Prostaglandin E_2 deficiency causes a phenotype of aspirin sensitivity that depends on platelets and cysteinyl leukotrienes

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Aspirin-exacerbated respiratory disease (AERD) is characterized by asthma, tissue eosinophilia, overproduction of cysteinyl leukotrienes (cysLTs), and respiratory reactions to nonselective cyclooxygenase (COX) inhibitors. Ex vivo studies suggest that functional abnormalities of the COX-2/microsomal prostaglandin (PG)E_2 synthase-1 system may underlie AERD. We demonstrate that microsomal PGE_2 synthase-1 null mice develop a remarkably AERD-like phenotype in a model of eosinophilic pulmonary inflammation. Lysine aspirin (Lys-ASA)–challenged PGE_2 synthase-1 null mice exhibit sustained increases in airway resistance, along with lung mast cell (MC) activation and cysLT overproduction. A stable PGE_2 analog and a selective E prostanoid $(EP)_2$ receptor agonist blocked the responses to Lys-ASA by ∼90%; EP_3 and EP_4 agonists were also active. The increases in airway resistance and MC products were blocked by antagonists of the type 1 cysLT receptor or 5-lipoxygenase, implying that bronchoconstriction and MC activation were both cysLT dependent. Lys-ASA–induced cysLT generation and MC activation depended on platelet-adherent granulocytes and T-prostanoid (TP) receptors. Thus, lesions that impair the inducible generation of PGE_2 remove control of platelet/granulocyte interactions and TP-receptor–dependent cysLT production, permitting MC activation in response to COX-1 inhibition. The findings suggest applications of antiplatelet drugs or TP receptor antagonists for the treatment of AERD.

Aspirin-exacerbated respiratory disease (AERD) affects 5–10% of all adults with asthma (1–3), ∼30% with severe asthma (4), and ∼40% with refractory chronic hyperplastic sinusitis (5). It involves severe eosinophilic respiratory tract inflammation and is defined by bronchoconstriction following the ingestion of nonselective COX inhibitors (6). Cysteinyl leukotrienes (cysLTs) (LTC_4, LTD_4, and LTE_4) drive these reactions, as well as some of the chronic features of AERD (7, 8). CysLTs derive from arachidonic acid metabolized by 5-lipoxygenase (5-LO) to LTA_4, conjugated to reduced glutathione by leukotriene C_4 synthase (LTC_4S) to LTC_4 in mast cells (MCs), eosinophils, basophils, macrophages, and granulocyte–platelet complexes (9). After export, LTC_4 is converted sequentially to LTD_4 and LTE_4. CysLTs induce bronchoconstriction (10, 11), tissue eosinophilia (12), and remodeling (13) through G-protein–coupled receptors (GPCRs) expressed by structural and hematopoietic cells (14–16). Individuals with AERD display higher urinary levels of LTE_4 than do aspirin-tolerant asthmatic (ATA) control subjects (17). Reactions to aspirin or other nonselective COX inhibitors are accompanied by marked further increases in urinary levels of LTE_4 and can be blocked by pretreatment with the 5-LO inhibitor zileuton or with antagonists of the type 1 receptor for cysLTs ($CysLT_1R$) (18, 19). The dependency on COX products to maintain homeostasis over 5-LO activity is a unique feature of AERD. Remarkably, subjects with AERD can tolerate selective antagonists of COX-2 (20), suggesting that the homeostatic prostaglandins derive principally from COX-1.

Prostaglandin E_2 (PGE_2) forms from COX-dependent conversion of arachidonic acid to PGH_2, which is metabolized to PGE_2 by three PGE_2 synthases (PGESs), termed "cytosolic PGES" (21) and "microsomal PGES" (mPGES)-1 (22) and -2 (23), respectively. mPGES-1 expression is up-regulated simultaneously with COX-2 (24, 25), permitting increased PGE_2 generation during inflammatory responses. PGE_2 signals through E prostanoid $(EP)_1$, EP_2, EP_3, and EP_4 receptors, respectively. EP_2 and EP_4 receptors activate protein kinase A (PKA), which phosphorylates 5-LO and suppresses its function (26, 27). PKA also phosphorylates and desensitizes the T-prostanoid (TP) receptor (28). Inhaled PGE_2 blocks both bronchoconstriction and increases in urinary LTE_4 that occur with aspirin challenge of subjects with AERD (29). Cromone drugs that block MC activation have effects similar to inhaled PGE_2 (30, 31). Thus, endogenous PGE_2 may control 5-LO activity in AERD, and COX-1 inhibition causes both LT production and MC activation. Neither the basis for the unique requirement for PGE_2 in AERD nor the sequence of molecular events culminating in MC activation when COX-1 is inhibited is known.

Nasal polyps from subjects with AERD show reduced expression of COX-2 mRNA (32) and hypermethylation of the PGE_2 synthase (*PTGES*) gene (33), and contain less PGE_2 than nasal polyps from aspirin-tolerant controls (34). Although these findings suggest impaired up-regulation of PGE_2 synthesis with inflammation, their causality in AERD is unproven. We now show that impaired induction of PGE_2 synthesis causes AERD-like features when respiratory tract inflammation is superimposed. Mice lacking mPGES-1 ($ptges^{-/-}$ mice) treated intranasally (i.n.) with an extract (*Df*) from the dust mite *Dermatophagoides farinae* develop marked eosinophilic bronchovascular inflammation compared with wild-type control animals (28, 35). *Df*-treated $ptges^{-/-}$ mice exhibit airflow obstruction, cysLT production, and lung MC activation in response to aspirin. The airflow obstruction and MC activation both depend on cysLTs and are blocked by EP_2 receptor signaling. TP receptors and platelet-adherent granulocytes are essential for all features of aspirin sensitivity. Failure to

Significance

Aspirin-exacerbated respiratory disease (AERD) is a common, severe variant of asthma, which is associated with overproduction of cysteinyl leukotrienes (cysLTs) and respiratory reactions to drugs that block cyclooxygenase 1. We demonstrate that mice selectively lacking the capacity to up-regulate the generation of prostaglandin E_2 with inflammation develop an AERD-like phenotype that depends critically on platelets and thromboxane receptors, which drive transcellular synthesis of cysLTs, which, in turn, activate mast cells with aspirin challenge. The findings suggest a role for antiplatelet or thromboxane-selective antagonists as treatments for AERD.

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appropriately increase PGE_2 production with inflammation permits TP receptor-dependent cysLT generation by platelet-adherent granulocytes, providing the cysLTs that drive MC activation in AERD when residual PGE_2 is depleted.

Results

***Df*-Treated *ptges*$^{-/-}$ Mice Develop Aspirin Sensitivity.** WT and *ptges*$^{-/-}$ mice were challenged through a ventilator circuit with Lysine aspirin (Lys-ASA) (12 μL of a 100-mg/mL solution) or diluent 24 h after their last doses of *Df* or saline. Lung resistance (R_L) increased markedly in the *Df*-treated *ptges*$^{-/-}$ mice challenged with Lys-ASA compared with the WT mice and to the saline-treated *ptges*$^{-/-}$ controls (Fig. 1*A*). R_L in the *Df*-treated *ptges*$^{-/-}$ mice increased by 9 min after Lys-ASA challenge, and was sustained throughout the 45-min period of observation (Fig. 1*B*). Inhaled ketorolac also increased R_L in the *Df*-treated *ptges*$^{-/-}$ mice, exceeding the increase in response to celecoxib challenge (Fig. S1).

To determine whether the response to Lys-ASA was associated with LT generation, MC activation, and depletion of endogenous PGE_2, bronchoalveolar lavage (BAL) fluid was collected after challenge with nebulized Lys-ASA or diluent and analyzed for histamine, mouse MC protease-1 (mMCP-1) (36), cysLTs, LTB_4, and PGE_2. After treatment with *Df*, BAL fluid levels of mMCP-1, histamine, and cysLTs in diluent-challenged *ptges*$^{-/-}$ mice exceed those in WT controls (Fig. 1*C*). LTB_4 did not increase significantly with *Df* treatment and was not different between the genotypes (32 ± 6 pg/mL vs. 32 ± 5 pg/mL in saline-treated WT and *ptges*$^{-/-}$ mice, respectively; 33 ± 4 pg/mL vs. 39 ± 8 pg/mL in the corresponding *Df*-treated groups, $n = 5$). Lys-ASA challenge of the WT mice did not alter the mediator levels in BAL fluids. In contrast, the concentrations of cysLTs, mMCP-1, and histamine all increased significantly in the BAL fluids from Lys-ASA–challenged *ptges*$^{-/-}$ mice that had received *Df* previously (Fig. 1*C*). LTB_4 levels tended to increase (to 57 ± 8 pg/mL) in the challenged *ptges*$^{-/-}$ mice, but did not reach significance compared with WT mice (36 ± 6 pg/mL). PGE_2 levels increased in WT mice after treatment with *Df*, but did not increase in the *ptges*$^{-/-}$ mice (Fig. 1*D*). Challenge with Lys-ASA reduced PGE_2 levels in the BAL fluid of *Df*-treated WT mice by ∼50% and nearly completely depleted PGE_2 in the BAL fluid of both saline and *Df*-treated *ptges*$^{-/-}$ mice (Fig. 1*D*).

EP Receptor Agonists Block Reactions to Lys-ASA in PGE_2-Deficient Mice. *Df*-treated *ptges*$^{-/-}$ mice received single intranasal doses of either the stable PGE_2 mimic 16,16-dimethyl PGE_2 or selective agonists of the EP_1 (DI-004), EP_2 (AE1-259-01), EP_3 (AE-248), and EP_4 receptors (AE-329) (all at 5 nmol) at 1 h before Lys-ASA challenge. Both 16,16-dimethyl PGE_2 and the EP_2 receptor agonist blocked the increase in R_L by ∼90% (Fig. 2*A*). The EP_3 and EP_4 agonists also modestly reduced the change in R_L induced by Lys-ASA (Fig. 2*A*). Treatment with either 16,16-dimethyl PGE_2 or the EP_2 receptor agonist significantly reduced the levels of cysLTs, histamine, and mMCP-1 (Fig. 2*B*). The EP_4 receptor agonist also decreased the levels of mMCP-1 and histamine, whereas the EP_3 receptor agonist decreased histamine levels. The 1-h exposure to the EP agonists did not alter the histologic appearance of the lungs of the *ptges*$^{-/-}$ mice.

Deletion of Hematopoietic EP_2 Receptors Partially Reproduces the Phenotype of Lys-ASA Sensitivity. To determine whether EP_2 receptor deletion reproduced the aspirin-sensitive phenotype, we studied *ptger2*$^{-/-}$ mice and WT controls. Compared with WT controls, *Df*-treated *ptger2*$^{-/-}$ mice had higher total BAL fluid cell numbers and eosinophils (Fig. S2*A*), more extensive bronchovascular cellular infiltration (Fig. S2*C*), and more vascular smooth muscle cells and thicker smooth muscle layers than WT controls (Fig. S2*B*). The hyperplastic arterioles appeared similar to those observed in the *Df*-treated *ptges*$^{-/-}$ mice (Fig. S2*C*). When challenged with Lys-ASA, the *ptger2*$^{-/-}$ mice showed increases in R_L that were smaller than those observed in comparably treated *ptges*$^{-/-}$ mice (Fig. S2*D*). Concentrations of cysLTs (279 ± 57 pg/mL vs. 89 ± 23 pg/mL) and histamine (12.6 ± 3.6 nM vs. 5.2 ± 1.2 nM) were higher in the BAL fluids of Lys-ASA–challenged, *Df*-treated *ptger2*$^{-/-}$ mice than in the identically treated WT controls, but neither reached significance ($n = 5$). mMCP-1 levels (286 ± 57 pg/mL vs. 273 ± 50 pg/mL) were similar between the groups. After Lys-ASA challenge, the levels of PGE_2 detected in the BAL fluids from the saline-treated (117 ± 19 pg/mL) and *Df*-treated (179 ± 26 pg/mL) *ptger2*$^{-/-}$ mice were similar to those found in the corresponding groups of WT control mice (99 ± 20 pg/mL and 198 ± 33 pg/mL, respectively, $n = 5$). To identify the most important sites of action of EP_2 receptor signaling, we transferred bone marrow cells from *ptger2*$^{-/-}$ mice into lethally irradiated WT mice and vice versa. WT and *ptger2*$^{-/-}$ mice engrafted with *ptger2*$^{-/-}$ bone marrow developed much higher total cell counts and eosinophil counts in their BAL fluid after treatment with *Df* than did mice from either genotype receiving WT marrow (Fig. S2*E*).

Lys-ASA Sensitivity and MC Activation Depend on CysLTs. *Df*-treated *ptges*$^{-/-}$ mice received the $CysLT_1R$ antagonist montelukast overnight in the drinking water, or a single i.p. dose of the 5-LO inhibitor zileuton on the day before Lys-ASA challenge. Both drugs attenuated the increase in R_L occurring in response to Lys-ASA (Fig. S3*A*). Both also reduced the concentrations of BAL fluid histamine and mMCP-1 from Lys-ASA–challenged mice (Fig. S3*B*). Zileuton reduced the levels of cysLTs in BAL fluid by ∼60% and LTB_4 by ∼40% compared with Lys-ASA–challenged controls not receiving drug. Bronchovascular infiltrates and goblet cells were not altered by the LT antagonists.

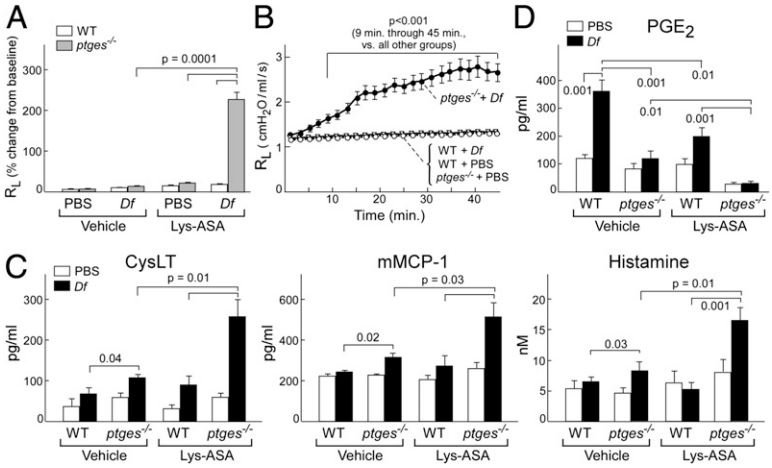

Fig. 1. PGE_2-deficient mice develop aspirin sensitivity after induction of allergic airway inflammation. WT and *ptges*$^{-/-}$ mice were treated intranasally with *Df* (3 μg) or PBS on six occasions over 17 d. Mice were tracheostomized and mechanically ventilated 24 h after the last dose and challenged with Lys-ASA or vehicle. (*A*) Peak change in lung resistance (R_L) developing in response to challenges with Lys-ASA or vehicle in mice of the indicated genotypes. (*B*) Time course of change in R_L in the indicated Lys-ASA challenged groups. (*C*) Levels of cysLTs (*Left*), mMCP-1 (*Center*), and histamine (*Right*) BAL fluid collected from the mice after measurement of R_L. (*D*) Levels of PGE_2 in the BAL fluids collected from the same mice as in *C*. Results in *A* and *B* are from 30 mice per group. Results in *C* and *D* are from at least 10 mice per group.

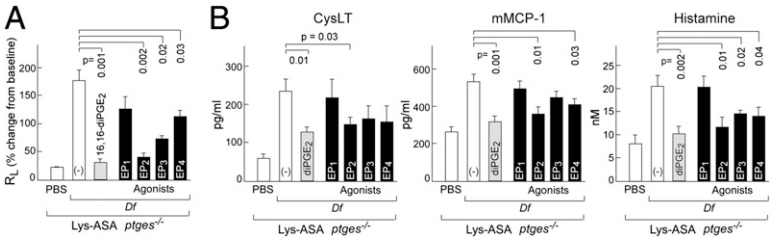

Fig. 2. EP receptor agonists attenuate the response of PGE_2-deficient mice to Lys-ASA challenge. *Df*-treated *ptges*$^{-/-}$ mice received single intranasal doses of 16,16-dimethyl PGE_2 or selective agonists for the indicated EP receptors (5 nmol each) or vehicle (–) 1 h before Lys-ASA challenge. (*A*) Peak R_L developing in the indicated Lys-ASA–challenged groups. (*B*) Effects of the EP receptor agonists on BAL fluid levels of cysLTs (*Left*), mMCP-1 (*Center*), and histamine (*Right*) recovered from the same mice as in *A*. Results are from 10–15 mice per group.

Platelets and Platelet-Adherent Leukocytes Are Required for Aspirin Sensitivity. By flow cytometry, the blood of WT mice contained distinct populations of CD41$^-$ and CD41$^+$ (platelet adherent) granulocytes, with ∼50% of the cells in each category (representative sample, Fig. 3*A*, *Left*). In contrast, the blood granulocytes of *ptges*$^{-/-}$ mice were essentially all CD41$^+$ (Fig. 3*A*, *Left*). The median channel fluorescence MFI for CD41 was approximately fivefold higher on the granulocytes in the blood of the *ptges*$^{-/-}$ mice than in the blood of the WT controls (Fig. 3*A*, *Right*). The PBS-treated mice did not differ from the *Df*-treated mice in either strain. Numerous CD41$^+$ platelets, some colocalizing with infiltrating leukocytes, were present in the bronchovascular bundles of the *Df*-challenged *ptges*$^{-/-}$ mice (and to a lesser extent in the WT mice) (Fig. S4).

To determine the importance of platelet-adherent granulocytes in our model, *Df*-treated mice received i.p. doses of either the anti–IL-5 Ab TRFK5 (to deplete eosinophils), anti-Gr1/Ly6G/C Ab (to deplete neutrophils and a subset of monocytes), or anti-CD41 Ab (to deplete platelets and platelet-adherent granulocytes) (37) 1 d before Lys-ASA. TRFK5 did not significantly block the change in R_L induced by Lys-ASA (Fig. 3*B*). Anti-Gr1 reduced the magnitude of change in R_L by ∼40%. Mice treated with the anti-platelet Ab were markedly protected from Lys-ASA–induced changes in lung function (Fig. 3*B*). Treatment with TRFK5 modestly reduced the levels of mMCP-1 and histamine, whereas treatment with anti-Gr1 significantly reduced all three mediators (Fig. 3*C*). BAL fluids from mice treated with anti-CD41 showed markedly reduced concentrations of all three mediators. Anti-CD41 also sharply reduced the level of LTB_4 in the BAL fluid (from 57 ± 6 pg/mL to 25 ± 6 pg/mL, $P = 0.02$). Anti-TRFK5 reduced the numbers of circulating eosinophils by ∼70%, without altering neutrophils or free platelets, compared with the isotype control. The anti-Gr1 Ab depleted neutrophils by ∼90% and eosinophils by ∼40% with no effect on free platelets. Treatment with anti-CD41 depleted free platelets by ∼90% and also depleted neutrophils by ∼40% and eosinophils by ∼50%, the latter likely reflecting reductions in the platelet-adherent granulocyte subsets. BAL fluid myeloperoxidase (MPO) levels in diluent-challenged *Df*-treated *ptges*$^{-/-}$ mice were equivalent to the levels in WT controls and increased with Lys-ASA only in the *ptges*$^{-/-}$ mice (Fig. S5). Treatments with anti-Gr1 and anti-CD41 both significantly reduced the levels of BAL fluid MPO in response to Lys-ASA. The levels of eosinophil peroxidase in the BAL were at or near the limits of detection under all conditions.

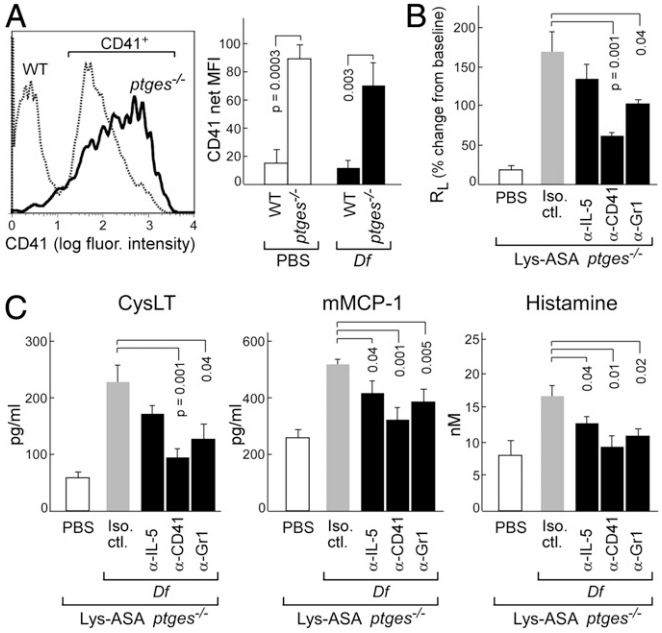

Fig. 3. Platelet-adherent granulocytes are important for Lys-ASA sensitivity of PGE_2-deficient mice. (*A*) Representative histograms (*Left*) of CD45$^+$ granulocytes in the peripheral blood of PBS-treated WT (dotted tracing) and *ptges*$^{-/-}$ (bold tracing) mice stained for CD41 to identify the platelet-adherent subsets. Net MFI of CD41 staining in granulocyte gate in the blood of PBS- or *Df*-treated WT and *ptges*$^{-/-}$ mice (4–5 mice per group, *Right*). (*B*) Peak change in R_L after Lys-ASA challenge of *Df*-treated *ptges*$^{-/-}$ mice that received single i.p. doses (50 μg per mouse) of anti–IL-5, anti-CD41, anti-Gr1, or an isotype control at 24 h before challenge. (*C*) The concentrations of cysLTs (*Left*), mMCP-1 (*Center*), and histamine (*Right*) in the BAL fluids of the same mice as in *B*. Results in *B* and *C* are from 10 mice per group.

TP Receptors Mediate Lys-ASA Sensitivity. We challenged *Df*-treated *ptges*/*tpr* knockout (DKO) mice with Lys-ASA. Compared with the *ptges*$^{-/-}$ mice, the DKO mice were protected from Lys-ASA–induced increases in R_L (Fig. 4*A*). The levels of mediators in BAL fluid following Lys-ASA challenge of the DKO mice were significantly lower than their levels in the BAL fluids of Lys-ASA–challenged *ptges*$^{-/-}$ mice (Fig. 4*C*). PGE_2 levels in the BAL fluid of DKO mice were similar to those found in the *ptges*$^{-/-}$ single knockout mice after Lys-ASA challenge (29 ± 8 pg/mL vs. 30 ± 6 pg/mL, $n = 5$). The BAL fluid levels of the stable thromboxane $(TX)A_2$ metabolite, TXB_2, were similar in the saline-treated groups of the two genotypes, showed similar (approximately twofold) increases with *Df* treatment and similar reductions in response to Lys-ASA challenge (Fig. S6).

To determine whether TP receptor blockade altered reactions to Lys-ASA in mice with airway inflammation, we administered the TP receptor-selective antagonist SQ29.548 (38) to *Df*-treated *ptges*$^{-/-}$ mice 24 h before Lys-ASA challenge. SQ29.548 significantly reduced the increase in R_L in response to Lys-ASA (Fig. 4*B*) and markedly decreased the levels of cysLTs, histamine, and mMCP-1 in the BAL fluid of the Lys-ASA–challenged *ptges*$^{-/-}$ mice (Fig. 4*C*). Short-term TP receptor blockade had no effect on histologic indices of inflammation, whereas *tpr* deletion markedly impaired the development of these features as in our previous study (28).

Discussion

AERD accounts disproportionately for severe asthma and chronic rhinosinusitis (3, 4). Although the mechanisms responsible for the disease have been evasive (6), the pathognomonic reactions to COX-1–active drugs can be attenuated by inhibitors of 5-LO and $CysLT_1R$ (39) and by drugs that block MC activation (30, 31). PGE_2 suppresses 5-LO activity (39) and MC activation (26, 40) in vitro. Inhaled PGE_2 inhibits aspirin-induced bronchoconstriction and cysLT production in subjects with AERD

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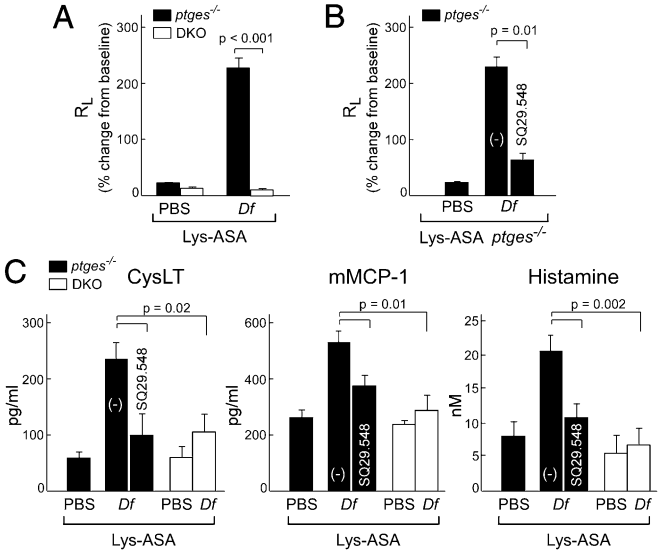

Fig. 4. Deletion or blockade of TP receptors attenuates aspirin sensitivity in PGE_2-deficient mice. (*A*) Peak change in R_L occurring in response to Lys-ASA challenge of *ptges*$^{-/-}$ or *ptges/tpr*$^{-/-}$ (DKO) mice 24 h after their final treatment with PBS or *Df*. (*B*) Peak change in R_L in *ptges*$^{-/-}$ mice receiving two doses of the TP receptor selective antagonist SQ29.548 before challenge with Lys-ASA. (*C*) Levels of cysLTs (*Left*), mMCP-1 (*Center*), and histamine (*Right*) in BAL fluids from the same mice as in *B*. Results are from 10 mice per group.

(29). Pharmacologic studies (41) suggest that as much as 70% of urinary PGE_2 metabolites in ATA and healthy subjects derive from COX-2, a largely aspirin-resistant enzyme (42) that pairs functionally with mPGES-1 (22). The impaired expression of COX-2 in nasal polyps from subjects with AERD, combined with hypermethylation of the *PTGES* gene (33), predict that subjects with AERD may not sustain PGE_2 in the respiratory tissue when COX-1 is inhibited. This prediction was supported by early ex vivo studies of excised, aspirin-treated nasal polyps (43). However, no study had directly addressed whether these lesions are causative, and which functional perturbations were essential to manifest the response to COX-1 inhibitors. We used *ptges*$^{-/-}$ mice to determine whether the selective loss of inducible PGE_2 permitted AERD-like physiology to develop in a model of pulmonary inflammation, and if so, to define the mechanisms responsible.

The absence of mPGES-1 impairs the up-regulation of PGE_2 production in mice. *Ptges*$^{-/-}$ mice develop marked eosinophil-dominated bronchovascular cellular infiltrates (>75% of BAL fluid cells) with lesser numbers of neutrophils (28, 35). AERD is also associated with marked eosinophilia of the respiratory mucosa (44), often without evidence of sensitization to allergens. Because there is no method to elicit eosinophilic inflammation in mice independently of allergen, we elicited eosinophilic pulmonary inflammation in WT and *ptges*$^{-/-}$ mice with *Df* and challenged with Lys-ASA to determine whether impaired inducible PGE_2 generation permitted AERD-like physiology. Lys-ASA challenge caused a significant increase in R_L (Fig. 1 *A* and *B*) in *Df*-treated *ptges*$^{-/-}$ mice, but not in WT controls, while causing the releases of histamine and mMCP-1 (two markers of MC activation) and cysLTs (Fig. 1*C*). BAL fluid from diluent-challenged *ptges*$^{-/-}$ mice contained higher levels of cysLTs than WT mice (Fig. 1*C*), but LTB_4 levels were identical, implying specific dysregulation of cysLT pathway activity. The marked depletion of residual PGE_2 by Lys-ASA in the *ptges*$^{-/-}$ mice (Fig. 1*D*) suggests that mPGES-1 sustains PGE_2 generation in the face of COX-1 inhibition. Whereas our previous studies showed that the impaired induction of PGE_2 synthesis dysregulates the extent of *Df*-induced inflammation (28), our current findings demonstrate that the failure to maintain PGE_2 production when COX-1 is inhibited results in bronchoconstriction, MC activation, and release of cysLTs.

Polymorphic variants of *PTGER2* (45) and reduced EP_2 receptor expression (46, 47) are reported in AERD. We used both pharmacologic and transgenic approaches to test the role of EP_2 receptors in our model. The EP_2 receptor agonist nearly completely blocked the Lys-ASA–mediated increase in R_L in the *ptges*$^{-/-}$ mice (Fig. 2*A*) and attenuated the increases in mediator levels in the BAL fluid (Fig. 2*B*). EP_3 and EP_4 agonists were also active. In our previous studies, the long-term intranasal administrations of the EP_2 or EP_3 agonists suppressed *Df*-induced pulmonary eosinophilia in the *ptges*$^{-/-}$ mice with equal efficacy (35). Deletion of EP_2 receptors mirrored the increase in *Df*-induced inflammation and remodeling of the pulmonary vasculature (Fig. S2 *A–C*) observed in *Df*-treated *ptges*$^{-/-}$ mice (28), reflecting the absence of this receptor on hematopoietic cells (Fig. S2*E*). Notably, the absence of EP_3, but not EP_2 receptors, potentiated airway eosinophilia in a previous study (48). The differences between that study and ours may relate to different allergens (*Df* vs. ovalbumin) and routes of sensitization (intranasal vs. intraperitoneal).The modest response of *ptger2*$^{-/-}$ mice to Lys-ASA (Fig. S2*D*) likely reflects their capacity to maintain PGE_2 production and compensatory signaling through EP_3 and EP_4 receptors during aspirin challenge (Fig. 2*A*). Thus, EP_2 receptor defects identified in AERD (46, 47) may potentiate granulocyte infiltration of the airways, but may not be sufficient to cause a full "aspirin sensitive" phenotype, which depends on deficiency of inducible PGE_2. Compensatory functions of EP_3 and EP_4 may account for the fact that inhaled PGE_2 prevents reactions to aspirin in subjects with AERD (29), even if EP_2 receptor protein on the target cells is reduced.

The 5-lipoxygenase is serine phosphorylated and inhibited by cAMP-dependent PKA (27). EP_2 receptors activate PKA and suppress the formation of cysLTs (26). This likely explains the constitutively high levels of BAL fluid cysLTs in the *ptges*$^{-/-}$ mice (Fig. 1*C*), and the suppression of aspirin-induced cysLT formation in vivo by PGE_2 in our study (Fig. 2) and in humans with AERD (29). Consistent with the pharmacology of AERD (49), short-term blockade of $CysLT_1R$ or inhibition of 5-LO markedly attenuated the increase in R_L in response to Lys-ASA (Fig. S3*A*). Unexpectedly, both agents also blocked MC activation (Fig. S3*B*). Oral administration of zileuton to subjects with AERD blocked the release of tryptase into the nasal lavage fluid following intranasal Lys-ASA challenge (50). MCs express all of the known CysLTRs (14–16, 51), and blockade of $CysLT_1R$ with montelukast mirrored the effect of zileuton on the release of MC activation products in our study. Thus, AERD involves a prominent autocrine and/or paracrine signaling loop in which cysLTs promote MC activation. Such a mechanism has been inferred by in vitro studies of human MCs harvested from nasal polyps (52, 53). It is possible that a lack of PGE_2 input through EP receptors on MCs (and/or other cells) permits this cysLT-driven activation pathway in vivo.

We next sought to identify the sources of the cysLTs in our model. Activated granulocytes, particularly neutrophils, generate LTA_4 in excess of the capacity for their terminal enzymes to convert to LTB_4 (or LTC_4 in eosinophils). Platelets lack 5-LO, but possess LTC_4S and convert unmetabolized LTA_4 to LTC_4 when they adhere to the granulocyte surface via P-selectin (54). We previously demonstrated that platelet-adherent eosinophils and neutrophils are more frequent in the peripheral blood and sinonasal tissues from patients with AERD than in samples from aspirin-tolerant controls (9). Adherent platelets were the dominant source of LTC_4S activity in granulocytes from subjects with AERD and they correlate with urinary LTE_4 levels (9). The increased numbers of platelets adherent to the granulocytes in the blood (Fig. 3*A*) and large numbers of extravasated platelets in lung tissue (Fig. S4) of *ptges*$^{-/-}$ mice compared with WT control mice suggest that impaired PGE_2 synthesis (or reduced EP receptor signaling) disturbs control of platelet–leukocyte complex formation. Adherence to platelets also primes granulocyte integrin

function (55) and chemotaxis (56). Thus, the platelet-adherent granulocytes in the blood of *ptges*$^{-/-}$ mice likely increase their susceptibility to *Df*-induced inflammation. Moreover, Ab-mediated cell depletion studies support a central contribution from platelet-adherent granulocytes to the physiologic response to Lys-ASA (Fig. 3*B*), likely by providing cysLTs that facilitate MC activation (Fig. 3*C*). The release of MPO in response to Lys-ASA, and its reduction by platelet and granulocyte depletions (Fig. S5), supports the role of intrapulmonary platelet–neutrophil complexes. The comparatively modest effect of anti–IL-5 may reflect incomplete depletion of eosinophils from the airway (as described in humans with asthma) (57). Alternatively, eosinophils may contribute to baseline cysLT generation (44), whereas other cells provide cysLTs during the reaction.

Platelets generate TXA_2 primarily from COX-1 (58). TXA_2 signaling through TP receptors facilitates the formation of platelet–leukocyte complexes (59, 60) and induces endothelial cells to express ICAM-1 (61). In the lung, TP receptor signaling is restrained by PKA (28, 62). Although TXA_2 production by *ptges*$^{-/-}$ mice was similar to WT controls (Fig. S6), the loss of EP receptor-dependent cross-regulation amplifies the contributions of the TXA_2/TP pathway to *Df*-induced pulmonary inflammation (28). Because TP receptor deletion essentially eliminates *Df*-induced pulmonary pathology in *ptges*$^{-/-}$ mice, the fact that the DKO mice failed to respond to Lys-ASA was not surprising (Fig. 4*A*). However, the profound protective effect of the TP receptor antagonist SQ29.548 with short-term administration (Fig. 4*B*) was not associated with a change in bronchovascular inflammation, implying that TP receptors are essential for platelet-adherent granulocytes to generate the pathogenetic cysLTs in this model, likely by facilitating cross-talk between platelets and granulocytes. Moreover, the findings in the DKO mice further highlight that airway inflammation is a prerequisite for the AERD-like phenotype, even when inducible PGE_2 is deficient.

Our findings support the causative nature of lesions reported in AERD (32, 33) that impair PGE_2 generation in the inflamed respiratory tract. These lesions limit basal EP receptor signaling that normally prevents the severe persistent respiratory mucosal eosinophilia. Impaired EP receptor input compromises control of TP receptor signaling by PKA (28), increasing the adherence of platelets to granulocytes, inducing endothelial ICAM-1 (28), activating 5-LO, and enabling platelet-adherent leukocytes to mediate LT generation (9). The residual local PGE_2 derives principally from COX-1, which may explain why only COX-1 active drugs provoke clinical reactions (6) and why COX-2–selective drugs are well tolerated by these patients (20). The MC activation typical of clinical reactions (50, 63, 64) may be due to the agonistic effects of cysLTs derived from intrapulmonary platelet–granulocyte complexes when residual PGE_2 is depleted. Importantly, reactions are followed by a state of desensitization, during which time urinary levels of TXB_2 decline, lung function recovers, urinary LTE_4 levels return to their prechallenge baselines (63), and subjects are refractory to subsequent challenges. Our study suggests that COX-1 products have a dual role in AERD; whereas aspirin depletes PGE_2 in the respiratory tissue to cause clinical reactions, it also depletes TXA_2, promoting resolution of the reaction and initiating therapeutic desensitization. Our findings suggest potential applications for drugs that target TP receptors, platelets, and EP receptors, each of which is a component of a hierarchical system culminating in AERD when perturbed by inflammation without sufficient PGE_2 generation.

Methods

Mice. C57BL6 *ptges*$^{-/-}$ mice were from Satoshi Uematsu (Osaka University, Osaka) (25). TP receptor knockout (*tpr*$^{-/-}$) mice were from Thomas Coffman (Duke University, Durham, NC) (65). DKO mice were derived by intercrossing as described (28). C57BL/6 *ptger2*$^{-/-}$ mice were from Beverly Koller (University of North Carolina, Chapel Hill, NC) (66). Six- to 8-wk-old males were used. Studies were approved by the Animal Care and Use Committee of the Dana–Farber Cancer Institute. Bone marrow transfers and induction of airway inflammation were described previously (28).

Reagents. *Df* was from Greer Laboratories (XPB81D3A25) (35). Selective agonists of the EP_1 (DI-004), EP_2 (AE1-259-01), EP_3 (AE-248), and EP_4 (AE-329) receptors were from ONO Pharmaceuticals (67). Zileuton, 16,16-dimethyl PGE_2, selective antagonist of the TP receptor (SQ29.548), and ketorolac were purchased from Cayman Chemical. Montelukast and clopidogrel were obtained from the hospital pharmacy. The mMCP-1 enzyme immunoassay (EIA) kit was purchased from eBiosciences, and MPO and eosinophil peroxidase kits were from Abcam. Histamine, PGE_2, TXB_2, LTB_4, and cysLT EIA kits were from Cayman Chemical. Celecoxib was purchased from Sigma-Aldrich.

Lys-ASA Challenge and Measurement of Airway Resistance. R_L was assessed with an invasive pulmonary function device (Buxco). Briefly, mice were tracheostomized and ventilated. After allowing R_L to reach a stable baseline, Lys-ASA (12 μL of 100 mg/mL), ketorolac (12 μL of 100 mg/mL), or celecoxib (24 μL of 50 mg/mL) was delivered to the lung via nebulizer, and R_L was recorded for 45 min. The results are expressed as percentage change of R_L from baseline. Some mice were treated with montelukast (6.7 μg/mL in drinking water, per os 24 h before Lys-ASA), zileuton (2 mg per mouse, i.p. 24 h before Lys-ASA), EP agonists (5 nmol, i.n. 1 h before Lys-ASA), or SQ29.548 (50 μg per mouse, i.p. 24 h before Lys-ASA).

Ab-Mediated Cell Depletions. Twenty-four hours before the Lys-ASA challenge, mice received 50 μg rat anti-mouse IL-5 Ab (clone TRFK4; Biolegend), 50 μg of rat anti-mouse CD41 (clone MWReg30; Biolegend) (38), or 50 μg of rat anti-mouse Gr1 (clone RB6-8C5; Biolegend). Rat IgG2b kappa isotype was used as control (clone RTK4530; Biolegend). Each Ab was diluted in 50 μL of sterile saline.

Histology. Smooth muscle actin staining was performed as previously described (28). For detection of platelets, lungs were embedded in optimal cutting temperature compound, flash frozen, and sections were prepared, which were incubated in rabbit IgG anti-mouse CD41 (Ab H-160; Santa Cruz Biotechnology) or nonimmune rabbit IgG for 1 h at room temperature. Ab binding was visualized with an avidin-biotin complex (Santa Cruz Biotechnology), and sections were counterstained with hematoxylin.

Flow Cytometry. Blood was drawn into 4% (wt/vol) sodium citrate and incubated with Allophycocyanin-conjugated rat anti-mouse CD41 and with phycoerythrine-cyanine 7 conjugated rat anti-mouse CD45 Abs or isotype controls (BD Biosciences) for 20 min and fixed in 1% paraformaldehyde. At least 20,000 $CD45^+$ cells were recorded for each sample on a BD FACSAria flow cytometer and analyzed with FlowJo version 7.6.5. $CD45^+$ granulocytes were identified based on light scatter and assessed for the presence of adherent platelets by relative expression of CD41. Results were expressed as the net MFI and percent positive for CD41.

Statistical Analysis. Data are expressed as ±SEM from at least 10 mice from at least two experiments, except where otherwise indicated. Differences between treatment groups were determined with the Student *t* test.

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