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GPR17 Regulates Immune Pulmonary Inflammation Induced by House Dust Mites

Akiko Maekawa, Wei Xing, K. Frank Austen, and Yoshihide Kanaoka

Antagonists of the type 1 cysteinyl leukotriene receptor (CysLT1R) are efficacious for bronchoconstriction in humans with bronchial asthma; however, the clinical response to these drugs is heterogeneous. In particular, how CysLT1R expression and function are constitutively regulated in vivo is not known. In this study, we show that a seven-transmembrane receptor, GPR17, negatively regulates the CysLT1R-mediated inflammatory cell accumulation in the bronchoalveolar lavage fluid and lung, the levels of IgE and specific IgG1 in serum, and Th2/Th17 cytokine expression in the lung after intranasal sensitization and challenge with the house dust mite (extract of Dermatophagoides farinae [Df]) in mice. Sensitization of naive wild-type recipients with Df-pulsed bone marrow-derived dendritic cells of each genotype or sensitization of each genotype with Df-pulsed wild-type bone marrow-derived dendritic cells and Df challenge revealed markedly increased pulmonary inflammatory and serum IgE responses for GPR17-deficient mice as compared with wild-type mice and reduced responses in the genotypes lacking CysLT1R. These findings reveal a constitutive negative regulation of CysLT1R functions by GPR17 in both the Ag presentation and downstream phases of allergic pulmonary inflammation. The Journal of Immunology, 2010, 185: 1846–1854.

The cysteinyl leukotrienes (cys-LTs), leukotriene (LT) C4, LTD4, and LTE4, are proinflammatory lipid mediators (1, 2) that are particularly implicated in human bronchial asthma for their potent bronchoconstrictive activity (3, 4) via their type 1 receptor, CysLT1R (5). CysLT1R-selective antagonists, termed “lukastas,” are the only mechanism-targeted small molecules that have been approved and widely used for the treatment of asthma (6, 7). However, their efficacy is most apparent among subsets of patients with asthma (8, 9). One might speculate that CysLT1R expression and function are tightly regulated, and thus in some patients, CysLT1R function is already turned off or obscured. Although in vitro studies have addressed ligand-initiated internalization of CysLT1R and regulation by protein kinase C (10, 11), there is little information on constitutive regulation of CysLT1R function in vivo.

Studies of mouse models with pharmacologic inhibition and targeted gene disruption of the cys-LT pathway have revealed that the cys-LTs/CysLT1R axis plays a role not only in mast cell-mediated smooth muscle contraction in the microvasculature (12–14) but also in immune inflammatory cell functions in OVA-induced allergic pulmonary inflammation (15–17). Indeed, the CysLT1R is expressed not only on smooth muscle cells of airways and microvasculature but also on bone marrow-derived cells of the innate and adaptive immune systems, such as mast cells, macrophages, eosinophils, basophils, and dendritic cells (DCs) (18–20). Thus, a potential involvement of the cys-LT/CysLT1R pathway in innate and adaptive immune systems, such as mast cells, macrophages, eosinophils, basophils, and dendritic cells (DCs) (18–20). Thus, a potential involvement of the cys-LT/CysLT1R pathway in innate and adaptive immune systems, such as mast cells, macrophages, eosinophils, basophils, and dendritic cells (DCs) (18–20).

We hypothesized that Gpr17−/− mice would exhibit increased CysLT1R-dependent responses beyond the microvasculature and reveal cellular targets of the cys-LTs in complex models such as Ag-induced pulmonary inflammation. We generated a mouse strain lacking both GPR17 and CysLT1R (Gpr17/Cysltr1−/−) to determine the extent to which any cellular signal in the Gpr17−/− mice was attributable to CysLT1R. Additionally, we turned to sensitization and challenge with house dust mites, extract of Dermatophagoides farinae (Df), to examine the role of cys-LTs and their receptors in pulmonary inflammation elicited by an allergen most relevant to human asthma and dominant in homes (26). Finally, we used not only active sensitization and challenge but also a passive approach to sensitization by administering Df-pulsed bone marrow-derived dendritic cells (BMDCs), which allowed us to assess both the allergen and efferent aspects of the CysLT1R-mediated response to challenge in the presence and absence of GPR17. In this study, we show in both the active and passive sensitization and challenge models that in the absence of GPR17, CysLT1R mediates expansion of the draining lymph node...
(LN), inflammatory cell infiltration in the bronchoalveolar lavage (BAL) fluid and the bronchovascular bundles of lung tissue, marked increases in total IgE and Df-specific IgG1, and Th2/Th17 cytokine expression in the lung. In wild-type (WT) mice, these responses are modest but are dependent on CysLT1-R. These findings reveal the role of CysLT1-R in WT C57BL/6 mice and emphasize the separate negative regulatory effects of GPR17 on CysLT1-R functions in DCs and in the downstream cellular and humoral responses of allergic pulmonary inflammation.

Materials and Methods

Mice

Cysltr1−/− mice were originally generated from embryonic stem cells from C57BL/6 mice (12) and were maintained by breeding with C57BL/6 mice; 15 generations (N15) were used. Gpr17+/+ mice (Delta- gen, San Mateo, CA) were established from 129-derived embryonic stem cells, were backcrossed onto the C57BL/6 background for seven to eight generations, and N7 and N8 generations were used (23). To generate GPR17-deficient mice (Gpr17/Cyslt1−/−), Cysltr1−/− male and Cysltr1−/− females were bred to obtain Gpr17+/−/Cysltr1−/− mice and Gpr17−/−/Cysltr1−/− females. The Gpr17+/−/Cysltr1−/− and Gpr17−/−/Cysltr1−/− females were further bred to obtain Gpr17+/−/Cysltr1−/− males and Gpr17−/−/Cysltr1−/− females. The Gpr17+/−/Cysltr1−/− males and Gpr17−/−/Cysltr1−/− females were viable and had no apparent abnormalities up to at least 12 mo of age. WT littermates from breeding for Cysltr1−/−, Gpr17+/−, and Gpr17+/−/Cysltr1−/− strains were used. Eight- to 12-wk-old male and female mice were used. All animal studies were approved by the Animal Care and Use Committee of the Dana-Farber Cancer Institute.

Active sensitization and challenge with repeated intranasal injections of Df

Mice received 1 μg Df (Greer Laboratories, Lenoir, NC) intranasally twice per week for 3 wk as described (27). Two days after the last injection, mice were killed by i.p. injection of pentobarbital.

BAL fluid cell analysis

Two days after the last intranasal injection, the trachea was cannulated and BAL fluid was obtained by three repeated lavages with 0.75 ml Ca2+- and Mg2+-free PBS with 1 mM EDTA. The BAL fluid was centrifuged at 200 × g for 5 min, Cells were resuspended in 0.2 ml PBS with 1% BSA, and the total cells were counted manually with a hemocytometer. For the differential cell counts of macrophages, neutrophils, eosinophils, and lymphocytes, the cells were cytopsins on a glass slide and stained with Diff-Quik (Dade Behring, Newark, DE), and cell types in a total of 200 cells were identified by morphologic criteria.

Histology

The lung tissues were excised, and the left lung was fixed and stained as described previously (16). For general morphology, tissue sections were stained with H&E. The extent of cellular infiltration in the bronchovascular bundles was assessed in a blinded manner. Congo red staining was used to stain with H&E. The extent of cellular infiltration in the bronchovascular bundles was assessed in a blinded manner. Congo red staining was used to identify eosinophils, and periodic acid-Schiff staining was used to assess mucus and goblet cells. To assess vascular smooth muscle hyperplasia, some tissue sections were stained with mouse anti-α-smooth muscle actin mAb (Clone 1A4; Sigma, St. Louis, MO) and with the LSAB+ system (Dako, Carpinteria, CA) according to the manufacturers’ instructions. The total smooth muscle cell numbers and the thickness of arteriolar medial muscular layer (tunica media) was quantified by ImageJ (National Institutes of Health, Bethesda, MD) on smooth muscle actin-stained sections as previously described (27). Briefly, digital images of five medium-sized arterioles with a diameter of 30–50 μm were taken from each slide, and the intima-to-medial ratio was presented as the mean number of smooth muscle cells per 100-μm basement membrane and the mean thickness of the medial arteriolar walls in five arterioles from three to five mice per group.

For immunohistochemistry to detect CysLT1-R, mouse lung tissues were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) and cut into 5-μm-thick sections. Frozen sections were fixed with 4% paraformaldehyde, washed, and incubated with polyclonal rabbit anti-CysLT1-R IgG (RB34, against the conserved sequence DEKNTMCE-FFPQNN of extracellular loop 3 of both mouse and human CysLT1-R, 30 μg/ml) (23). Immunoreactivities were visualized with a rabbit ABC peroxidase staining system (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer’s instructions. The slides were analyzed with a Leica DM LB2 microscope (Leica Microsystems, Wetzlar, Germany). The images were taken with a Nikon digital camera DXM 1200 with Nikon ACT-1 (version 2.70) image acquisition software.

Measurement of total IgE and Df-specific IgG1

Sera were collected by cardiac puncture 2 d after the last intranasal injection. Total IgE was determined with an ELISA kit (BD Biosciences, San Jose, CA). Df-specific IgG1 was measured as described (28). Briefly, 96-well plates were coated with a 5 μg/ml solution of Df and incubated with diluted serum followed by alkaline phosphatase-conjugated anti-mouse IgG1 (SouthernBiotech, Birmingham, AL) and p-nitrophenyl phosphate substrate (Sigma-Aldrich, St. Louis, MO).

Measurement of cytokine mRNA expression in the lung

Total RNA was isolated from the right lungs with TRIzol reagent (Invi- trogen, Carlsbad, CA) according to the manufacturer’s protocol. Quantities of mRNA for IL-4, IL-5, IL-13, IL-17A, and IFN-γ were measured relative to GAPDH using the Mx3000P real-time PCR system (Agilent Technologies, Santa Clara, CA) with gene-specific primers.

Flow cytometry

Macrophages were harvested by peritoneal lavage with ice-cold PBS, washed, and incubated in RPMI 1640 medium containing 10% FBS for 3 h at 37°C in a humidified atmosphere with 5% CO2. Adherent cells were detached with 4 mg/ml lidocaine and 5 mM EDTA in PBS and were incubated with polyclonal rabbit anti-CysLT1-R IgG (RB34, 5 μg/ml) and allophycocyanin-conjugated donkey anti-rabbit IgG. Non-specific rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a control. Analyses were performed on a FACS Canto flow cytometer (BD Biosciences), and data were analyzed with the FlowJo software (Tree Star, Ashland, OR).

Cytokine production by parabronchial LN cells after ex vivo restimulation with Df

Two days after the last intranasal injection, three parabronchial LNs were excised from each mouse and homogenized. The cell suspensions were filtered through a 70-μm cell strainer, centrifuged at 300 × g for 5 min at room temperature, and resuspended in RPMI 1640 medium containing heat-inactivated 10% FBS. After the total number of cells was counted for each mouse, cells were cultured at 2 × 105 cells/ml (100 μl) in the presence of 20 μg/ml Df in a 96-well plate for 72 h. The concentrations of IL-4, IL-5, IL-17A, and IFN-γ in the supernatants were measured with ELISA kits (eBioscience, San Diego, CA).

Transfer of Df-pulsed BMDCs into mice and Df challenge

Adoptive transfer of Df-pulsed BMDCs into naive mice was carried out as described (29) with modifications. Bone marrow cells were harvested from femurs and tibiae of each mouse and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml l-glutamine, 50 μM 2-ME, and recombinant mouse GM-CSF as described (30). Floating cells were harvested on day 8 and pulsed with either PBS or 100 μg/ml Df at a concentration of 1 × 105 cells/ml in a 35-mm culture dish (Sumilon Celltix G; Sumitomo Bakelite, Tokyo, Japan) for 24 h. The next day, the cells were washed twice with PBS and resuspended in PBS. Cells (1 × 105) in 50 μl were transferred intranasally to recipients that were briefly anesthetized with isoflurane. At days 0 and 14 after DC transfer, recipient mice were challenged with 1 μg Df intranasally. Two days after the last challenge, mice were killed by i.p. injection of pentobarbital. BAL fluid analysis, lung histology, and assessment of cytokine production in the LN cells were performed as described above.

Statistical analysis

Results are expressed as means ± SEM. A Student unpaired, two-tailed t test was used for the statistical analysis in cases in which the variance was homogeneous, and Welch’s test was used when the variance was hetero- geneous. A p < 0.05 was considered significant.

Results

GPR17 deficiency increases CysLT1-R-mediated Df-induced pulmonary inflammation, serum levels of total IgE and Df-specific IgG1, and Th2/Th17 cytokine expression in lungs

We examined Df-elicted allergic pulmonary inflammation in WT, Cysltr1−/−, Gpr17+/−, and Gpr17+/−/Cysltr1−/− mice to assess the
respective roles of CysLT, R and GPR17. Mice received intranasal injections of 1 µg of Df or PBS twice per week for 3 wk and were killed 48 h after the last injection (27). Df-challenged WT mice had an increased cellular infiltration in BAL fluid composed of monocytes/macrophages, neutrophils, eosinophils, and lymphocytes as compared with PBS-injected WT mice. Df-challenged Cysltr1/−/− mice showed no increase in any of these cell types in BAL fluid as compared with Df-challenged WT mice. In contrast, Df-challenged Gpr17/−/− mice had significant and marked increases in BAL fluid cell numbers of neutrophils, eosinophils, and lymphocytes as compared with Df-challenged WT mice. This exaggerated cellular influx into the BAL fluid of Gpr17/−/− mice was completely abrogated in Df-challenged Gpr17/Cysltr1/−/− mice (Fig. 1A).

Histologic analysis of the bronchovascular bundles in the lung reflected the cellular influx in the BAL fluid. There was a modest increase in cellular infiltration in WT mice that was largely absent in Cysltr1/−/− mice, and there was a marked increase in the cellular infiltration in Gpr17/−/− mice that was abolished in the Gpr17/Cysltr1/−/− mice (Fig. 1B, H&E). Congo red staining showed that the cellular infiltration in WT and especially in Gpr17/−/− mice was enriched with eosinophils as compared with the infiltration in the absence of CysLT, R (Supplemental Fig. 1A, CR). Goblet cell metaplasia with mucus hyperproduction was particularly prominent in Df-challenged Gpr17/−/− mice compared with similarly challenged WT mice as assessed by periodic acid-Schiff staining and was abolished in the Cysltr1/−/− and Gpr17/Cysltr1/−/− mice (Supplemental Fig. 1A, PAS). Unexpectedly, Df-challenged Gpr17/−/− mice had strikingly increased smooth muscle cells in the vasculature as assessed by immunohistochemical staining for α-smooth muscle actin (Fig. 1B, SMA). The numbers of α-smooth muscle actin-positive cells as well as thickness of the α-smooth muscle actin-staining layer were significantly increased in Df-challenged Gpr17/−/− mice as compared with Df-challenged WT mice (Supplemental Fig. 1B).

After sensitization and challenge with Df, Cysltr1/−/− mice had significantly less total serum IgE and Df-specific IgG1 as compared with similarly treated WT mice (Fig. 2). In contrast, Df-challenged Gpr17/−/− mice had significantly increased levels of total IgE and Df-specific IgG1 as compared with WT controls, and these increases were completely abrogated in Df-challenged Gpr17/Cysltr1/−/− mice. PBS-injected Gpr17/−/− mice also had a significantly increased total IgE level at baseline as compared with WT controls. The levels of Df-specific IgG2a in all serum samples were below the detection limit of the ELISA.

To assess the Th2/Th17/Th1 cell cytokine expression in the lungs after sensitization and challenge with Df, total RNA was isolated from the right lungs of WT, Cysltr1/−/−, Gpr17/−/−, and Gpr17/−/− mice.
Cysltr1−/− mice, and quantitative RT-PCR was performed. In WT mice, sensitization and challenge with Df significantly increased the expression of mRNAs for IL-4, IL-5, and IL-13, but not of mRNAs for IL-17A or IFN-γ, as compared with PBS-injected controls (Fig. 3). Df-challenged Gpr17−/− mice had a significant further increase in expression of IL-4, IL-5, and IL-13, along with a significant increase in IL-17A expression and a decrement in IFN-γ expression, as compared with Df-challenged WT mice; each of these changes was completely abrogated in Df-challenged Gpr17/Cysltr1−/− mice (Fig. 3). The aggregate findings for pulmonary inflammation, for levels of serum total IgE and Df-specific IgG1, and for cytokine expression in lungs with Df sensitization and challenge reveal a core role of CysLT1R in an integrated Th2 type response of WT mice that is markedly exaggerated and includes IL-17A in the absence of regulation by GPR17.

GPR17 deficiency upregulates Df-induced CysLT1R expression in inflammatory cells in lungs

As we had previously observed increased expression of CysLT1R by flow cytometry as well as increased function in in vitro bone marrow-derived macrophages subjected to GPR17 knockdown (23), we used flow cytometry to identify CysLT1R expression on...
resting peritoneal macrophages. There was significant CysLT₁R expression in the macrophages from WT mice but no expression in the cells from Cysltr¹⁻/⁻ mice (Supplemental Fig. 2A), thereby showing the specificity of the Ab. There was further increased CysLT₁R expression in the macrophages from Gpr17⁻/⁻ mice as compared with those from WT mice (Supplemental Fig. 2B).

We next performed immunohistochemical staining for CysLT₁R in the lungs from PBS-injected or Df-sensitized and challenged WT, Cysltr¹⁻/⁻, Gpr17⁻/⁻, and Gpr17/Cysltr¹⁻/⁻ mice. Many macrophages and polymorphonuclear neutrophils (PMNs), but not eosinophils or lymphocytes, in the lung tissue of Df-challenged WT mice stained positively for CysLT₁R as compared with the lack of staining in lung tissue from PBS-injected WT mice or from Df-challenged Cysltr¹⁻/⁻ or Gpr17/Cysltr¹⁻/⁻ mice. Furthermore, there was stronger positive staining for CysLT₁R in macrophages and PMNs in Df-challenged Gpr17⁻/⁻ mice (Fig. 4) as well as slight but definite staining in bronchial smooth muscle cells in lung of this genotype (data not shown). The fact that the eosinophils and lymphocytes were not stained in Df-challenged lungs of WT or Gpr17⁻/⁻ mice suggests that their presence is part of an integrated cytokine/chemokine-directed inflammatory response rather than cys-LT-dependent migration. We were not

**FIGURE 4.** GPR17 deficiency upregulates Df-induced CysLT₁R expression in inflammatory cells in lungs. Immunohistochemistry for CysLT₁R in lungs of WT, Cysltr¹⁻/⁻, Gpr17⁻/⁻, and Gpr17/Cysltr¹⁻/⁻ mice sensitized and challenged with PBS or Df. Lungs were stained with polyclonal rabbit anti-CysLT₁R IgG and developed with ABC-peroxidase reagent visualized in brown. Data are representative of results from two of the experiments depicted in Fig. 1. Magnified images of representative macrophages, PMNs, eosinophils, and lymphocytes from Df-challenged groups are shown in lower panels. Scale bar, 50 μm (original magnification ×400). Eos, eosinophils; Lym, lymphocytes; MΦ, macrophages.

**FIGURE 5.** Sensitization of WT mice with Df-pulsed GPR17-deficient BMDCs enhances pulmonary, LN, and Ig responses upon challenge. A, Inflammatory cell counts in BAL fluid. BMDCs from each genotype of mice were pulsed with Df at 100 μg/ml for 24 h, and 10⁴ cells were administered intranasally for sensitization into WT recipients. Recipients were challenged with 1 μg of Df intranasally at days 10 and 14 and were killed at day 16 for analyses. Total and differential cell counts for BAL fluid monocytes/macrophages, neutrophils, eosinophils, and lymphocytes are shown. B, Parabronchial LN cell numbers. Parabronchial LN cells were harvested and counted, and total cell numbers are shown. C, Levels of total IgE and Df-specific IgG1 in serum. Total IgE and Df-specific IgG1 in sera of Df-challenged mice were measured by ELISA. Values are the means ± SEM (n = 12–15) combined from three independent experiments. *p < 0.01. MΦ, macrophages.
able to detect CysLT₁R expression on the alveolar walls or vasculature of the bronchovascular bundles of any genotype.

**Sensitization of WT mice with Df-pulsed GPR17-deficient BMDCs enhances pulmonary, LN, and Ig responses upon challenge**

To determine whether the exaggerated inflammatory phenotype in the lungs of Gpr17⁻/⁻ mice involved the Df-presenting function and afferent aspect of the integrated response, we adoptively transferred 1 × 10⁴ Df-pulsed BMDCs from the four genotypes (WT, Cysltr₁⁻/⁻, Gpr17⁻/⁻, and Gpr17/Cysltr₁⁻/⁻) to WT recipients, challenged them with 1 µg of Df at days 10 and 14, and killed them for assessment at day 16 (29). The numbers of neutrophils and eosinophils in BAL fluid with Df challenge were increased in WT mice sensitized with Df-pulsed WT BMDCs (WT BMDCs→WT mice), whereas these inflammatory cells were absent with Df challenge of WT mice receiving PBS-treated WT BMDCs (data not shown) or Df-pulsed BMDCs from Cysltr₁⁻/⁻ mice (Cysltr₁⁻/⁻ BMDCs→WT mice) (Fig. 5A). The mere sensitization of WT mice with Df-pulsed BMDCs from Gpr17⁻/⁻ mice slightly increased the numbers of neutrophils and eosinophils in the BAL fluid (data not shown), and these numbers rose 2- to 3-fold with Df challenge in association with lymphocytes (Gpr17⁻/⁻ BMDCs→WT mice) (Fig. 5A). This augmentation of cell recruitment to BAL fluid was lost with sensitization by Df-pulsed BMDCs from Gpr17/Cysltr₁⁻/⁻ mice (Gpr17/Cysltr₁⁻/⁻ BMDCs→WT mice). These results suggest that GPR17 is a negative regulator for CysLT₁R-dependent, DC-mediated allergic pulmonary inflammation.

We harvested the lungs for histology and the LNs for cell counts and restimulation from these mice after BAL. Histologic analysis of the bronchovascular bundles revealed that the cellular infiltrates were moderate in the Gpr17⁻/⁻ BMDCs→WT mice (data not shown). The LN cells were dissociated, counted, and restimulated at a concentration of 2 × 10⁶ cells/ml with 20 µg/ml Df for 72 h, and the cytokine concentrations in the supernatant were measured with ELISAs. The total number of LN cells was decreased in Cysltr₁⁻/⁻ BMDCs→WT mice and significantly increased in Gpr17⁻/⁻ BMDCs→WT mice as compared with WT BMDCs→WT mice (Fig. 5B). The total numbers of LN cells from Gpr17/Cysltr₁⁻/⁻ BMDCs→WT mice were minimal and comparable to the numbers for Cysltr₁⁻/⁻ BMDCs→WT mice (Fig. 5B). Df restimulation of LN cells from WT mice that received PBS-treated BMDCs from any genotype of donor mice did not yield any detectable cytokines (data not shown). LN cells of WT BMDCs→WT mice responded to ex vivo restimulation with Df with increased levels of IL-17A and IFN-γ, as well as minimal levels of IL-5. IL-5 was selectively increased in ex vivo Df-restimulated LN cells from Gpr17⁻/⁻ BMDCs→WT mice (Supplemental Fig. 3). We did not detect IL-4 production. Of note was the finding that restimulation of LN cells of Cysltr₁⁻/⁻ BMDCs→WT mice or Gpr17/Cysltr₁⁻/⁻ BMDCs→WT mice failed to produce any detectable cytokines even though the cell numbers for restimulation were equivalent.

We assessed humoral Th2 immune responses in this cohort by measuring total IgE and Df-specific IgG1. As shown in Fig. 5C, Cysltr₁⁻/⁻ BMDCs→WT mice had significantly less total IgE and Df-specific IgG1 as compared with WT BMDCs→WT mice. In contrast, Gpr17⁻/⁻ BMDCs→WT mice had slightly increased levels of total IgE and Df-specific IgG1 as compared with WT BMDCs→WT mice, and these increases were significantly reduced in Gpr17/Cysltr₁⁻/⁻ BMDCs→WT mice (Fig. 5C). Thus,
the contribution of CysLT1R to the function of Df-pulsed BMDCs in mediating sensitization for effective Df challenge is apparent by the cell number of the draining LNs and the levels of humoral IgE/IgG1 and the cellular influx into the BAL fluid and lung on the efferent side.

Sensitization of GPR17-deficient mice with Df-pulsed WT BMDCs enhances pulmonary, LN, and Ig responses upon challenge

To determine whether the CysLT1R-mediated recipient effector functions are also increased in the absence of GPR17, we sensitized WT, Cysltr1−/−, Gpr17−/−, and Gpr17/Cysltr1−/− mice with Df-pulsed BMDCs from WT mice, challenged them with Df, and assessed for pulmonary and associated LN and Ig responses. As compared with WT recipients, the numbers of neutrophils and eosinophils in BAL fluid were significantly decreased in Cysltr1−/− recipients (Fig. 6A). In contrast, the numbers of neutrophils and eosinophils along with lymphocytes were significantly increased in Gpr17−/− recipients as compared with WT recipients, and this response was abolished in Gpr17/Cysltr1−/− recipients. Histologic analysis of the bronchovascular bundles revealed that the cellular infiltrates were minimal in WT recipients, absent in Cysltr1−/− and Gpr17/Cysltr1−/− mice, and significantly increased in Gpr17−/− mice (data not shown).

Moreover, as compared with WT recipients, the total number of LN cells from Cysltr1−/− recipients was significantly decreased, and that from Gpr17−/− recipients was strikingly increased. This increased LN cell number was absent in Gpr17/Cysltr1−/− recipients (Fig. 6B). The restimulated LN cells from WT recipients generated IL-5, IL-17A, and IFN-γ, and the amounts of these cytokines were 4- to 10-fold higher per cell in Gpr17−/− recipients. There was no generation of cytokines with Df restimulation of LN cells from Cysltr1−/− and Gpr17/Cysltr1−/− recipients, again on a per cell basis (Supplemental Fig. 4).

A

B

C

FIGURE 7. GPR17-deficient mice sensitized with Df-pulsed GPR17-deficient BMDCs. A, Inflammatory cell count in BAL fluid. BMDCs from Gpr17−/− and Gpr17/Cysltr1−/− mice were pulsed with Df at 100 μg/ml for 24 h, and 10⁶ cells were administered intranasally for sensitization of Gpr17−/− recipients. Recipients were challenged with 1 μg of Df intranasally at days 10 and 14 and killed at day 16 for analysis. Total and differential cell counts for BAL fluid monocytes/macrophages, neutrophils, eosinophils, and lymphocytes are shown. B, Parabronchial LN cell numbers. Parabronchial LN cells were harvested and counted, and total cell numbers are shown. C, Levels of total IgE and Df-specific IgG1 in serum. Total IgE and Df-specific IgG1 in sera of Df-challenged mice were measured by ELISA. Values are the means ± SEM (n = 10) combined from two independent experiments. *p < 0.01. Mφs, macrophages.
The concomitant increased expression of CysLT1R is evident when CysLT1R was also absent. When the GPR17-regulated, Df2 mice showed that the CysLT1R transcripts and proteins are expressed when CysLT1R was also absent. Thus, in the absence of GPR17, cys-LTs/CysLT1R might produce mat... 

Discussion

We find that the absence of GPR17 allows a constitutive increased expression of CysLT1R, R that is further markedly upregulated with an inflammatory response that follows sensitization and challenge with Df. With sensitization and challenge, the phenotype of WT mice includes local cellular infiltration of the lung and BAL fluid, a systemic further increase in total IgE with appearance of Df-specific IgG1, and increased Th2 cytokine transcripts in the lung. In the Gpr17-/-mice, there is a further significant increase in cellular infiltration of lung, in total IgE and Df-specific IgG1 in serum, and in Th2 cell cytokine transcripts, along with an increase in transcript for IL-17A and a decrement of transcript for IFN-γ. The concomitant increased expression of CysLT1R, R is evident in the infiltrating pulmonary macrophages and neutrophils in WT mice and is exaggerated in the lungs of Gpr17-/-mice. The remarkable distributions of CysLT1R effects to both the Df presentation step and the downstream cellular responses were recognized when the four genotypes of mice were sensitized by Df-pulsed BMDCs and Df challenged. When the Df-pulsed BMDCs lacked GPR17, the response of WT recipients was marked but abolished when CysLT1R, R was also absent. Similarly, when the Df-pulsed BMDCs were WT, the local and systemic responses of GPR17-deficient recipients were again marked but abolished when CysLT1R, R was also absent. When the GPR17-regulated, CysLT1R, R-mediated functions were reintegrated by sensitizing Gpr17-/-mice with Df-pulsed Gpr17-/-BMDCs and challenging them with Df, the magnitude of responses resembled that of WT BMDCs → Gpr17-/-mice. These findings reveal that the downstream regulatory functions of GPR17 are profound but under the control of the gate-opening sensitization function of CysLT1R, R on DCs.

A systemic phenotype in naive mice lacking GPR17 is observed as a significant increase in total serum IgE, reminiscent of our previous observation that total serum IgE was reduced in naive BALB/c mice lacking LTC4 synthase, the critical enzyme for cys-LT biosynthesis (16). An in vitro study has shown that stimulation of IL-4 and CD40-activated human B cells with LTC4 induces mature epsilon transcripts and increases IgE production (31). Thus, in the absence of GPR17, cys-LT/cysLT1R might promote the generation of IgE-secreting B cells in vivo.

There was slightly increased expression of CysLT1R, R in bronchial smooth muscle cells of Df-challenged Gpr17-/- mice but not WT mice. Increased expression of CysLT1R, R in bronchial smooth muscle cells had been noted with OVA-induced chronic pulmonary inflammation with remodeling in the BALB/c strain (17). Previous reports on molecular characterization of the human CysLT1R showed that the CysLT1R transcripts and proteins are expressed in human lung smooth muscle cells and lung macrophages as assessed by in situ hybridization (5) and by immunohistochemistry (18), respectively. The same group further identified the expression of CysLT1R transcripts and proteins in monocytes/macrophages, eosinophils, and a subset of neutrophils in nasal lavage fluid from patients with seasonal allergic rhinitis (19).

We were surprised to observe that Df remobilization of lung-draining LN cells from mice presenting an induced Th2-like pulmonary and systemic response revealed an unselective cytokine response that was, however, entirely CysLT, R-dependent. In a pharmacologic study in BALB/c mice sensitized by adoptive transfer of Df-pulsed BMDCs and challenged with Df, the recruitment of eosinophils and the levels of IL-5 in BAL fluid were increased by costimulation with LTβD and suppressed by the presence of a CysLT1R antagonist (29). Another pharmacologic study showed that the treatment of BALB/c mice with a CysLT1R antagonist during OVA sensitization reduced the production of IL-4, IL-5, and IFN-γ in BAL fluid after OVA challenge without affecting the levels of OVA-specific IgE and IgG in serum (32). Whereas these pharmacologic studies suggest that CysLT1R activation may augment DC function in both Th2- and Th1-biased immune responses, our findings reveal a role in Th2 and Th17 immune responses in the absence of GPR17. CysLT1R, R can be upregulated after TCR activation on mouse T cells in vitro and mediates LTD4-elicited calcium flux and migration toward LTD4 (33). An Affymetrix GeneChip analysis with various human T cell subsets has revealed that CysLT1R expression is upregulated in Th2 and effector memory T cells as compared with Th1 and central memory T cells (34). Thus, our future studies may find that Th2 cells as well as Th17 cells preferentially migrate from the regional LNs to lung after Df challenge in Gpr17-/-mice.

Another surprising finding was the appearance of smooth muscle cell hyperplasia in the pulmonary vasculature of the Gpr17-/-mice. The abrogation of this finding in the Gpr17/Cysltr1-/-mice indicates a critical mitogenic role for CysLT1R, R in vascular smooth muscle cells when GPR17 dampening is lacking. Vascular remodeling is one of the key features in allergen-induced pulmonary inflammation in mice, such as intense inhalation challenge with OVA (35) or Df (36). In vitro studies have shown that LTD4 can potentiate epidermal growth factor-driven human airway smooth muscle cell proliferation (37) and that LTD4 can promote TGF-β- or IL-13-primed human airway smooth muscle cell proliferation in a CysLT1R-dependent manner (38). Recently, Lundequist et al. reported in a similar Df active sensitization and challenge protocol that mice lacking microsomal PGE synthase-1 show an enhanced vascular smooth muscle hyperplasia (27). Thus, both the antiproliferative function of PGE2 and the GPR17 downregulation of CysLT1R, R can mitigate the action of mitogenic factors for smooth muscle cells in allergic pulmonary inflammation.

Our findings that C57BL/6 Cysltr1-/- and Gpr17/Cysltr1-/- mice were protected from pulmonary inflammation both in active Df sensitization and challenge and in Df-pulsed BMDC transfer models with challenge reveal a broad and dose-related role for CysLT1R in Ag presentation and downstream responses. These Th2-like pulmonary and systemic responses mediated by CysLT1R are profoundly downregulated by GPR17 expression. It is conceivable that the homologous human GPR17 may contribute to the heterogeneous responses to CysLT1R antagonists in patients with bronchial asthma due to variation in GPR17 regulation of the CysLT1R at particular or multiple cell sites.

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Disclosures

The authors have no financial conflicts of interest.

References


Corrections


The columns were incorrectly identified in the legend for Fig. 2. The second sentence of the legend to Fig. 2 should read as follows: “Total IgE and *Df*-specific IgG1 in serum of PBS-injected (open columns) or *Df*-challenged (filled columns) mice depicted in Fig. 1 are shown.” The results and conclusions of the article remain unchanged.

The online version of this article has been corrected and now differs from the print version as originally published.

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Fig. S1. GPR17 deficiency increases CysLT1R-mediated Df-induced pulmonary inflammation. (A) Further histologic analyses of the lung samples shown in Figure 1B stained with Congo red (CR) or periodic acid-Schiff (PAS). Scale bars = 50 μm for CR and 100 μm for PAS. Arrows in CR panels depict eosinophils in the bronchovascular bundles. (B) Quantitative analysis of vascular smooth muscle cell numbers (left) and thickness (right). The mean number of α-smooth muscle actin-positive cells per 100-μm basement membrane (BM) and the mean thickness of the medial arteriolar walls in lung sections of PBS-injected (white columns) or Df-challenged (black columns) mice are shown. Results are the mean ± SEM (n = 3-5 mice per group) from 2 of the experiments depicted in Figure 1B. *P < 0.01.
Figure S2. Flow cytometry for CysLT1R in peritoneal MΦs. Adherent cells from peritoneal lavage fluid of resting WT (solid line) and Cysltr1−/− (dotted line) mice (A) or of resting WT (thin line) and Gpr17−/− (thick line) mice (B) were incubated with polyclonal rabbit anti-CysLT1R IgG (RB34, 5 μg/ml) and allophycocyanin-conjugated donkey anti-rabbit IgG. Nonspecific rabbit IgG was used as a control (shaded histogram).
Figure S3. Cytokine production after ex vivo restimulation with Df. Parabronchial LN cells harvested from WT recipients depicted in Figure 5 were counted and restimulated with Df for 72 h. The concentrations of IL-5, IL-17A, and IFN-γ in the culture supernatants are shown as the mean ± SE (n = 12-15). *p < 0.01.
Figure S4. Cytokine production after ex vivo restimulation with Df. Parabronchial LN cells harvested from WT, Cysltr1−/−, Gpr17−/−, and Gpr17/Cysltr1−/− recipients depicted in Figure 6 were counted and restimulated with Df for 72 h. The concentrations of IL-5, IL-17A, and IFN-γ in the culture supernatants are shown as the mean ± SE (n = 5-12). *p < 0.01, #p < 0.05.
Figure S5. Effects of sensitization by adoptive transfer of Df-pulsed BMDCs from Gpr17<sup>−/−</sup> and Gpr17/Cysltr1<sup>−/−</sup> mice into Gpr17<sup>−/−</sup> recipients and subsequent challenge. (A) Histologic analyses of the lung from mice depicted in Figure 7 stained by hematoxylin and eosin. Scale bars = 100 μm. (B) Cytokine production after ex vivo restimulation with Df. Parabronchial LN cells harvested from Gpr17<sup>−/−</sup> recipients depicted in Figure 7 were counted and restimulated with Df for 72 h. The concentrations of IL-5, IL-17A, and IFN-γ in the culture supernatants are shown as the mean ± SE (n = 10). *p < 0.01.