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GPR17 Regulates Immune Pulmonary Inflammation Induced By House Dust Mite

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Abstract

Antagonists of the type 1 cysteinyl leukotriene receptor ($CysLT_1R$) are efficacious for bronchoconstriction in humans with bronchial asthma; however, the clinical response to these drugs is heterogeneous. In particular, how $CysLT_1R$ expression and function are constitutively regulated in vivo is not known. Here we show that a 7-transmembrane receptor GPR17 negatively regulates the $CysLT_1R$-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 house dust mite (*Df*) in mice. Sensitization of naïve wild-type recipients with *Df*-pulsed bone marrow-derived dendritic cells (BMDCs) of each genotype or sensitization of each genotype with *Df*-pulsed wild-type BMDCs and *Df* challenge revealed markedly increased pulmonary inflammatory and serum IgE responses for GPR17-deficient mice as compared to wild-type mice and reduced responses in the genotypes lacking $CysLT_1R$. These findings reveal a constitutive negative regulation of $CysLT_1R$ functions by GPR17 in both the antigen presentation and downstream phases of allergic pulmonary inflammation.

Keywords

Knockout Mice; Lipid Mediators; Allergy; Inflammation; Lung

INTRODUCTION

The cysteinyl leukotrienes (cys-LTs), leukotriene (LT) C_4, LTD_4 and LTE_4, are proinflammatory lipid mediators (1, 2), particularly implicated in human bronchial asthma for their potent bronchoconstrictive activity (3, 4) via their type 1 receptor, $CysLT_1R$ (5). $CysLT_1R$-selective antagonists, termed "lukasts", 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 $CysLT_1R$ expression and function are tightly regulated and thus in some patients, $CysLT_1R$ function is already turned off or obscured. Although in vitro studies

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have addressed ligand-initiated internalization of $CysLT_1R$ and regulation by protein kinase C (10, 11), there is little information on constitutive regulation of $CysLT_1R$ function in vivo.

Studies of mouse models with pharmacologic inhibition and targeted gene disruption of the cys-LT pathway revealed that the cys-LTs/$CysLT_1R$ axis plays a role not only in mast cell-mediated smooth muscle constriction in the microvasculature (12-14) but also in immune inflammatory cell functions in OVA-induced allergic pulmonary inflammation (15-17). Indeed, the $CysLT_1R$ 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/$CysLT_1R$ pathway in the infiltrating cells of the allergic inflammatory response has been suggested, but how the cys-LT/$CysLT_1R$ pathway is regulated at the antigen-presenting phase and/or at the downstream responses remains to be elucidated.

Human, rat, and mouse GPR17, which are modestly homologous to $CysLT_1R$, were identified as dual receptors for uracil nucleotides and the cys-LTs, LTC_4 and LTD_4, based on [^{35}S]GTPγS binding assays and single-cell calcium imaging with transfectants (21, 22). Neither we (23) nor others (24, 25) found binding or agonist function for the cys-LTs in GPR17-transfected cell lines. In contrast, we observed that mouse GPR17 is a negative regulator of $CysLT_1R$. Cotransfection of GPR17 with $CysLT_1R$ in several cell lines suppressed $CysLT_1R$-mediated calcium flux in response to LTD_4, and knockdown of GPR17 in mouse bone marrow-derived macrophages markedly increased the calcium flux in response to LTD_4 in a dose-dependent manner (23).

We hypothesized that *Gpr17*$^{-/-}$ mice would exhibit increased $CysLT_1R$-dependent responses beyond the microvasculature and reveal cellular targets of the cys-LTs in complex models such as antigen-induced pulmonary inflammation. We generated a mouse strain lacking both GPR17 and $CysLT_1R$ (*Gpr17*/*Cysltr1*$^{-/-}$) to determine the extent to which any cellular signal in the *Gpr17*$^{-/-}$ mice was attributable to $CysLT_1R$. In addition, we turned to sensitization and challenge with house dust mite, *Dermatophagoides farinae*, extract (*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) that allowed us to assess both the afferent and efferent aspects of the $CysLT_1R$-mediated response to challenge in the presence and absence of GPR17. Here we show in both the active and passive sensitization and challenge models that in the absence of GPR17, $CysLT_1R$ 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 WT mice, these responses are modest but again dependent on $CysLT_1R$. These findings reveal the role of $CysLT_1R$ in WT C57BL/6 mice and emphasize the separate negative regulatory effects of GPR17 on $CysLT_1R$ functions in DCs and in the downstream cellular and humoral responses of allergic pulmonary inflammation.

MATERALS AND METHODS

Mice

Cysltr1$^{-/-}$ mice were originally generated from embryonic stem cells from C57BL/6 mice (12), were maintained by breeding with C57BL/6 mice, and N15 generations were used. *Gpr17*$^{-/-}$ mice (Deltagen, San Mateo, CA) were established from 129-derived embryonic stem cells, were backcrossed onto the C57BL/6 background for 7-8 generations, and N7 and N8 generations were used (23). To generate GPR17/CysLT$_1$R double-deficient (*Gpr17/Cysltr1*$^{-/-}$) mice, *Gpr17*$^{-/-}$ males (N7) and *Cysltr1*$^{-/-}$ females were bred to obtain *Gpr17*$^{+/-}$*Cysltr1*$^{-}$ males and *Gpr17*$^{+/-}$ *Cysltr1*$^{+/-}$ females. The *Gpr17*$^{+/-}$*Cysltr1*$^{-}$ males and *Gpr17*$^{+/-}$*Cysltr1*$^{+/-}$ females were further bred to obtain *Gpr17*$^{-/-}$*Cysltr1*$^{-}$ males and *Gpr17*$^{-/-}$*Cysltr1*$^{-/-}$ females. The *Gpr17/Cysltr1*$^{-/-}$ mice were viable and had no apparent abnormalities up to at least 12 months of age. WT littermates from breeding for *Cysltr1*$^{-/-}$, *Gpr17*$^{-/-}$, and *Gpr17/Cysltr1*$^{-/-}$ strains were used. 8- 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 of *Df* (Greer Laboratories, Lenoir, NC) intranasally twice per wk for 3 wks as described (27). 2 d after the last injection, mice were killed by i.p. injection of pentobarbital.

BAL Fluid Cell Analysis

2 d after the last intranasal injection, the trachea was cannulated and BAL fluid was obtained by three repeated lavages with 0.75 ml of Ca^{2+}- and Mg^{2+}-free PBS with 1 mM EDTA. The BAL fluid was centrifuged at 500 x *g* for 5 min. Cells were resuspended in 0.2 ml of 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 cytospun onto a glass slide and stained with Diff-Quik, 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 hematoxylin and eosin (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-smooth muscle actin mAb (clone 1A4, Covance, Princeton, NJ) and with LSAB+ System-alkaline phosphatase (Dako, Carpinteria, CA) according to the manufacturer's instructions. The total smooth muscle cell numbers and the thickness of arteriolar medial muscular layer (tunica media) were quantified by Image J (National Institutes of Health image analysis software) on smooth muscle actin-stained sections as previously described (27). Briefly, digital pictures of 5 medium-sized arterioles with a diameter of 30- to 50-μm were taken from each slide, and the

readouts are presented as the mean number of smooth muscle cells per 100-μm basement membrane and the mean thickness of the medial arteriolar walls in 5 arterioles from 3-5 mice per group.

For immunohistochemistry to detect $CysLT_1R$, mouse lung tissues were embedded in Tissue-Tek® OCT compound and cut into 5-μm-thick sections. Frozen sections were fixed with 4% paraformaldehyde, washed, and incubated with polyclonal rabbit anti-$CysLT_1R$ IgG (RB34, against the conserved sequence DEKNNTKCFEPPQNN of extracellular loop 3 of both mouse and human $CysLT_1R$, 30 μg/ml) (23). Immunoreactivities were visualized with the 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, Germany). The pictures were taken by 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 (Invitrogen, 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 medium containing 10% FBS for 3 h at 37°C in a humidified atmosphere with 5% CO_2. Adherent cells were detached with 4 mg/ml lidocaine and 5 mM EDTA in PBS and were incubated with polyclonal rabbit anti-$CysLT_1R$ IgG (RB34, 5 μg/ml) and allophycocyanin-conjugated donkey anti-rabbit IgG. Nonspecific rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a control. Analyses were performed on a FACSCanto 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*

2 d 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 x *g* for 5 min at room temperature, and resuspended in RPMI1640 medium containing heat-inactivated 10% FBS. After the total number of cells was counted for each mouse, cells were cultured at 2×10^6 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 (eBiosciences, San Diego, CA).

Transfer of *Df*-pulsed BMDCs into Mice and Df Challenge

Adoptive transfer of *Df*-pulsed BMDCs into naïve 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 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM 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 of *Df* at a concentration of 1×10^6 cells/ml in a 35-mm culture dish (Sumilon Celltight X, Sumitomo Bakelite, Japan) for 24 h. The next day, the cells were washed twice with PBS and resuspended in PBS. 1×10^4 cells in 50 μl were transferred intranasally to recipients that were briefly anesthetized with isoflurane. At days 10 and 14 after DC transfer, recipient mice were challenged with 1 μg *Df* intranasally. 2 d 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 were expressed as means ± SEM. Student's 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 heterogeneous. A value of $p < 0.05$ was considered significant.

RESULTS

GPR17 Deficiency Increases CysLT$_1$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*-elicited allergic pulmonary inflammation in WT, *Cysltr1*$^{-/-}$, *Gpr17*$^{-/-}$, and *Gpr17/Cysltr1*$^{-/-}$ mice to assess the respective roles of CysLT$_1$R and GPR17. Mice received intranasal injections of 1 μg *Df* or PBS twice per wk for 3 wks 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 to PBS-injected WT mice. *Df*-challenged *Cysltr1*$^{-/-}$ mice showed no increase in any of these cell types in BAL fluid as compared to *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 to *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. 1*A*).

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. 1*B*, H&E). Congo

red staining showed that the cellular infiltration in WT and especially in *Gpr17*$^{-/-}$ mice was enriched with eosinophils as compared to the infiltration in the absence of $CysLT_1R$ (Supplemental Fig. 1*A*, CR). Goblet cell metaplasia with mucus hyperproduction was particularly prominent in *Df*-challenged *Gpr17*$^{-/-}$ mice compared to similarly challenged WT mice as assessed by periodic acid-Schiff staining and was abolished in the *Cysltr1*$^{-/-}$ and *Gpr17/Cysltr1*$^{-/-}$ mice (Supplemental Fig. 1*A*, 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. 1*B*, 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 to *Df*-challenged WT mice (Supplemental Fig. 1*B*).

After sensitization and challenge with *Df*, *Cysltr1*$^{-/-}$ mice had significantly less total serum IgE and *Df*-specific IgG1 as compared to 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 to 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 to 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/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 to PBS-injected controls (Fig. 3). *Df*-challenged *Cysltr1*$^{-/-}$ mice had no increase in mRNA expression of IL-4, IL-5, IL-13, IL-17A, and IFN-γ as compared to PBS-injected controls. *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 to *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 $CysLT_1R$ 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 $CysLT_1R$ Expression in Inflammatory Cells in Lungs

As we had previously observed increased expression of $CysLT_1R$ 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 $CysLT_1R$ expression on resting peritoneal macrophages. There was significant $CysLT_1R$ expression in the macrophages from WT mice but no expression in the cells from *Cysltr1*$^{-/-}$ mice (Supplemental Fig. 2*A*), thereby showing the specificity of the antibody. There was further increased $CysLT_1R$

expression in the macrophages from *Gpr17*$^{-/-}$ mice as compared to those from WT mice (Supplemental Fig. 2*B*).

We next performed immunohistochemical staining for $CysLT_1R$ in the lungs from PBS-injected or *Df*-sensitized and challenged WT, *Cysltr1*$^{-/-}$, *Gpr17*$^{-/-}$, and *Gpr17/Cysltr1*$^{-/-}$ mice. Many macrophages and PMNs, but not eosinophils or lymphocytes, in the lung tissue of *Df*-challenged WT mice stained positively for $CysLT_1R$ as compared to 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 $CysLT_1R$ 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 able to detect $CysLT_1R$ 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 1×10^4 *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. 5*A*). 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. 5*A*). 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 $CysLT_1R$-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 2×10^6 cells/ml with 20 μg/ml of *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 to WT BMDCs→WT mice (Fig. 5*B*). 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. 5*B*). *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, and minimal 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 for humoral Th2 immune responses in this cohort by measuring total IgE and *Df*-specific IgG1. As shown in Figure 5C, *Cysltr1*$^{-/-}$ BMDCs→WT mice had significantly less total IgE and *Df*-specific IgG1 as compared to WT BMDCs→WT mice. In contrast, *Gpr17*$^{-}$/− BMDCs→WT mice had slightly increased levels of total IgE and *Df*-specific IgG1 as compared to WT BMDCs→WT mice, and these increases were significantly reduced in *Gpr17/Cysltr1*$^{-/-}$ BMDCs→WT mice (Fig. 5*C*). Thus, the contribution of $CysLT_1R$ 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 $CysLT_1R$-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 to WT recipients, the numbers of neutrophils and eosinophils in BAL fluid were significantly decreased in *Cysltr1*$^{-/-}$ recipients (Fig. 6*A*). In contrast, the numbers of neutrophils and eosinophils along with lymphocytes were significantly increased in *Gpr17*$^{-/-}$ recipients as compared to 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 to 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. 6*B*). 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).

As shown in Figure 6C, *Cysltr1*$^{-/-}$ recipients had significantly less total IgE and *Df*-specific IgG1 as compared to WT recipients. In contrast, *Gpr17*$^{-/-}$ recipients had about 2-fold and 1.5-fold increases in total IgE and *Df*-specific IgG1, respectively, as compared to WT recipients, and these increases were significantly reduced in *Gpr17/Cysltr1*$^{-/-}$ recipients

(Fig. 6*C*). Thus, these results reveal additional GPR17-regulated $CysLT_1R$ functions that are downstream of the DC antigen presentation phase but yet also influence the inflammatory response in lungs, the draining LNs, and Th2-regulated Igs.

GPR17-deficient Mice Sensitized with *Df*-pulsed GPR17-deficient BMDCs

Finally, we adoptively transferred *Df*-pulsed *Gpr17*$^{-/-}$ or *Gpr17/Cysltr1*$^{-/-}$ BMDCs into *Gpr17*$^{-/-}$ recipients to see if DC $CysLT_1R$ functions can be apparent in *Gpr17*$^{-/-}$ recipients. The BAL fluid in *Gpr17*$^{-/-}$ BMDCs→*Gpr17*$^{-/-}$ mice was enriched for eosinophils, neutrophils, and lymphocytes, and this augmented response of *Gpr17*$^{-/-}$ recipients was abrogated in *Gpr17/Cysltr1*$^{-/-}$ BMDCs→*Gpr17*$^{-/-}$ mice (Fig. 7*A*). Histologic analysis revealed that the cellular infiltrates were markedly increased in *Gpr17*$^{-/-}$ BMDCs→*Gpr17*$^{-/-}$ mice, and the increase was reduced in *Gpr17/Cysltr1*$^{-/-}$ BMDCs→*Gpr17*$^{-/-}$ mice (Supplemental Fig. 5*A*). The increased total LN cell number of *Gpr17*$^{-/-}$ BMDCs→*Gpr17*$^{-/-}$ mice was significantly reduced in *Gpr17/Cysltr1*$^{-/-}$ BMDCs→*Gpr17*$^{-/-}$ mice (Fig. 7*B*). The levels of IL-5, IL-17A, and IFN-γ with restimulation of LN cells (Supplemental Fig. 5*B*) and of total IgE and *Df*-specific IgG1 in serum in *Gpr17*$^{-/-}$ BMDCs→*Gpr17*$^{-/-}$ mice was also reduced in *Gpr17/Cysltr1*$^{-/-}$ BMDCs→*Gpr17*$^{-/-}$ mice (Fig. 7*C*). On inspection, the magnitude of the cellular infiltrate in the BAL fluid, the cell numbers in the draining LNs, and the levels of serum IgE/IgG1 were comparable to the findings for *Df*- pulsed WT BMDCs adoptively transferred into *Gpr17*$^{-/-}$ recipients (Fig. 6) and greater than those for *Gpr17*$^{-/-}$ BMDCs adoptively transferred into WT recipients (Fig. 5).

DISCUSSION

We find that the absence of GPR17 allows a constitutive increased expression of $CysLT_1R$ 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 both 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 $CysLT_1R$ is evident in the infiltrating pulmonary macrophages and neutrophils in WT mice and exaggerated in the lungs of *Gpr17*$^{-/-}$ mice. The remarkable distribution of $CysLT_1R$ effects to both the *Df* presentation step and the downstream cellular responses were recognized when the 4 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 if $CysLT_1R$ 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 if $CysLT_1R$ was also absent. When the GPR17-regulated, $CysLT_1R$-mediated functions were re-integrated 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 $CysLT_1R$ on DCs.

A systemic phenotype in naïve 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 naïve BALB/c mice lacking LTC_4 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 LTD_4 induces mature epsilon transcripts and increases IgE production (31). Thus, in the absence of GPR17, cys-LTs/$CysLT_1R$ might promote the generation of IgE-secreting B cells in vivo.

There was slightly increased expression of $CysLT_1R$ in bronchial smooth muscle cells of *Df*-challenged *Gpr17*$^{-/-}$ mice but not WT mice. Increased expression of $CysLT_1R$ 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 $CysLT_1R$ showed that the $CysLT_1R$ 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 $CysLT_1R$ 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* restimulation 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 $CysLT_1R$-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 co-stimulation with LTD_4 and suppressed by the presence of a $CysLT_1R$ antagonist (29). Another pharmacologic study showed that the treatment of BALB/c mice with a $CysLT_1R$ 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 $CysLT_1R$ 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. $CysLT_1R$ can be upregulated after TCR activation on mouse T cells in vitro and mediates LTD_4-elicited calcium flux and migration towards LTD_4 (33). An Affymetrix GeneChip analysis with various human T cell subsets has revealed that $CysLT_1R$ expression is upregulated in Th2 and effector memory T cells as compared to 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 $CysLT_1R$ 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 LTD_4 can potentiate epidermal growth factor-driven human airway smooth muscle cell proliferation (37) and that LTD_4 can promote TGF-β- or IL-13-primed human airway smooth muscle cell proliferation in a $CysLT_1R$-dependent manner (38). Recently, Lundequist et al. reported in a similar *Df* active sensitization and challenge protocol that mice lacking microsomal prostaglandin E synthase-1 show an enhanced vascular smooth muscle hyperplasia (27). Thus, both the anti-proliferative function of prostaglandin E_2 and the GPR17 downregulation of $CysLT_1R$ 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 $CysLT_1R$ in antigen presentation and downstream responses. These Th2-like pulmonary and systemic responses mediated by $CysLT_1R$ are profoundly downregulated by GPR17 expression. It is conceivable that the homologous human GPR17 may contribute to the heterogeneous responses to $CysLT_1R$ antagonists in patients with bronchial asthma due to variation in GPR17 regulation of the $CysLT_1R$ at particular or multiple cell sites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

BAL	bronchoalveolar lavage
BMDC	bone marrow-derived dendritic cell
cys-LT	cysteinyl leukotriene
$CysLT_1R$	type 1 receptor for cys-LTs
Df	extract of *Dermatophagoides farinae*
DC	dendritic cell
LN	lymph node
LT	leukotriene
WT	wild-type

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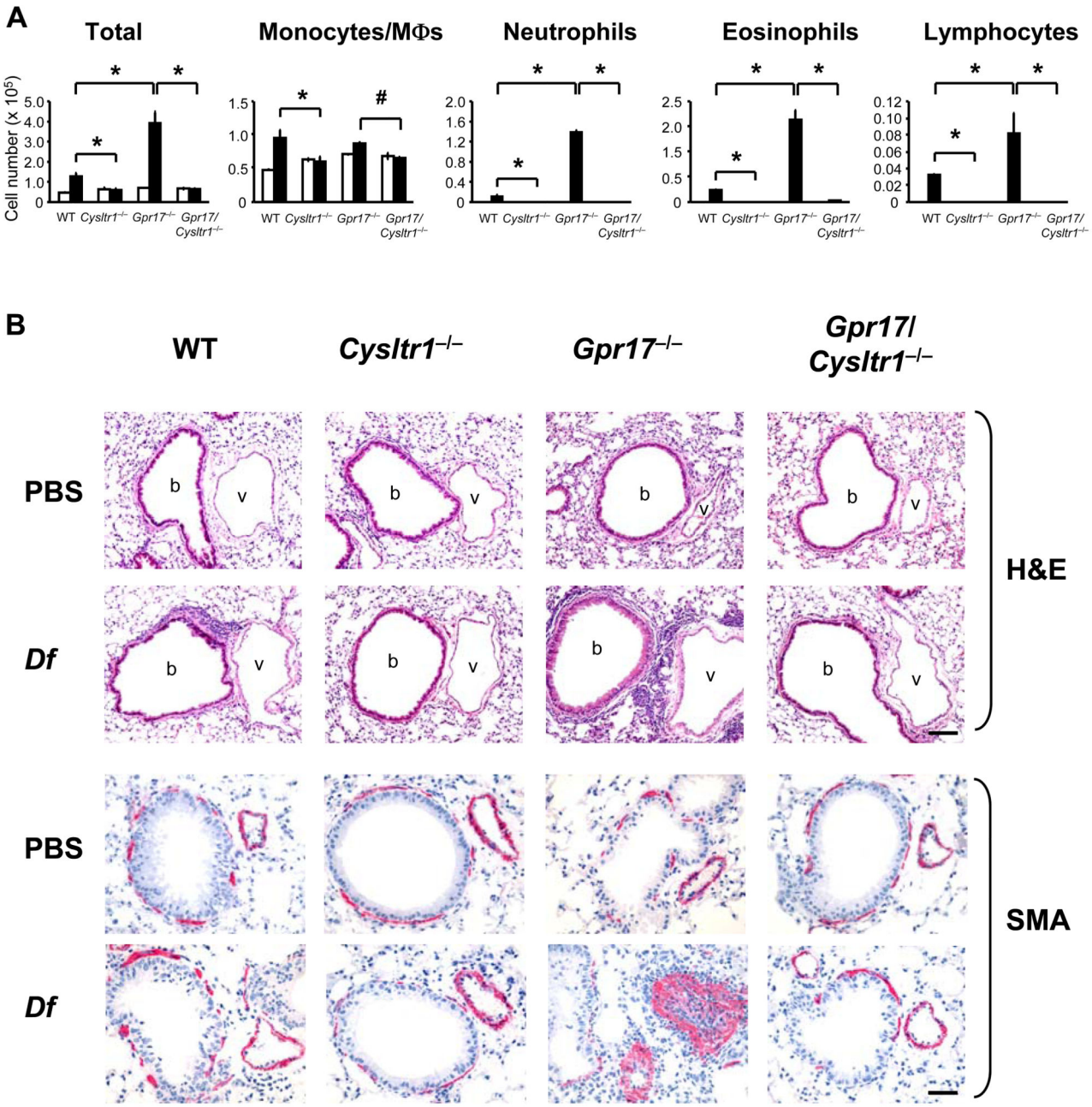

FIGURE 1. GPR17 Deficiency Increases CysLT$_1$R-mediated *Df*-induced Pulmonary Inflammation

(A) Inflammatory cell counts in BAL fluid. For active sensitization and challenge, mice received 1 μg of *Df* (black columns) or PBS (white columns) by intranasal injection twice per wk for 3 wks, and BAL was performed 2 d after the last injection. Total and differential cell counts for monocytes/macrophages (MΦs), neutrophils, eosinophils, and lymphocytes are shown. Values are the mean ± SEM (n = 8-10) combined from 3 independent experiments. $^{*}P < 0.01$, $^{\#}P < 0.05$. (B) Histologic analyses of the lung. After BAL, lung tissues were fixed with paraformaldehyde and stained with H&E. b, bronchi, v, vessels.

Immunohistochemistry was performed with mouse mAb to α-smooth muscle actin (SMA) and ABC-alkaline phosphatase reagent, visualized in red. Scale bars = 100 μm for HE, 50 μm for SMA.

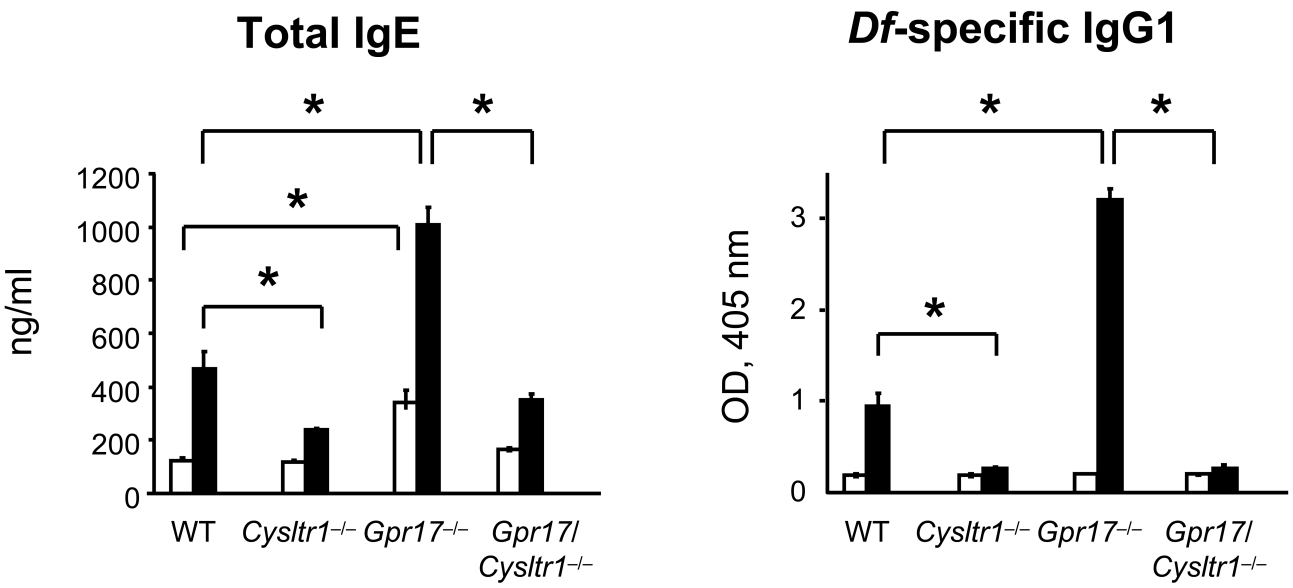

FIGURE 2. GPR17 Deficiency Increases Serum Levels of Total IgE and *Df*-specific IgG1

Total IgE and *Df*-specific IgG1 in serum of PBS-injected (white columns) or *Df*-challenged (black columns) mice depicted in Figure 1 are shown. Values are the mean ± SEM (n = 8-10) combined from 3 independent experiments. *$P < 0.01$.

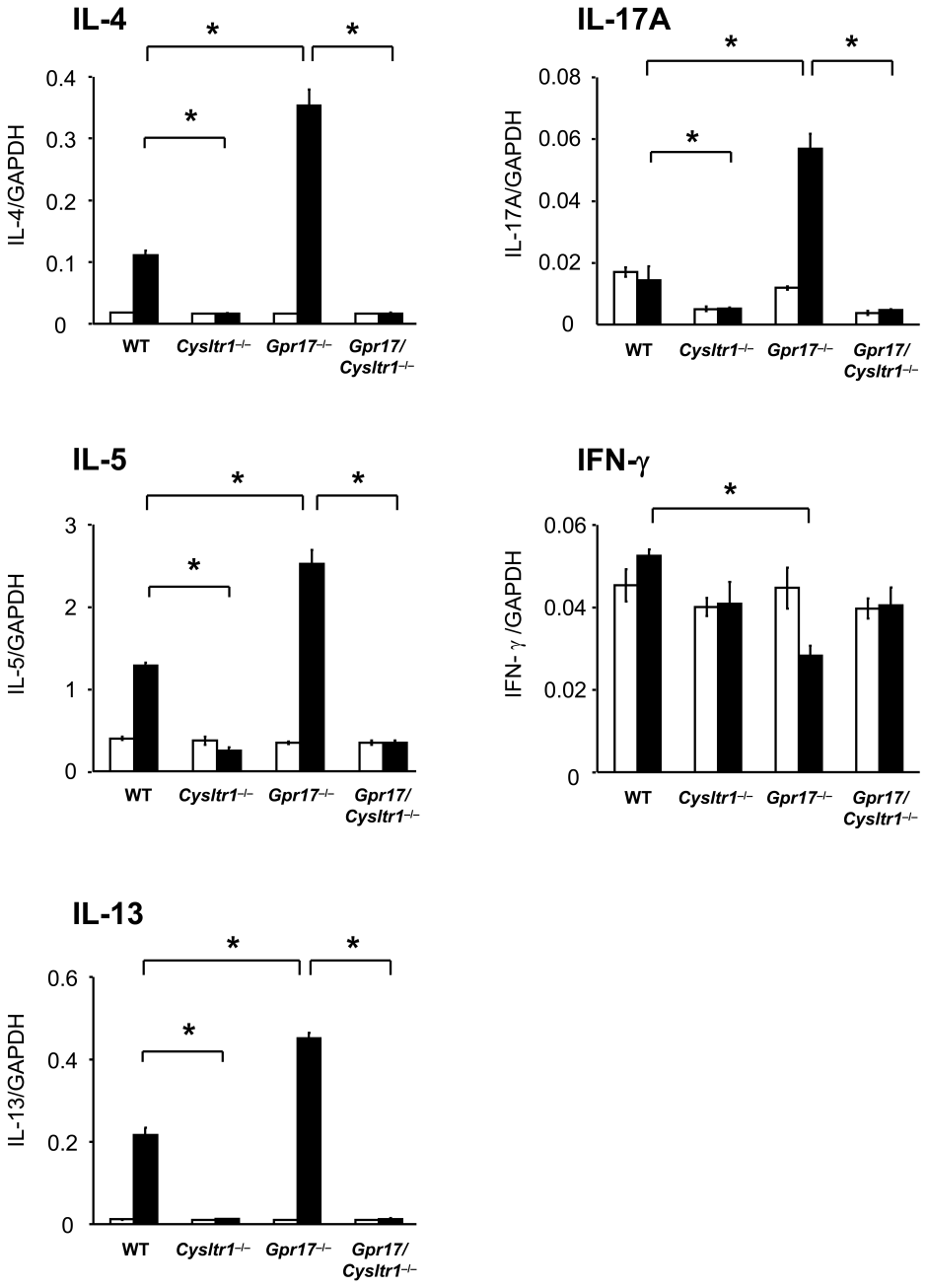

FIGURE 3. GPR17 Deficiency Increases Th2/Th17 Cytokine Generation in the Lung

Relative expression of mRNA for IL-4, IL-5, IL-13, IL-17A, and IFN-γ to GAPDH in lungs of PBS-injected (white columns) or *Df*-challenged (black columns) mice from 2 of the experiments depicted in Figure 1 was assessed by quantitative RT-PCR. Values are the mean ± SEM (n = 5-7). *P < 0.01.

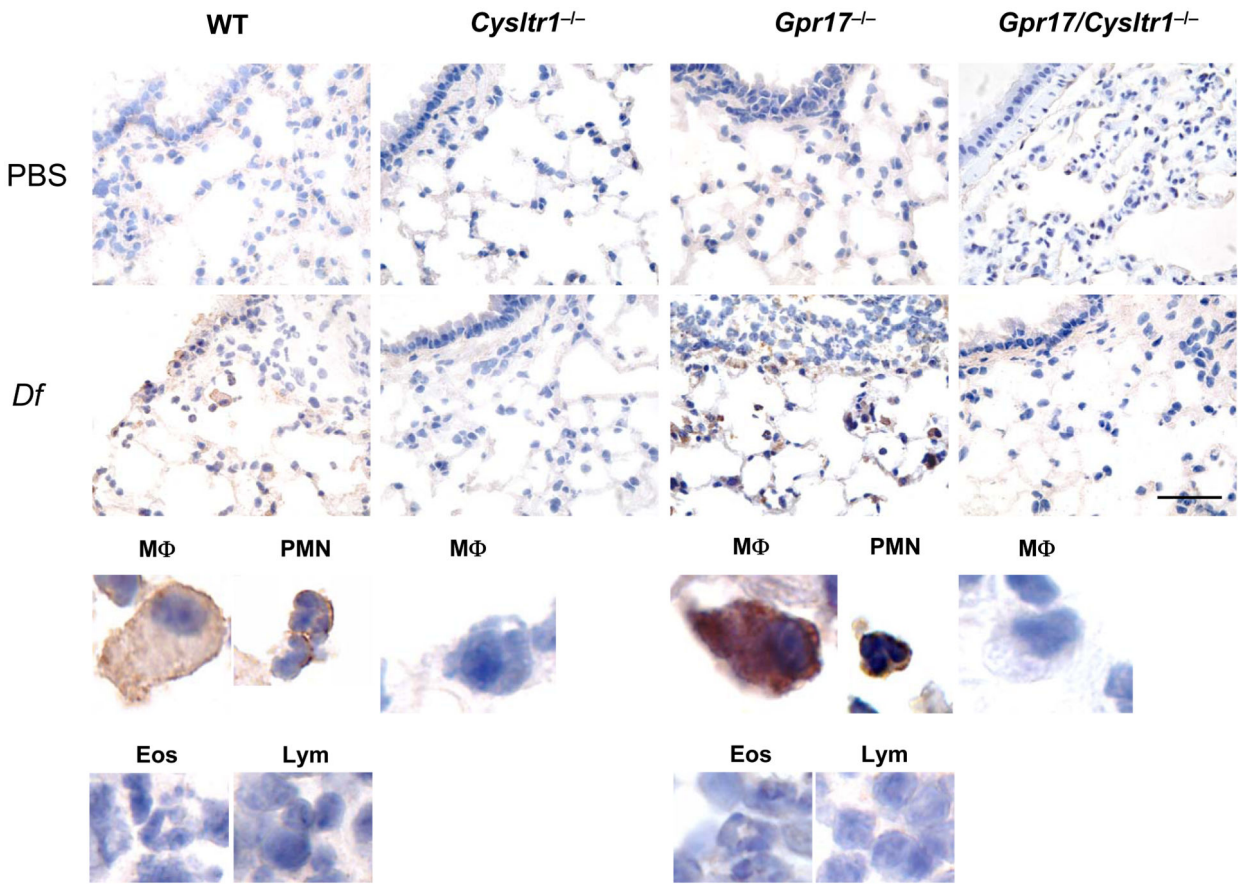

FIGURE 4. GPR17 Deficiency Upregulates *Df*-induced CysLT$_1$R Expression in Inflammatory Cells in Lungs

Immunohistochemistry for CysLT$_1$R in lungs of WT, *Cysltr1*$^{-/-}$, *Gpr17*$^{-/-}$, and *Gpr17/Cysltr1*$^{-/-}$ mice sensitized and challenged with PBS or *Df*. Lungs were stained with polyclonal rabbit anti-CysLT$_1$R IgG and developed with ABC-peroxidase reagent visualized in brown. Scale bar = 50 μm. Data are representative of results from 2 of the experiments depicted in Figure 1. Magnified pictures of representative macrophages (MΦ), PMNs, eosinophils (Eos), and lymphocytes (Lym) from *Df*-challenged groups are shown in lower panels.

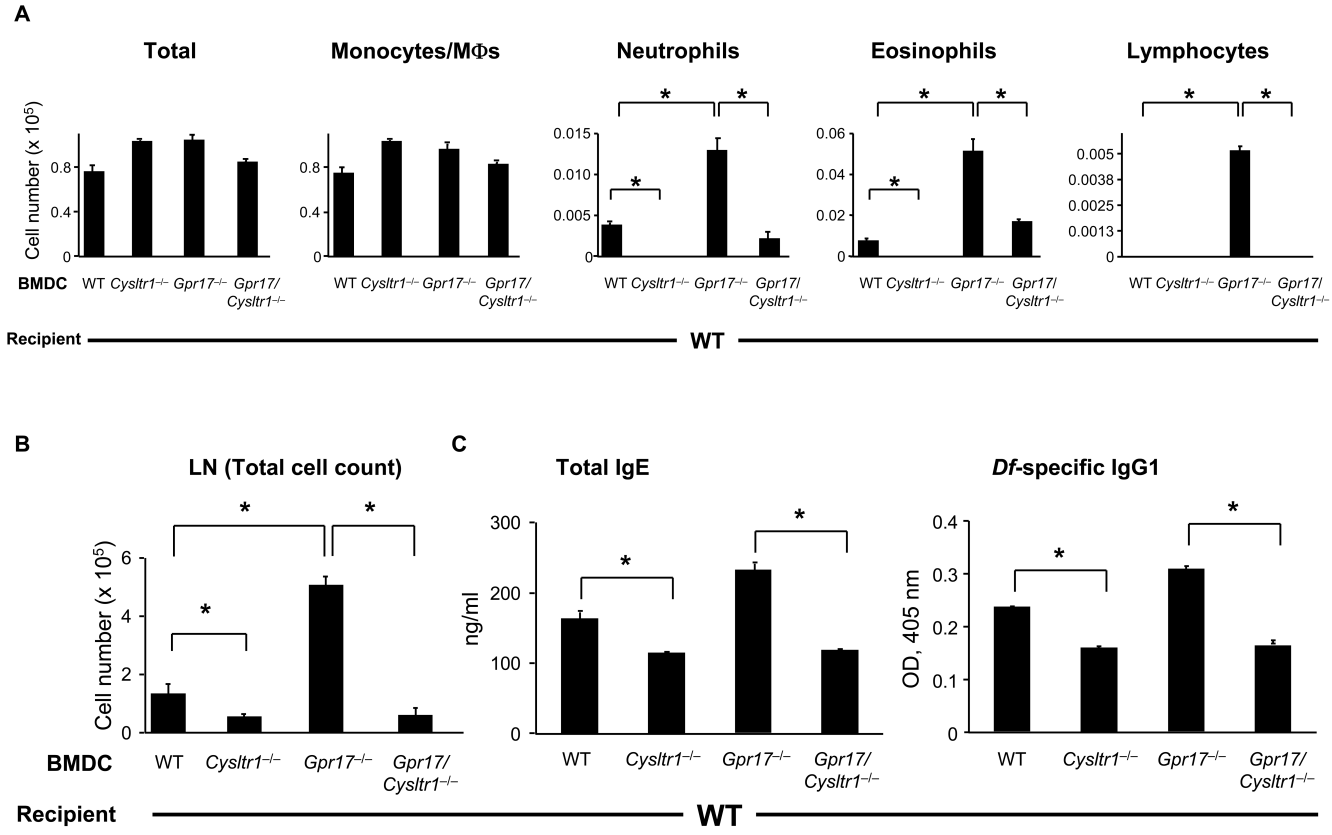

FIGURE 5. Sensitization of WT Mice with *Df*-pulsed GPR17-deficient BMDCs Enhances Pulmonary, LN, and Ig Responses upon Challenge

(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 10^4 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 (MΦs), 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 mean ± SEM (n = 12-15) combined from 3 independent experiments. *$P < 0.01$.

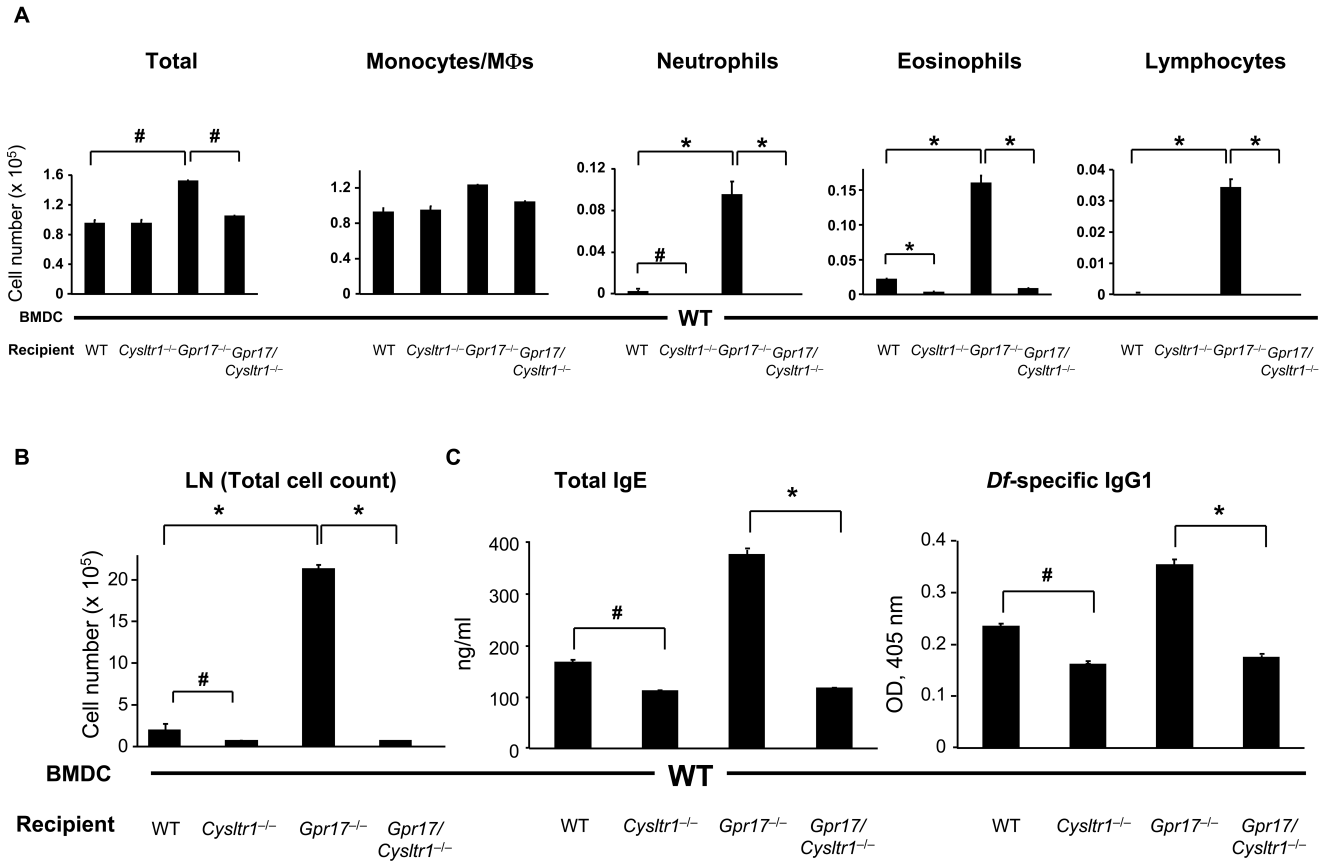

FIGURE 6. Sensitization of GPR17-deficient Mice with *Df*-pulsed WT BMDCs Enhances Pulmonary, LN, and Ig Responses upon Challenge

(A) Inflammatory cell counts in BAL fluid. BMDCs from WT mice were pulsed with *Df* at 100 μg/ml for 24 h, and 10^4 cells were administered intranasally for sensitization into recipients of 4 genotypes. 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 (MΦs), 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 as the mean ± SEM (n = 5-12) combined from 3 independent experiments. $^*P < 0.01$, $^{\#}P < 0.05$.

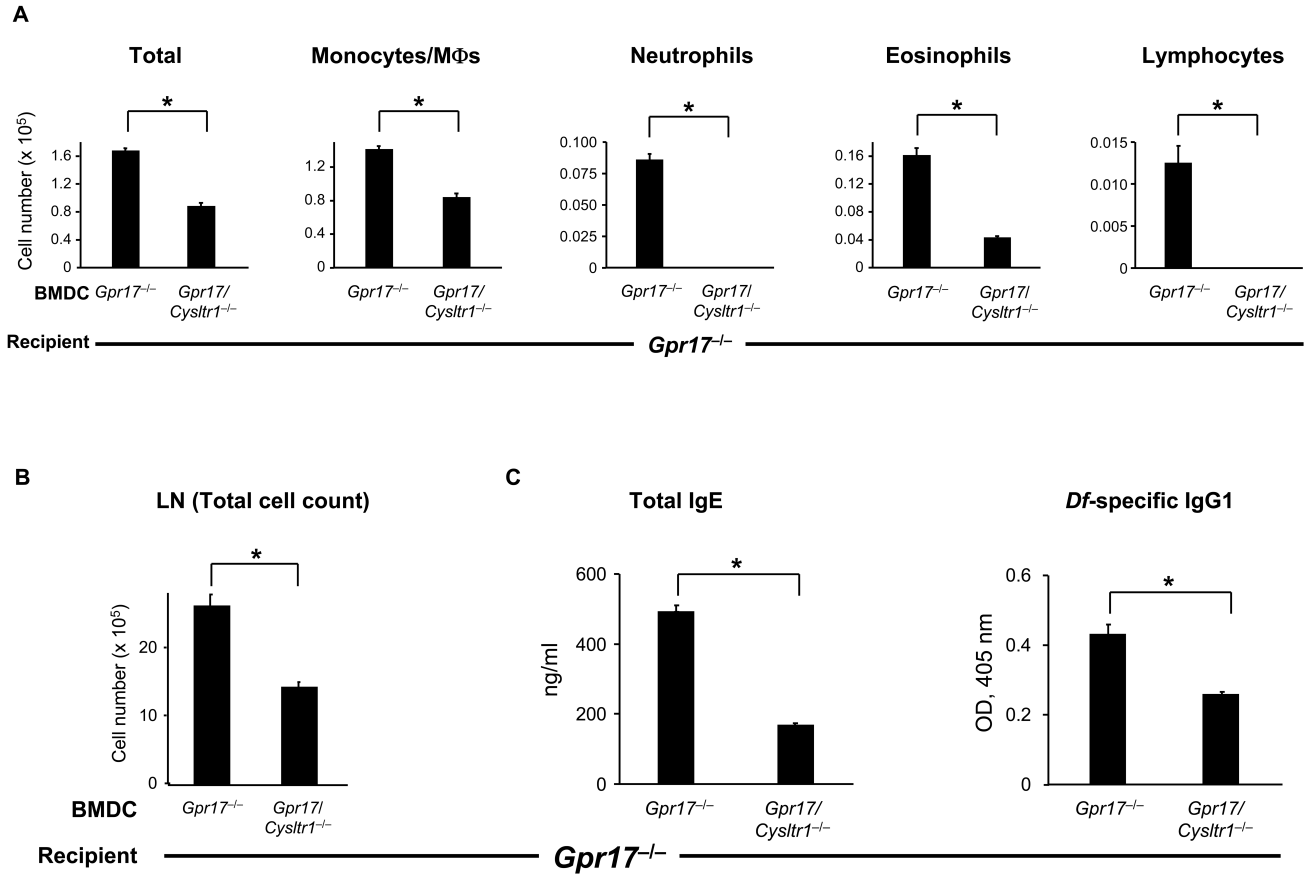

FIGURE 7. GPR17-deficient Mice Sensitized with *Df*-pulsed GPR17-deficient BMDCs

(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 10^4 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 (MΦs), 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 mean ± SEM (n = 10) combined from 2 independent experiments. *$P < 0.01$.