We did not detect GPR99 expression in the vascular endothelium, but note the limited sensitivity of this technique.

Intranasal injection of LTE_4 induced a dose-dependent increase in submucosal swelling in WT mice (Fig. 4*D*), reaching a plateau at 0.1 nmol. Similar to *Alternaria*, LTE_4 caused expansion of the extracellular space and dilatation of the submucosal vessels with no detectable cellular infiltration at 1 h. Both *Cysltr1*$^{-/-}$ and *Gpr99*$^{-/-}$ mice were protected from LTE_4-elicited submucosal edema. LTE_4 also induced EpC mucin release in a dose-dependent fashion with a plateau at 0.1 nmol (Fig. 4*E*). Strikingly, only *Gpr99*$^{-/-}$ mice were protected from mucin release, demonstrating that GPR99 is the dominant CysLTR for LTE_4-elicited EpC secretory function.

Discussion

The role(s) of LTE_4 in airway biology has received little attention, in part because of its poor activity at the classical cysLT receptors, $CysLT_1R$ and $CysLT_2R$ (5, 19), in transfected cells. Here we find that the high-affinity receptor for LTE_4, GPR99, is expressed on airway EpCs and controls EpC mucin release and submucosal swelling in response either to exogenous LTE_4 or to endogenously generated cysLTs elicited by *Alternaria.* Moreover, we find reduced nasal GC numbers in both naive *Ltc4s*$^{-/-}$ and *Gpr99*$^{-/-}$ mice, but not in *Cysltr1*$^{-/-}$ or *Cysltr2*$^{-/-}$ mice. These results demonstrate a selective role for GPR99 in EpC homeostasis and secretory function. Furthermore, they suggest that targeting GPR99, which is resistant to commercially available $CysLT_1R$ antagonists, may have therapeutic benefit in airway diseases characterized by abnormalities in mucin secretion and clearance.

LTE_4 elicited mucin release at 0.01 nmol in WT mice and reached a plateau at 0.1 nmol. The dose of LTE_4 required for mucin release was one log lower than that required to elicit

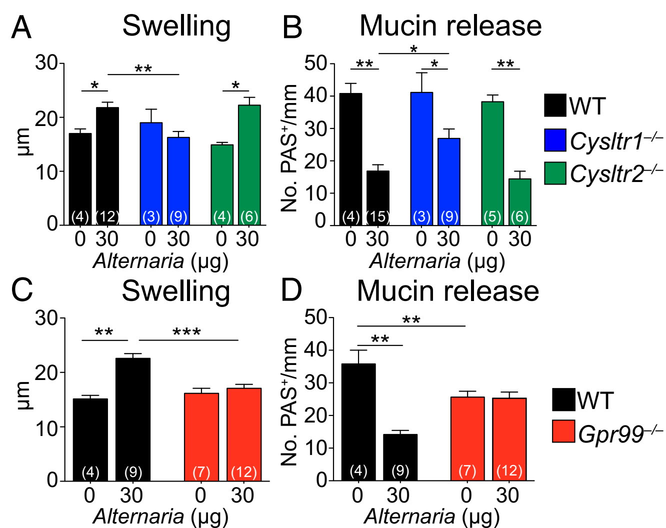

Fig. 3. GPR99 controls *Alternaria*-elicited EpC mucin release and baseline EpC composition. (*A*–*D*) *Alternaria* (30 μg) or PBS (0 μg) was administered to WT (black bars), *Cysltr1*$^{-/-}$ (blue bars), *Cysltr2*$^{-/-}$ (green bars), and *Gpr99*$^{-/-}$ (red bars) mice. (*A* and *C*) Swelling and (*B* and *D*) mucin release were measured in histological samples from the nasal mucosa 1 h after i.n. challenge, as in Fig. 1. Results are means ± SEM pooled from three independent experiments with the numbers of mice per group indicated on each bar in parentheses. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

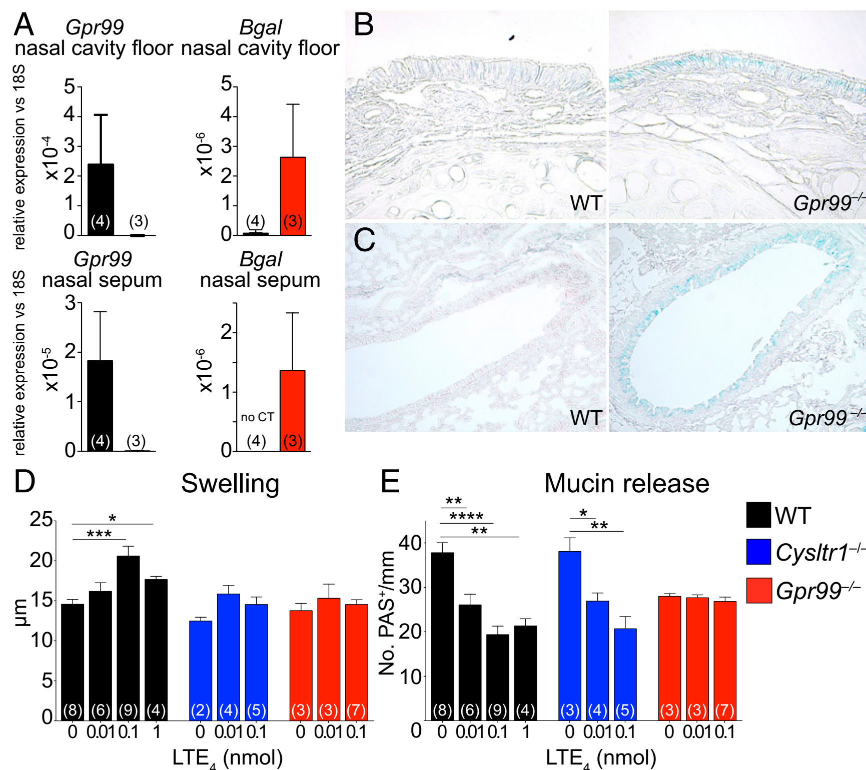

Fig. 4. GPR99 is expressed in respiratory EpCs and mediates LTE_4-elicited submucosal swelling and mucin release. (*A*) Quantitative RT-PCR analysis of expression of *Gpr99* and β-galactosidase (*Bgal*) genes in the nasal cavity floor (*Upper* panels) and nasal mucosa of the septum and side wall (*Lower* panels) compared with 18S rRNA. Results are means ± SEM pooled from two independent experiments with the numbers of mice per group indicated in parentheses in each column. (*B* and *C*) X-gal staining in the nasal mucosa of *Gpr99*$^{-/-}$ mice (*Right* panels) and WT mice (*Left* panels) in the (*B*) nasal cavity (magnification: 630×) and (*C*) large bronchi (magnification: 200×). Positive X-gal reactivity produces a blue precipitate. (*D* and *E*) LTE_4 (0.01, 0.1, or 1 nmol) or PBS (0 nmol) was administered i.n. to WT (black bars), *Cysltr1*$^{-/-}$ (blue bars), and *Gpr99*$^{-/-}$ (red bars) mice. (*D*) Submucosal swelling and (*E*) mucin release were measured 1 h after LTE_4 administration in histological samples from the nasal mucosa. Data are means ± SEM pooled from three independent experiments with the numbers of mice per group indicated on each bar in parentheses. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

submucosal swelling, demonstrating a dominant effect for LTE_4 on airway EpCs that was solely mediated by GPR99 in the presence of the classical receptors. By contrast, LTE_4-induced submucosal swelling, elicited at higher doses, was reduced in both *Cysltr1*$^{-/-}$ and *Gpr99*$^{-/-}$ mice, indicating nonredundant functions for these receptors in control of the nasal microvasculature. In response to a fixed dose of *Alternaria*, we again saw the nonredundant effects of these receptors for swelling and did find that mucin release was partially reduced in *Cysltr1*$^{-/-}$ mice. A role for $CysLT_1R$ may reflect LTE_4 action at $CysLT_1R$ at higher doses.

The decreased numbers of PAS-reactive EpCs in both PBS-treated *Ltc4s*$^{-/-}$ and *Gpr99*$^{-/-}$ mice, and the intact numbers in *Cysltr1*$^{-/-}$ or *Cysltr2*$^{-/-}$ mice, seem a credible functional coupling of ligand production and a receptor-selective response. Remarkably, a reduced number of GCs was also seen in unmanipulated MC-deficient Mcpt5/DTA mice, but not in *Fcer1g*$^{-/-}$, suggesting an innate function of MCs in controlling EpC composition in the absence of specific activation. Moreover, we found that IgE-independent MC activation controlled EpC mucin release in response to airway *Alternaria* challenge. These findings are in keeping with an innate immune cell that is present in sea squirts, preceding adaptive immunity, and which expresses a conserved transcriptional signature between mouse and human (30). Our findings surprisingly suggest that some allergen-elicited EpC pathways may be regulated indirectly by tissue-resident constitutive mast cells.

GPR99 was initially reported as an oxoglutarate receptor (31). As GPR99 belongs to the same nucleotide P2Y receptor family as $CysLT_1R$ and $CysLT_2R$ and recognizes dicarboxylic acids, it was identified as a candidate LTE_4 receptor and subsequently demonstrated to mediate cutaneous swelling in response to low doses of LTE_4 (25). Our findings for GPR99 control of mucin secretion and EpC homeostasis indicate a central role for this CysLTR at low doses of LTE_4. Although we do find GPR99 expressed in both nasal and lung EpCs, a function for GPR99 in other cell types cannot be excluded. There are no reports of GPR99 function in human airways; however, LTE_4 elicits mucin release from both human conjunctival GCs (32) and human airway EpCs (33). Notably, asthmatic patients are more sensitive to LTE_4-elicited airflow obstruction than healthy controls, a hyperresponsiveness not seen with either LTC_4 or LTD_4 (20–22, 34). This suggests a mechanism for LTE_4-mediated airflow obstruction that is distinct from the bronchoconstricting effects of the other CysLTRs and that depends on precedent lung disease. As mucus secretion can contribute to airway obstruction (35, 36), it is possible that the sensitivity to LTE_4-induced obstruction reflects a contribution of GPR99 in the setting of the GC expansion seen in asthmatic lung. Furthermore, targeting GPR99, which is resistant to commercially available CysLTR antagonists, may have therapeutic benefit in airway diseases characterized by abnormalities of mucin secretion and clearance. In sum, our findings define GPR99 as the airway receptor for LTE_4 and identify a function for this third CysLTR in airway epithelial integrity and secretion that is distinct from the classical receptors.

Materials and Methods

Mice. $Ltc4s^{-/-}$ mice were generated on a 129Sv background (37) and backcrossed for 15 generations onto the C57BL/6 background. $Cysltr1^{-/-}$ and $Cysltr2^{-/-}$ mice were generated on a C57BL/6 background as reported previously (38, 39). C57BL/6 $Gpr99^{+/-}$ mice were obtained from the National Institutes of Health Knock-Out Mouse Project and intercrossed to obtain $Gpr99^{-/-}$ mice (25). $Fcer1g^{-/-}$ mice (B6;129P2-Fcer1g^{tm1Rav}/J) were purchased from Jackson Laboratories. Mcpt5/DTA mice were generated by crossing mice with MC-specific expression of Cre recombinase (40) (kindly provided by Axel Roers, Technische Universitaet Dresden) on a C57BL/6 background with ROSA-diphtheria toxin-α mice (B6.129P2-Gt(ROSA)26Sor$^{tm1(DTA)Lky}$/J from Jackson Laboratories). WT littermates from the intercrossing of $Cysltr1^{-/-}$, $Cysltr2^{-/-}$, and Mcpt5/DTA mice (Cre$^+$DTA$^-$, Cre$^-$DTA$^+$, and Cre$^-$DTA$^-$) or age- and sex-matched C57BL/6 mice (Charles River Laboratories) were used as controls. In 18% of the Mcpt5/DTA mice, significant numbers of MCs were detectable in the nasal mucosa (41), and these mice were excluded. Male and female mice 3–8 mo of age were used. All mice were housed in a specific pathogen-free facility in groups of four to five mice per cage with a standard light/dark cycle of 12 h. Mice were provided food and water ad libitum. All mice except C57BL/6 mice purchased from Charles River Laboratories were bred in the Dana Farber Cancer Institute mouse facility, and pups were weaned between 19 and 28 d old. All experiments were performed during the day. The use of mice for these studies was in accordance with review and approval by the Animal Care and Use Committee of the Dana Farber Cancer Institute.

***Alternaria* and LTE_4 Administration.** Mice received a single application of *A. alternata* culture filtrate (Greer Laboratories) intranasally after anesthesia with an i.p. injection of ketamine (10 mg/kg) and xylazine (20 mg/kg) for full sedation. *Alternaria* culture filtrate was delivered in a total volume of 20 μL of sterile PBS. In a separate set of experiments, 0.01, 0.1, or 1 nmol of LTE_4 (Cayman Chemical) was dissolved and diluted in 20 μL PBS after ethanol was evaporated and immediately administered intranasally. Mice of a given genotype were randomized to treatment dose, and challenges were performed in groups organized by genotype and treatment dose. Mice were euthanized exactly 1 h after *Alternaria* or LTE_4 administration with isoflurane overdose, and tissues were harvested for histology and fixed with 4% (wt/vol) paraformaldehyde (PFA). NL was performed with 500 μL of PBS, and after acetone precipitation, PGD_2 was measured in the lipid fraction using a PGD_2–MOX ELISA kit (Cayman Chemical) with the following reported reactivity: Prostaglandin D_2-MOX (100%), Prostaglandin D_2 (0.2%), Prostaglandin E_2-MOX (<0.01%), 6-keto Prostaglandin $F_{1\alpha}$-MOX (<0.01%), Prostaglandin $F_{2\alpha}$ (<0.01%), Tetranor-PGEM (<0.01%), Tetranor-PGFM (<0.01%), and Thromboxane B_2-MOX (<0.01%) (42, 43).

In Vitro Culture and Stimulation. BMMCs were generated as previously described (37). In brief, bone marrow was collected from femurs and tibiae of mice and cultured for 4–6 wk in RPMI medium 1640 containing 10% (vol/vol) FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, penicillin (100 units/mL), streptomycin (100 μg/mL), and 1% culture supernatant from Chinese hamster ovary cells expressing mouse interleukin-3. The culture medium for the BMMC was changed every week, and the cell density was adjusted to 3×10^5/mL at every passage. After 4 wk, more than 97% of the cells were BMMCs as assessed by staining with Wright–Giemsa and toluidine blue. Human MCs were derived from fresh heparinized peripheral blood using a CD34$^+$ isolation kit (Miltenyi Biotec) and cultured in Iscove's modified Dulbecco's media with 4 mM L-glutamine, penicillin, streptomycin, 30% (vol/vol) charcoal-treated characterized FCS, and holo-Transferrin (50 μg/mL) in the presence of stem-cell factor (100 ng/mL) and GM-CSF (10 pg/mL).

BMMCs and human MCs were harvested and stimulated with *Alternaria* at the concentration of 0–100 μg/mL. After 15 min, the reaction was stopped by centrifugation at $120 \times g$ for 5 min at 4 °C, and the supernatants were retained for assays of β-hexosaminidase (β-HEX) and cysLTs. For β-HEX assay, a marker of MC degranulation, the cell pellets were suspended in Hanks' balanced salt solution and disrupted by repeated freeze–thawing. β-HEX was quantitated by spectrophotometric analysis of the hydrolysis of *p*-nitrophenyl-β-D-2-acetamido-2-deoxyglucopyranoside. The percentage release of β-HEX was calculated by the formula $[S/(S+P)] \times 100$, where S and P are the β-HEX contents of equal portions of supernatant and cell pellet, respectively.

cysLTs in the supernatants of BMMCs and human MCs were measured 30 min after stimulation with *Alternaria* culture filtrate by enzyme immunoassay according to the manufacturer's protocol (Amersham Biosciences) with a lower limit of detection at 60 pg/mL and the following reported reactivity: Leukotriene C4 (100%), Leukotriene D4 (100%), Leukotriene E_4 (91%), Leukotriene B_4 (<1.35%), Prostaglandin D_2 (<0.006%), Prostaglandin $F_{2\alpha}$ (<0.006%), Prostaglandin E_2 (<0.006%), 6-Keto-prostaglandin $F_{1\alpha}$ (<0.006%), Thromboxane B_2 (<0.006%), and Glutathione (<0.006%) (44).

RT-PCR. The nasal cavity floor was harvested by separating the palate with nasal cavity floor from the base of the skull. The remaining mucosa covering the nasal septum and side wall was scraped with a scalpel. Total RNA was isolated with TRIzol Reagent (Invitrogen) according to the manufacturer's protocol and further purified with Qiagen microcolumn (SABiosciences). Total RNA (1 μg) was reverse-transcribed with SuperScript III (Invitrogen), and quantitative RT-PCR was performed with primers specific for mouse GPR99, the *E. coli* β-galactosidase gene, mouse $CysLT_1R$, mouse 18S rRNA (SABiosciences), and for GAPDH and $CysLT_2R$ (Sigma-Aldrich) using the Mx3005P Real-Time PCR System (Agilent Technologies) under the following condition: 98 °C for 15 s, 58 °C for 30 s, 72 °C for 1 min, 40 cycles. The ratio of each mRNA relative to the 18S rRNA was calculated with the ddCt method.

Histochemistry and Quantitative Assessment of MC Degranulation, Mucin Release, and Submucosal Swelling. The nasal cavity was harvested from mouse snouts obtained from euthanized mice 1 h after i.n. administration of *Alternaria.* After separation from the spine, the skull skin was stripped, the lower jaw and brain were removed, and the tissue was fixed in 4% (wt/vol) PFA in PBS for 18 h, changed to PBS, and decalcified using 14% (wt/vol) EDTA in NH_4OH solution (pH 7.2–7.4) for 7–14 d. When the snouts were deemed to be sufficiently decalcified, they were rinsed in PBS and cross-sectioned behind the incisors and in between the first three palatal ridges, yielding four coronal sections through the nasal cavity. For histochemical evaluation, the tissues were embedded in glycolmethacrylate. Tissue sections, 2.5 μm thick, were assessed by PAS for quantitation of mucin-containing goblet cells and by CAE reactivity for quantitation of MC number and degranulation. Slides were counterstained with hematoxylin for general morphologic examination. All histologic assessments were done in a blinded fashion by a single investigator.

The number of PAS-reactive cells for each animal was enumerated from 6 to 8,200× digital photographs spanning a 4- to 8-mm basement membrane over the four coronal sections. Submucosal swelling for each animal was determined using ImageJ software (National Institutes of Health). The area of submucosal tissue between the epithelial basement membrane and nasal septum bone in two to four photographs spanning the 2- to 4-mm basement membrane over two coronal sections containing nasal septum was measured. This area was divided by the length of basement membrane to define the average thickness of the submucosal space. Degranulated and intact MCs were counted, as previously described (45), in 1.89 mm^2 over four coronal sections of the nasal cavity floor and septum.

X-gal staining was performed on mouse snouts from WT and $Gpr99^{-/-}$ mice. Briefly, tissues were fixed in 4% (wt/vol) PFA for 2 h, washed with PBS, and then incubated in X-gal staining solution for 48 h (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 2 mM $MgCl_2$, 5 mM EGTA, 0.02% IGEPAL, 0.01% sodium deoxycholate + 10 mM K_3FeCN_6, 10 mM K_4FeCN_6, 0.5 mg/mL X-gal). Subsequently, the mouse snouts were decalcified with 14% (wt/vol) EDTA solution and embedded in paraffin. Visulization was performed in 8-μm deparaffinized sections with a Leica DM LB2 microscope equipped with a Nikon DXM 1200 camera. For confocal microscopy, direct visualization of X-gal–stained tissue was achieved after excitation at 633 nm and recording fluorescence emission in the 650- to 770-nm range. Nuclei were stained with Hoechst 33342 nuclear stain (1:10,000; Sigma). Images were acquired with a Zeiss LSM 700 Laser Scanning Confocal microscope with a 63× Zeiss plan-APOCHROMAT oil, 1.4 N.A. objective. Transmitted and fluorescence images were overlayed using ImageJ (NIH).

Statistics. Analysis was performed with GraphPad Prism software (version 5.01, GraphPad). Nonparametric two-sided Mann–Whitney tests were used to determine significance. A value of $P < 0.05$ was considered significant. Sample sizes were not predetermined by statistical methods.

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1. Wenzel SE, Larsen GL, Johnston K, Voelkel NF, Westcott JY (1990) Elevated levels of leukotriene C4 in bronchoalveolar lavage fluid from atopic asthmatics after endobronchial allergen challenge. *Am Rev Respir Dis* 142(1):112–119.
2. Christie PE, et al. (1991) Urinary leukotriene E4 concentrations increase after aspirin challenge in aspirin-sensitive asthmatic subjects. *Am Rev Respir Dis* 143(5 Pt 1): 1025–1029.
3. Laidlaw TM, et al. (2012) Cysteinyl leukotriene overproduction in aspirin-exacerbated respiratory disease is driven by platelet-adherent leukocytes. *Blood* 119(16): 3790–3798.
4. Drazen JM, et al. (1992) Recovery of leukotriene E4 from the urine of patients with airway obstruction. *Am Rev Respir Dis* 146(1):104–108.
5. Lynch KR, et al. (1999) Characterization of the human cysteinyl leukotriene CysLT1 receptor. *Nature* 399(6738):789–793.
6. Figueroa DJ, et al. (2001) Expression of the cysteinyl leukotriene 1 receptor in normal human lung and peripheral blood leukocytes. *Am J Respir Crit Care Med* 163(1): 226–233.
7. Mechiche H, et al. (2003) Effects of cysteinyl leukotrienes in small human bronchus and antagonist activity of montelukast and its metabolites. *Clin Exp Allergy* 33(7): 887–894.
8. Leff JA, et al. (1998) Montelukast, a leukotriene-receptor antagonist, for the treatment of mild asthma and exercise-induced bronchoconstriction. *N Engl J Med* 339(3): 147–152.
9. Uzonyi B, et al. (2006) Cysteinyl leukotriene 2 receptor and protease-activated receptor 1 activate strongly correlated early genes in human endothelial cells. *Proc Natl Acad Sci USA* 103(16):6326–6331.
10. Jiang W, et al. (2008) Endothelial cysteinyl leukotriene 2 receptor expression mediates myocardial ischemia-reperfusion injury. *Am J Pathol* 172(3):592–602.
11. Shirasaki H, et al. (2013) Localization and up-regulation of cysteinyl leukotriene-2 receptor in human allergic nasal mucosa. *Allergol Int* 62(2):223–228.
12. Duah E, et al. (2013) Cysteinyl leukotrienes regulate endothelial cell inflammatory and proliferative signals through $CysLT_2$ and $CysLT_1$ receptors. *Sci Rep* 3:3274.
13. Yonetomi Y, et al. (2015) Leukotriene C4 induces bronchoconstriction and airway vascular hyperpermeability via the cysteinyl leukotriene receptor 2 in S-hexyl glutathione-treated guinea pigs. *Eur J Pharmacol* 754:98–104.
14. Sekioka T, et al. (2015) Expression of CysLT2 receptors in asthma lung, and their possible role in bronchoconstriction. *Allergol Int* 64(4):351–358.
15. Orning L, Kaijser L, Hammarström S (1985) In vivo metabolism of leukotriene C4 in man: Urinary excretion of leukotriene E4. *Biochem Biophys Res Commun* 130(1): 214–220.
16. Verhagen J, et al. (1987) The excretion of leukotriene E4 into urine following inhalation of leukotriene D4 by human individuals. *Biochem Biophys Res Commun* 148(2):864–868.
17. Lam S, Chan H, LeRiche JC, Chan-Yeung M, Salari H (1988) Release of leukotrienes in patients with bronchial asthma. *J Allergy Clin Immunol* 81(4):711–717.
18. Sala A, Voelkel N, Maclouf J, Murphy RC (1990) Leukotriene E4 elimination and metabolism in normal human subjects. *J Biol Chem* 265(35):21771–21778.
19. Heise CE, et al. (2000) Characterization of the human cysteinyl leukotriene 2 receptor. *J Biol Chem* 275(39):30531–30536.
20. Davidson AB, et al. (1987) Bronchoconstrictor effects of leukotriene E4 in normal and asthmatic subjects. *Am Rev Respir Dis* 135(2):333–337.
21. O'Hickey SP, Arm JP, Rees PJ, Spur BW, Lee TH (1988) The relative responsiveness to inhaled leukotriene E4, methacholine and histamine in normal and asthmatic subjects. *Eur Respir J* 1(10):913–917.
22. Arm JP, Spur BW, Lee TH (1988) The effects of inhaled leukotriene E4 on the airway responsiveness to histamine in subjects with asthma and normal subjects. *J Allergy Clin Immunol* 82(4):654–660.
23. Soter NA, Lewis RA, Corey EJ, Austen KF (1983) Local effects of synthetic leukotrienes (LTC4, LTD4, LTE4, and LTB4) in human skin. *J Invest Dermatol* 80(2):115–119.
24. Maekawa A, Kanaoka Y, Xing W, Austen KF (2008) Functional recognition of a distinct receptor preferential for leukotriene E4 in mice lacking the cysteinyl leukotriene 1 and 2 receptors. *Proc Natl Acad Sci USA* 105(43):16695–16700.
25. Kanaoka Y, Maekawa A, Austen KF (2013) Identification of GPR99 protein as a potential third cysteinyl leukotriene receptor with a preference for leukotriene E4 ligand. *J Biol Chem* 288(16):10967–10972.
26. Barrett NA, Maekawa A, Rahman OM, Austen KF, Kanaoka Y (2009) Dectin-2 recognition of house dust mite triggers cysteinyl leukotriene generation by dendritic cells. *J Immunol* 182(2):1119–1128.
27. Suram S, et al. (2006) Regulation of cytosolic phospholipase A2 activation and cyclooxygenase 2 expression in macrophages by the beta-glucan receptor. *J Biol Chem* 281(9):5506–5514.
28. Barrett NA, et al. (2011) Dectin-2 mediates Th2 immunity through the generation of cysteinyl leukotrienes. *J Exp Med* 208(3):593–604.
29. Doherty TA, et al. (2013) Lung type 2 innate lymphoid cells express cysteinyl leukotriene receptor 1, which regulates TH2 cytokine production. *J Allergy Clin Immunol* 132(1):205–213.
30. Dwyer DF, Barrett NA, Austen KF (2016) Transcriptional profiling of tissue resident mast cells reveals a unique identity within the immune system. *Nat Immunol*, 10.1038/ni.3445.
31. He W, et al. (2004) Citric acid cycle intermediates as ligands for orphan G-protein-coupled receptors. *Nature* 429(6988):188–193.
32. Dartt DA, et al. (2011) Conjunctival goblet cell secretion stimulated by leukotrienes is reduced by resolvins D1 and E1 to promote resolution of inflammation. *J Immunol* 186(7):4455–4466.
33. Shirasaki H, Kanaizumi E, Seki N, Himi T (2015) Leukotriene E4 induces MUC5AC release from human airway epithelial NCI-H292 cells. *Allergol Int* 64(2):169–174.
34. Arm JP, et al. (1990) Asthmatic airways have a disproportionate hyperresponsiveness to LTE4, as compared with normal airways, but not to LTC4, LTD4, methacholine, and histamine. *Am Rev Respir Dis* 142(5):1112–1118.
35. Evans CM, et al. (2015) The polymeric mucin Muc5ac is required for allergic airway hyperreactivity. *Nat Commun* 6:6281.
36. Kuyper LM, et al. (2003) Characterization of airway plugging in fatal asthma. *Am J Med* 115(1):6–11.
37. Kanaoka Y, Maekawa A, Penrose JF, Austen KF, Lam BK (2001) Attenuated zymosan-induced peritoneal vascular permeability and IgE-dependent passive cutaneous anaphylaxis in mice lacking leukotriene C4 synthase. *J Biol Chem* 276(25):22608–22613.
38. Beller TC, Maekawa A, Friend DS, Austen KF, Kanaoka Y (2004) Targeted gene disruption reveals the role of the cysteinyl leukotriene 2 receptor in increased vascular permeability and in bleomycin-induced pulmonary fibrosis in mice. *J Biol Chem* 279(44):46129–46134.
39. Maekawa A, Austen KF, Kanaoka Y (2002) Targeted gene disruption reveals the role of cysteinyl leukotriene 1 receptor in the enhanced vascular permeability of mice undergoing acute inflammatory responses. *J Biol Chem* 277(23):20820–20824.
40. Scholten J, et al. (2008) Mast cell-specific Cre/loxP-mediated recombination in vivo. *Transgenic Res* 17(2):307–315.
41. Dudeck A, et al. (2011) Mast cells are key promoters of contact allergy that mediate the adjuvant effects of haptens. *Immunity* 34(6):973–984.
42. Cayman Chemical Company (2015) *Prostaglandin D2-MOX ELISA Kit.* Available from: https://www.caymanchem.com/pdfs/512011.pdf.
43. Kelly RW, Deam S, Cameron MJ, Seamark RF (1986) Measurement by radioimmunoassay of prostaglandins as their methyl oximes. *Prostaglandins Leukot Med* 24(1): 1–14.
44. GE Healthcare (2015) *Amersham Leukotriene C4/D4/E4 Biotrak Enzymeimmunoassay (EIA) System*. Available at https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1314716762536/litdoc28953094AF_20110830172927.pdf. Accessed April 29, 2016.
45. Bankova LG, et al. (2014) Mouse mast cell proteases 4 and 5 mediate epidermal injury through disruption of tight junctions. *J Immunol* 192(6):2812–2820.