

Reduced expression of the prostaglandin E₂ receptor E-prostanoid 2 on bronchial mucosal leukocytes in patients with aspirin-sensitive asthma

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Background: Prostaglandin E₂ (PGE₂) is thought to play a role in the pathogenesis of aspirin-sensitive asthma (ASA).

Objective: We sought to extend our previous observations implicating impaired inflammatory cell responsiveness to PGE₂ as a pathogenetic mechanism in patients with aspirin-sensitive rhinosinusitis to the bronchial mucosa in patients with ASA.

Methods: Immunohistochemistry was used to enumerate inflammatory cells and their expression of cysteinyl leukotriene receptors 1 and 2 (CysLT₁ and CysLT₂) and the PGE₂ receptors E-prostanoid 1 to 4 (EP₁-EP₄) in bronchial biopsy specimens from patients with ASA, patients with aspirin-tolerant asthma, and control subjects (n = 15 in each group). Concentrations of PGE₂ in bronchoalveolar lavage fluid were measured by using ELISA. The effects of PGE₂ and EP receptor agonists on CD3/CD28-stimulated cytokine production by PBMCs were measured by using ELISA. Airways responsiveness to LTD₄ *in vivo* was measured in asthmatic patients by means of bronchial challenge.

Results: Compared with patients with aspirin-tolerant asthma, patients with ASA had increased bronchial mucosal neutrophil and eosinophil numbers but reduced percentages of T cells, macrophages, mast cells, and neutrophils expressing EP₂. Both groups showed increased bronchial sensitivity to inhaled LTD₄, but this did not correlate with mucosal expression of CysLT₁ or CysLT₂. Bronchoalveolar lavage fluid PGE₂ concentrations were comparable in all groups. *In vitro* PGE₂ inhibited cytokine production by PBMCs through EP₂ but not other PGE₂ receptors.

Conclusion: Our data are consistent with the hypothesis that impaired inhibition of inflammatory leukocytes by PGE₂ acting

through the EP₂ receptor has a role in the pathogenesis of ASA. (J Allergy Clin Immunol 2012;■■■■:■■■■-■■■■.)

Key words: Aspirin, prostaglandin E₂, E-prostanoid receptor, asthma

Aspirin-sensitive asthma (ASA), as first described by Widal et al¹ and extended by Samter and Beers² is a phenotype of typically late-onset severe asthma often preceded and accompanied by rhinosinusitis. It is slightly more common in female subjects and not related to atopic status.³ Clinically, patients experience acute exacerbation of chronic severe rhinosinusitis and asthma on exposure to aspirin or other drugs of the COX-1 inhibitor class.⁴ Prior exposure or “sensitization” is unnecessary. Some also experience acute urticaria and angioedema, suggesting mast cell degranulation.⁵ Aspirin-sensitive patients are overrepresented among those presenting with severe and life-threatening asthma exacerbations.⁶⁻⁸ Biochemically, current evidence suggests that ASA is characterized by abnormal eicosanoid metabolism, specifically overproduction of cysteinyl leukotrienes (CysLTs) by eosinophils and mast cells in the mucosa of the target organs, which is further augmented by aspirin exposure (as reviewed by Farooque and Lee⁴), increased expression of the CysLT₁ receptor⁹ on mucosal inflammatory cells, and abnormalities of prostanoid metabolism that might be further modified by drugs that inhibit COX-1. Interest has been particularly centered on the role of prostaglandin E₂ (PGE₂) because PGE₂ has been shown to block aspirin-induced bronchoconstriction, increased CysLT production, and mast cell degranulation¹⁰⁻¹³ in addition to exerting potential beneficial immunomodulatory and anti-remodeling activities.^{14,15} Indeed, the lung has been described as a “privileged site” for these wide-ranging beneficial effects.¹⁶

These observations have given rise to the hypothesis that ASA and aspirin-sensitive rhinosinusitis result at least partly from a critical deficiency in the synthesis/metabolism, actions, or both of PGE₂ such that the brake on excessive CysLT production exerted by PGE₂ is released in these patients on exposure to COX-1 inhibitors. Although there have been reports of deficient baseline PGE₂ production by epithelial and fibroblast cell lines outgrown from patients with aspirin-exacerbated respiratory disease (AERD) *in vitro*,^{17,18} *in vivo* global production of PGE₂ in these patients does not appear to be deficient.^{19,20}

In this study we pursue the alternative hypothesis that PGE₂ signaling is potentially defective in patients with AERD. PGE₂ ligates 4 G protein-coupled receptors designated E-prostanoid 1 to 4 (EP₁-EP₄).²¹ We previously reported reduced expression of the EP₂ receptor on nasal mucosal inflammatory cells in patients with

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Abbreviations used

AERD: Aspirin-exacerbated respiratory disease
 ASA: Aspirin-sensitive asthma
 BAL: Bronchoalveolar lavage
 Ct: Cycle threshold
 CysLT: Cysteinyl leukotriene
 EP: E-prostanoid
 HC: Healthy control subject
 LTE₄: Leukotriene E₄
 MBP: Major basic protein
 NASA: Non-aspirin-sensitive asthma
 PGE₂: Prostaglandin E₂

AERD-associated chronic rhinosinusitis.²² In addition, a functional single nucleotide polymorphism of the EP₂ gene, which is believed to be associated with reduced transcription of the receptor protein, has been reported to be associated with an increased risk of ASA.²³ Consequently, in the present study we hypothesized that expression of the EP₂ receptor is reduced on inflammatory cells in the bronchial mucosa of patients with ASA compared with that seen in patients with aspirin-tolerant asthma and control subjects and that the EP₂ receptor is of functional significance in mediating the anti-inflammatory effects of PGE₂. We also took the opportunity to extend our previous observation^{9,24} that increased percentages of inflammatory leukocytes in the nasal mucosa of patients with aspirin-sensitive rhinosinusitis express CysLT₁ to address the bronchial mucosa. Finally, we compared global expression of the CysLT receptors CysLT₁ and CysLT₂ in both patients with ASA and patients with non-aspirin-sensitive asthma (NASA) with their bronchoconstrictor responses to inhaled leukotriene D₄.

METHODS**Subjects and fiberoptic bronchoscopy**

The study was approved by the Guy's Hospital Ethics Committee and carried out in our clinical research facility. Each subject provided written informed consent. Endobronchial biopsy specimens were obtained from 11 patients with ASA, 15 patients with NASA, and 15 healthy control subjects (HCs). Asthmatic patients had a clear history of relevant symptoms, documented reversible ($\geq 12\%$) airways obstruction, and/or histamine PC₂₀ values of 8 mg/mL or less measured within 2 weeks before biopsy. None had ever smoked, and there was no history of other respiratory disease. All subjects were clinically free of respiratory tract infections and systemic glucocorticoid therapy for at least 1 month before the study. The diagnosis of aspirin sensitivity was made as previously described.^{9,22,24} Patients with ASA were defined by a positive aspirin challenge result (8/11) unless they had 1 or more obvious documented reactions in the past 6 months, which made challenge unnecessary and potentially dangerous (3/11). If a patient had taken aspirin or another COX-1 inhibitor drug at clinical dosages within the previous 6 months without any untoward reaction (exacerbation of rhinitis, asthma, or both; urticaria/angioedema; anaphylaxis; or nausea/vomiting/diarrhea within 4 hours after ingestion), he or she was considered aspirin tolerant. Atopy was defined by standard skin prick testing. HCs were healthy, lifelong nonsmoking volunteers who had no history of lung disease. Fiberoptic bronchoscopy was performed, and bronchoalveolar lavage (BAL) fluid and bronchial biopsy specimens were obtained and processed as previously described.^{25,26}

LTD₄ inhalation challenge

Subsets of patients with ASA and those with NASA were challenged incrementally with medical-grade LTD₄ diluted from a stock solution in 100%

ethanol in sterile PBS to a starting concentration of 0.1 $\mu\text{g/mL}$ (0.02 $\mu\text{mol/L}$). By using a breath-actuated dosimeter, saline (baseline) and then further incremental (doubling) concentrations were administered every 15 minutes until the baseline FEV₁ was reduced by 20% or greater. PC₂₀ concentrations were then extrapolated from the dose-response curve.

Single immunohistochemistry and image analysis

Macrophages (CD68, clone no. EBM11), mast cells (tryptase, clone no. AA1), neutrophils (elastase, clone no. NP57; Dako, High Wycombe, United Kingdom), T cells (CD3; Becton Dickinson, Cowley, Oxford, United Kingdom), and eosinophils (major basic protein [MBP]; a kind gift from A. B. Kay, National Heart & Lung Institute, Imperial College, London, United Kingdom) were identified in biopsy sections by staining with optimized dilutions of these primary antibodies and developing with alkaline phosphatase-antialkaline phosphatase, as previously described.^{22,24} The results were expressed as the number of individual immunoreactive cells per square millimeter of mucosa. CysLT₁, CysLT₂, and EP₁₋₄ immunoreactivity was detected by staining with specific antibodies against CysLT₁ (1:50), CysLT₂ (1:50; Cayman Chemical, Ann Arbor, Mich), EP₁ (N-20), EP₂ (H-75), EP₃ (R-18), and EP₄ (C-18; 1:50 dilution of each antibody, Santa Cruz Biotechnology, Autogen Bioclear UK Ltd, Wiltshire, United Kingdom), as previously described.^{9,22,24} The primary antibodies were detected with swine anti-rabbit biotinylated secondary antibody (Dako) used at a dilution of 1:200 and subsequent application of avidin/biotin/peroxidase complex solution (Dako). The immunoperoxidase reaction was developed with 3,3'-diaminobenzidine, producing a brown color, and the sections were counterstained with hematoxylin. Omission or substitution of the primary antibody with an irrelevant antibody of the same species and isotype was used as a negative control. The slides were observed in blind fashion on an Olympus BX40 microscope connected to a Zeiss Vision KS300 image system (Carl Zeiss, Oberkochen, Germany). Fully automated densitometric measurement of cysLT₁, cysLT₂, and EP receptor immunoreactivity was performed with the system software driven by a custom macro (Imaging Associates Ltd, Bicester, United Kingdom). Because some receptor immunoreactivity was expressed on structural tissue, global positive staining was quantified as the percentage of the total stainable area of the section defined by the hematoxylin counterstaining. Separation of immune- and hematoxylin-stained pixels was achieved by threshold adjustment of the hue-light saturation-scale image after setting a shading correction for each slide with a low-pass filtered blank image.

Sequential immunohistochemistry

To characterize the phenotypes of cells expressing cysLT₁, cysLT₂, and EP receptors, sequential immunohistochemical staining was performed as previously described.^{22,24} Briefly, avidin-biotin complex staining was performed first to detect the receptors. Before developing, the alkaline phosphatase-antialkaline phosphatase method was used to detect CD3⁺ T cells, CD68⁺ macrophages, elastase-positive neutrophils, MBP⁺ eosinophils, and tryptase-positive mast cells.²² Phenotypic markers were developed with Fast Red (red color; Sigma, Poole, United Kingdom), whereas EP staining was detected by using Fast 3,3'-diaminobenzidine (brown color, Sigma). Double-positive cells were stained brown/red.^{22,24} The results were expressed as the percentages of each cell type expressing cysLT₁, cysLT₂, EP₁, EP₂, EP₃, and EP₄ receptor immunoreactivity.

Isolation of human PBMCs

PBMCs were isolated and stimulated *in vitro* by using a published technique.²⁷ Briefly, PBMCs were obtained by means of density centrifugation of diluted blood (1 part blood to 1 part pyrogen-free saline) over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden), washed twice in PBS, and suspended in culture medium RPMI 1640. The cells were counted in a hemocytometer, and their concentration was adjusted to 1×10^6 cells per milliliter. Cells were stimulated by the addition of anti-CD3 (UCHT1) and anti-CD28 (CD28.2) mAbs (BD PharMingen, Cambridge, United Kingdom), each at 500 ng/mL in solution, as previously described.²⁷ PBMCs (5×10^5) in a

TABLE I. Clinical data on asthmatic patients and control subjects

	Age (y)	Sex (M/F)	Atopic	FEV ₁ (% predicted)	Methacholine PC ₂₀ (mg/mL)	Therapy*
Patients with NASA (n = 15)	28 (21-60)	8/7	15	92.0 (66.0-115.0) [†]	2.0 (0.25-8.0)	4 LABA, 5 IG 300 (200-1000)
Patients with ASA (n = 11)	29 (21-57)	1/10	8	83.0 (47.2-114.0) [‡]	0.376 (0.055-8.0)	4 LABA, 8 IG 300 (200-1000)
HCS (n = 15)	23 (19-36)	9/6	10	104.0 (83.0-122.0)	>16	

Data are expressed as medians (ranges).

F, Female; IG, inhaled glucocorticoid (figures show beclomethasone dosage equivalent in micrograms per day); LABA, long-acting β_2 -agonist; M, male.

*All patients were taking short-acting β_2 -agonists as required.

[†] $P < .05$.

[‡] $P < .01$ versus HCs (Mann-Whitney *U* test).

volume of 100 μ L per well were incubated at 37°C in round-bottomed 96-well plates (Greiner, Nurnberg, Germany) for 24 hours in the presence or absence of PGE₂ (Sigma-Aldrich, St Louis, Mo) or EP receptor agonists (EP₁: ONO-DI-004; EP₂: ONO-AE1-259-01; EP₃: ONO-AE-248 (EP3); and EP₄: ONO-AE1-329; ONO Pharmaceutical Company, Osaka, Japan).²⁸ After culture supernatants were collected and stored at -20°C until assayed.

MesoScale Discovery cytokine detection

The cytokines IL-2, IL-4, IL-5, and IFN- γ in supernatants from cultured PBMCs were measured with the MesoScale Discovery (Gaithersburg, Md) platform, according to the manufacturer's instructions. The raw data were measured as electrochemiluminescence (light) detected by using photodetectors and analyzed with the Discovery Workbench 3.0 software (MesoScale Discovery). A 4-parameter logistic fit curve was generated for each analyte by using standards, and the analyte concentrations in each sample were calculated.²⁹

RNA extraction and real-time PCR mRNA amplification

Total cellular RNA was extracted from PBMCs by using the RNeasy Mini kit (Qiagen, West Sussex, United Kingdom), as previously described.²⁵ Briefly, 2 μ g of RNA were DNase treated and then reverse transcribed according to kit instructions. Quantitative real-time PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems, Warrington, United Kingdom). Real-time PCR primers were 5'-GCGCGCTGGTGGCTGCTGTGACACTGCGG-3' and 5'-AGTGGCCGCTGCAGGGAGGTAGAGCTCCAG-3' for human EP₁, 5'-CTTCAGCCTGGCCACGATGCTCATGCTCTT-3' and 5'-CAGGAAGTTTGTGTTGCATCTTGTGTTCTT-3' for human EP₂, 5'-CGTGACCTGTCCAAGCAGCGTTGGGAGCA-3' and 5'-CCGTGTGTGCTTGCAGTGCTCAACTGATG-3' for human EP₃, and 5'-TCAACCATGCCATTCTACAGCCACTACG-3' and 5'-AGGTCTCTGATATTCGCAAAGTCTCAGTG-3' for human EP₄. mRNA copy numbers were calculated for each sample by using the cycle threshold (Ct) value. 18S rRNA (a housekeeping gene) was amplified in parallel with tested genes. The number of amplification steps required to reach an arbitrary intensity Ct was computed. The relative gene expression was represented as follows:

$$2^{-\Delta\Delta C_t}$$

where $\Delta\Delta C_t = \Delta C_{\text{Target gene}} - \Delta C_{18S}$.

Measurement of PGE₂ and leukotriene E₄ levels in BAL fluid

Concentrations of leukotriene E₄ (LTE₄) and PGE₂ in BAL fluid were measured by using published techniques.^{14,30} Briefly, cell-free BAL fluid was acidified with HCl 1N to pH 3.4 to 3.6 and slowly passed through an octadecylsilyl-silica column (SepPak C 18 column) prewashed with 10 mL of ethanol and 10 mL of water. After washing the column with 10 mL of water and 1 mL of ethanol (35%), the eicosanoid was eluted from the column with 2 mL of absolute ethanol, and the samples were dried under a stream of nitrogen. PGE₂ and LTE₄ concentrations were measured by using commercial EIA kits

with detection limits of 5.0 and 3.1 pg/mL, respectively (Cayman Chemical, Ann Arbor, Mich).

Statistical analysis

Data were analyzed with the aid of a commercially available statistical package (Minitab for Windows, Minitab Release 9.2; Minitab, Inc, State College, Pa). Between-group ANOVA (Kruskal-Wallis) was followed by the Mann-Whitney *U* test. A *P* value of less than .05 was accepted as statistically significant.

RESULTS

Clinical data

Clinical and demographic details of the study subjects are summarized in Table I.

Bronchial mucosal inflammatory cells

Single immunohistochemistry showed that the median numbers of bronchial mucosal total MBP⁺ eosinophils and elastase-positive neutrophils were significantly increased in the asthmatic patients (aspirin-sensitive and non-aspirin sensitive) compared with those seen in control subjects (Table II), with significantly higher median numbers of both cells in aspirin-sensitive patients ($P = .0014$ and $.0097$, respectively). In contrast, the median number of tryptase-positive mucosal mast cells was significantly lower in the patients with ASA compared with the patients with NASA ($P = .041$), although the overall variability between groups was not significant. There were no differences between the groups in the median numbers of CD3⁺ T lymphocytes and CD68⁺ macrophages (Table II).

CysLT₁, CysLT₂, and PGE₂ receptor immunoreactivity on structural cells in the bronchial mucosa

Cells showing immunoreactivity for cysLT₁, cysLT₂, and all 4 EP receptors were detected in bronchial biopsy sections from all subjects (Fig 1). The overall amount of staining for each receptor expressed as a percentage of the total stainable area of the sections (median and range) is summarized in Table II. Staining was observed on both structural and infiltrating inflammatory cells. Global CysLT₁ but not CysLT₂ expression showed significant variation between the groups and was significantly greater in patients with both ASA and NASA compared with that seen in HCs (patients with ASA vs HCs, $P = .002$; patients with NASA vs HCs, $P = .0019$; Table II). Global expression of EP₁ and EP₂ immunoreactivity was also significantly variable between the groups and clearly increased in both patients with

TABLE II. Numbers of inflammatory cells and global expression of immunoreactivity for CysLT₁, CysLT₂, EP₁, EP₂, EP₃, and EP₄ receptors in bronchial mucosal sections from patients with ASA, patients with NASA, and HCs

	Subjects			ANOVA
	Patients with ASA (n = 11)	Patients with NASA (n = 15)	HCs (n = 15)	
Cellular marker				
CD3	16.8 (2.0-77.9)	21.2 (2.0-94.0)	21.6 (2.0-93.0)	.57
CD68	23.3 (10.0-98.5)	27.8 (8.5-77.1)	24.4 (5.0-70.0)	.97
Tryptase	24.7* (4.0-73.0)	35.4 (10.0-67.1)	28.3 (0.0-89.0)	.27
MBP	45.7†‡ (10.0-117.0)	21.2† (2.0-77.0)	7.4 (0.0-32.5)	.0001
Elastase	39.0†‡ (7.2-92.5)	18.5§ (2.0-153.3)	11.3 (4.0-25.5)	.01
Receptor				
CysLT ₁	63.9¶ (45.0-80.0)	63.5¶ (33.4-90.0)	46.0 (14.0-67.6)	.03
CysLT ₂	39.0 (15.0-69.6)	41.3 (23.7-68.3)	32.7 (13.1-66.7)	.26
EP ₁	31.0¶ (9.5-43.6)	25.6 (10.4-43.0)	16.7 (5.2-44.9)	.02
EP ₂	46.9¶ (31.0-61.0)	50.0¶ (27.4-72.9)	34.0 (22.7-59.2)	.02
EP ₃	41.3 (27.7-64.9)	37.7 (18.1-69.0)	32.9 (15.9-63.4)	.07
EP ₄	28.6 (16.0-61.0)	23.2 (13.5-62.3)	23.1 (14.6-40.0)	.57

Cellular markers: median (range) numbers of cells per square millimeter of sections (right-hand column shows Kruskal-Wallis ANOVA among the 3 groups).

Receptors: median (range) percentages of total stainable areas of biopsy specimens showing immunoreactivity.

* $P < .05$ (vs patients with NASA).

† $P < .01$ (vs HCs).

‡ $P < .01$ (vs patients with NASA).

§ $P < .05$ (vs HCs), Mann-Whitney U test.

|| $P < .05$ (vs HCs).

¶ $P \leq .002$ (vs HCs), Mann-Whitney U test.

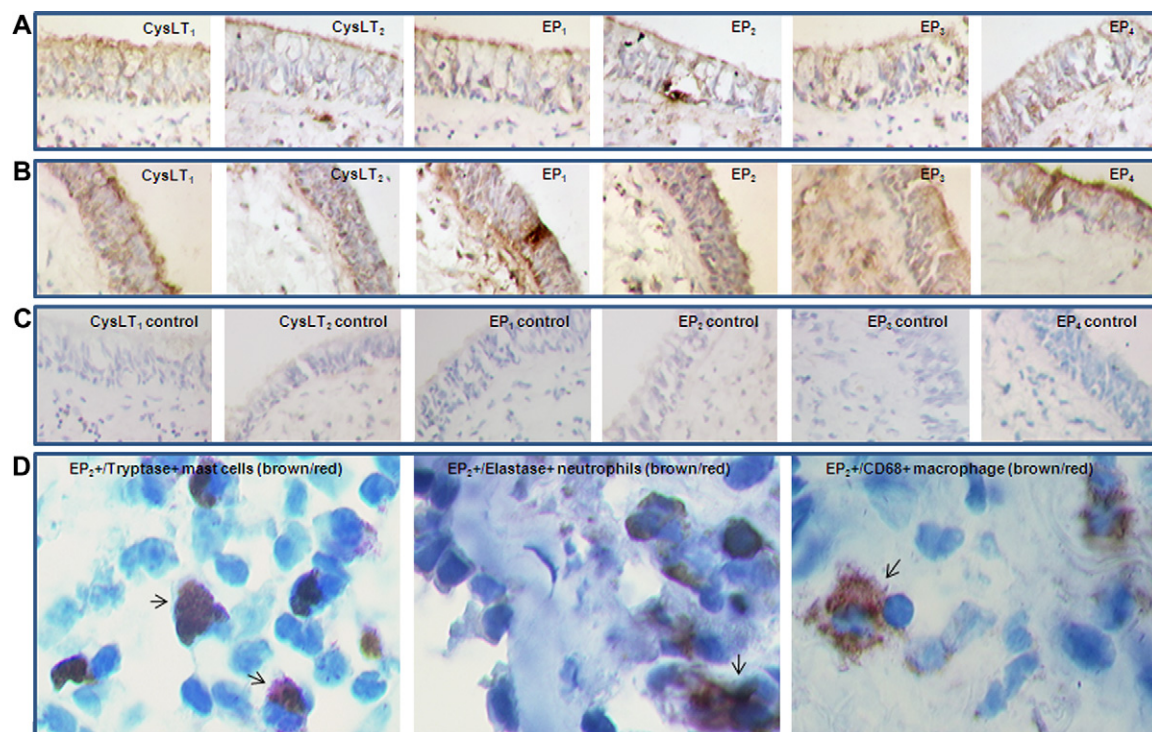


FIG 1. **A** and **B**, Typical patterns of immunoreactivity for CysLT₁, CysLT₂, EP₁, EP₂, EP₃, and EP₄ receptors in bronchial mucosal sections from patients with NASA (Fig 1, **A**) and ASA (Fig 1, **B**; brown, original magnification $\times 40$). **C**, Negative controls for each antibody. **D**, Some tryptase-positive mast cells (red), elastase-positive neutrophils (red), and CD68⁺ macrophages (red) expressing EP₂ (brown, arrows, original magnification $\times 100$).

ASA and patients with NASA compared with that seen in HCs (patients with ASA vs HCs: $P = .017$ vs $.017$; patients with NASA vs HCs: $P = .0465$ vs $.0161$, respectively). In contrast, global expression of EP₃ and EP₄ immunoreactivity did not

show significant variation among the 3 groups (Table II). Global expression of immunoreactivity for all 6 receptors did not significantly differ in the patients with ASA and those with NASA (Table II).

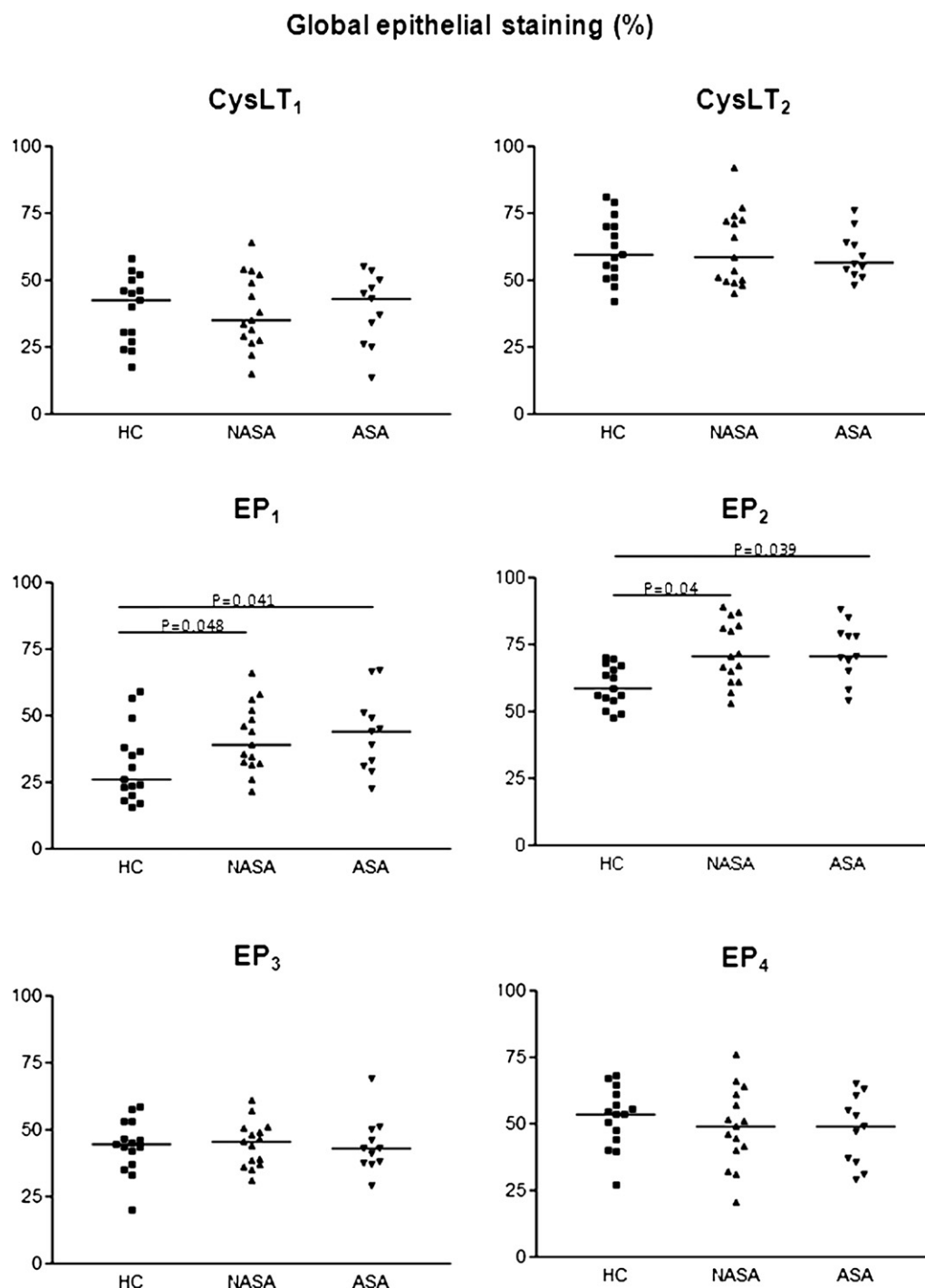


FIG 2. Global CysLT₁₋₂ and EP₁₋₄ receptor immunoreactivity (expressed as percentages of the total stainable areas) in the epithelium of bronchial mucosal sections from patients with ASA, patients with NASA, and HCs. The Mann-Whitney *U* test was used.

Substructural analysis showed that there was significantly increased expression of EP₁ and EP₂ but not CysLT₁, CysLT₂, EP₃, or EP₄ receptor immunoreactivity in the bronchial epithelium of patients with ASA and NASA compared with that seen in HCs (Fig 2), with no significant differences in expression in the asthma groups. In the submucosa global expression of immunoreactivity for CysLT₁, EP₁, and EP₂ but not CysLT₂ and EP₄

was observed in both patients with ASA and patients with NASA compared with that seen in HCs (Fig 3), with no significant differences in expression between the asthma groups. In addition, EP₃ immunoreactivity was significantly increased in the patients with ASA compared with that seen in HCs (Fig 3). There were no differences in the expression of any these receptors on smooth muscle or glandular tissue (data not shown).

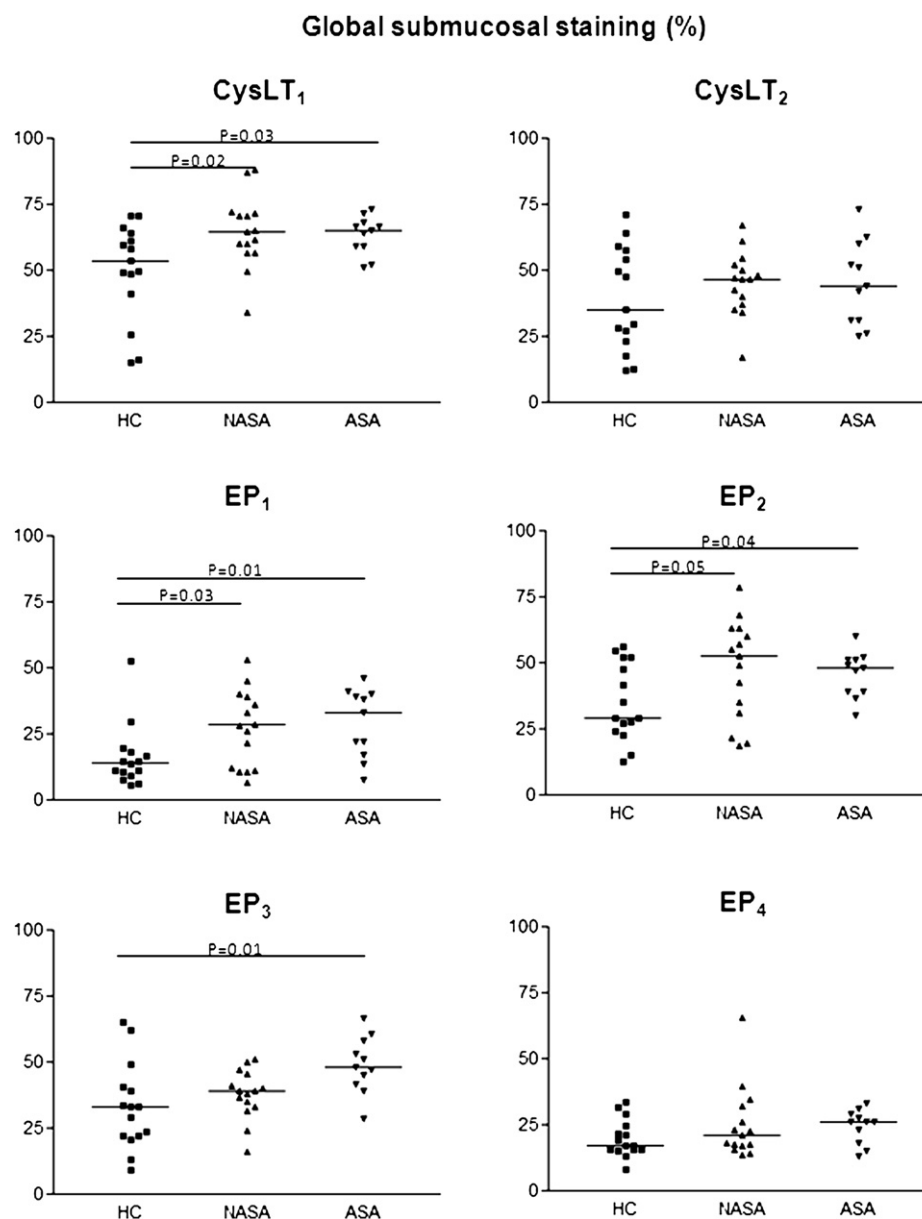


FIG 3. Global CysLT₁₋₂ and EP₁₋₄ receptor immunoreactivity (expressed as percentages of total stainable area stained) in the submucosa of bronchial mucosal sections from patients with ASA, patients with NASA, and HCs. The Mann-Whitney *U* test was used.

Bronchial reactivity to inhaled LTD₄

PC₂₀ LTD₄ levels were measured in 6 patients with NASA and 7 patients with ASA. As shown in Fig 4, LTD₄ PC₂₀ values did not correlate with global expression of CysLT₁ or CysLT₂ in the bronchial epithelium, the submucosa, or smooth muscle cells.

CysLT and EP receptor expression on inflammatory leukocytes

We further identified the phenotypes of mucosal inflammatory leukocytes expressing CysLT and EP receptors using sequential double immunostaining. As seen in Fig 5, the most striking finding was that significantly lower percentages of T cells, macrophages, mast cells, and neutrophils expressed the EP₂ receptor in the patients with ASA compared with those seen in patients

with NASA ($P = .023, .001, .001$, and $.005$, respectively). In contrast, the percentages of eosinophils expressing EP₂ and the percentages of all inflammatory leukocytes expressing CysLT₁, EP₁, EP₃, and EP₄ did not significantly differ between the 2 groups of asthmatic patients. Increased percentages of T cells, macrophages, mast cells, and neutrophils but not eosinophils expressed CysLT₁ in the asthmatic patients, regardless of aspirin sensitivity, compared with those seen in HCs, although these differences did not always attain statistical significance (Fig 5). In contrast, significantly lower percentages of T cells, mast cells, and neutrophils expressed CysLT₂ in the patients with ASA and, in the case of neutrophils, in both groups of asthmatic patients compared with HCs. Increased percentages of T cells, macrophages, eosinophils, and neutrophils, but not mast cells, expressed EP₁ in both groups of asthmatic patients compared

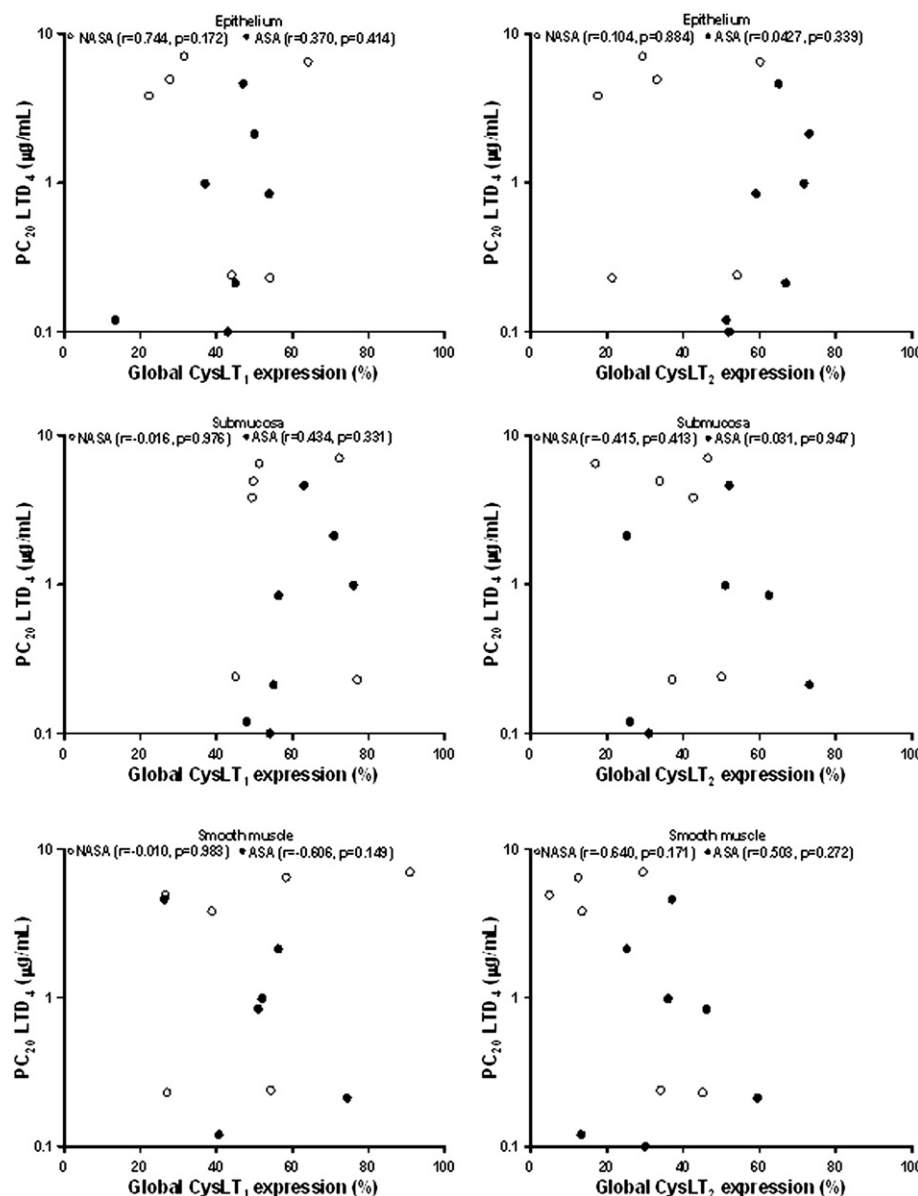


FIG 4. Correlations between LTD₄ PC₂₀ values and global expression of CysLT₁ and CysLT₂ receptor immunoreactivity in the epithelium (*top*), submucosa (*middle*), and smooth muscle (*bottom*) of patients with NASA (open circles) and patients with ASA (filled circles).

with those seen in HCs, whereas the percentages of eosinophils expressing EP₄ were reduced.

Effects of PGE₂ and EP receptor agonists on cytokine production by PBMCs

We investigated the ability of PGE₂ and agonists of all 4 of its receptors to inhibit the production of signal T_H1 and T_H2 cytokines by peripheral blood T cells from patients with ASA and patients with NASA in response to a pan-T-cell stimulus. As seen in Fig 6, both PGE₂ and EP₂ receptor agonist inhibited the production of IFN- γ , IL-2, IL-4, and IL-5 in a concentration-dependent fashion. In contrast, EP₁, EP₃, and EP₄ agonists were ineffective in this regard (data not shown). Quantitative PCR analysis verified that these same cells expressed equivalent amounts of mRNA

encoding all 4 EP receptors in both the patients with ASA and those with NASA (data not shown).

Concentrations of LTE₄ and PGE₂ in BAL fluid

The median concentration of PGE₂ in BAL fluid was slightly but significantly increased in patients with NASA but not the patients with ASA compared with those seen in the control group ($P = .0469$; median: patients with ASA, 12.3 pg/mL [range, 0-22.3 pg/mL]; patients with NASA, 20.3 pg/mL [range, 0-32.6 pg/mL]; HCs, 19.3 pg/mL [range, 0-23.8 pg/mL]), but overall, the range of concentrations of BAL fluid PGE₂ broadly overlapped between the groups, and there was no significant difference in these concentrations between the asthmatic groups ($P = .079$). No significant differences in median BAL fluid

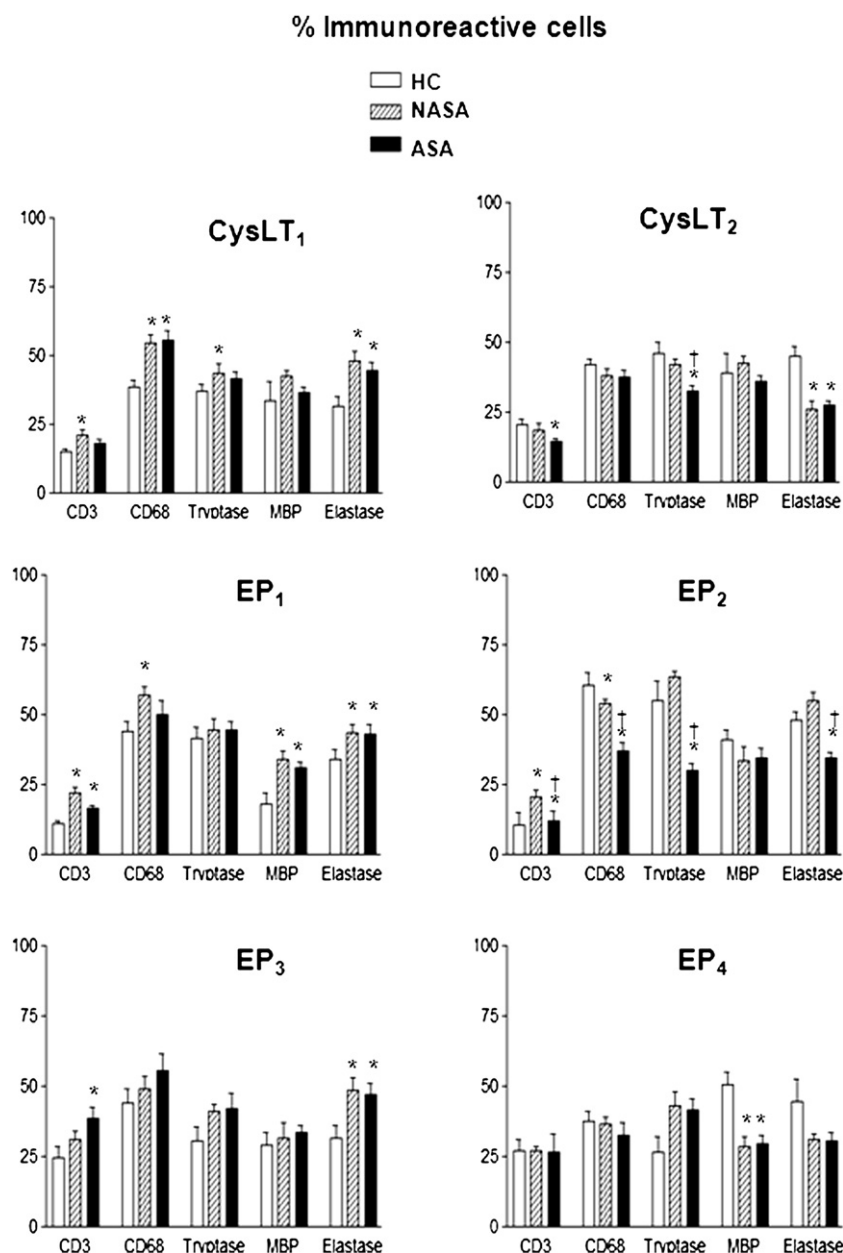


FIG 5. Percentages (means $\pm$ SEMs) of inflammatory leukocytes (CD3⁺ T cells, CD68⁺ macrophages, tryptase-positive mast cells, MBP⁺ eosinophils, and elastase-positive neutrophils) expressing immunoreactivity for CysLT₁₋₂ and EP₁₋₄ receptors in patients with ASA (n = 11), patients with NASA (n = 15), and HCs (n = 15). The Mann-Whitney U test was used. **P* < .05 (vs HCs) and †*P* < .05 (vs patients with NASA).

concentrations of LTE₄ were observed among the 3 groups (median: patients with ASA, 37 pg/mL [range, 8.3-668.2 pg/mL]; patients with NASA, 66.0 pg/mL [range, 21.4-421.2 pg/mL]; HCs, 69.0 pg/mL [range, 49.8-292.2 pg/mL]).

DISCUSSION

We believe this is the first systematic study of CysLT and EP receptor expression in the bronchial mucosa of patients with ASA. It follows from our previous studies on patients with aspirin-sensitive rhinosinusitis.^{22,24} Our data accord with these previous studies in demonstrating a striking deficiency of expression of the EP₂ receptor on a range of airway mucosal

inflammatory cells (T cells, macrophages, mast cells, and neutrophils) in patients with ASA and aspirin-sensitive rhinosinusitis. The possible pathophysiologic significance of this deficiency is underlined by our finding that, of the 4 EP receptors, only EP₂ appears to mediate PGE₂-induced inhibition of T cells. Furthermore, we saw no differences in the expression of these receptors, at least at the level of global mRNA expression, in blood mononuclear cells, suggesting (with the caveat that the relative proportion of T cells, B cells, and monocytes in mononuclear cell populations from individual patients might have differed) that the defect could be confined to the target mucosa. This might also explain why the differences in the inhibitory effects of EP₂ agonist on blood cells from the patients with ASA

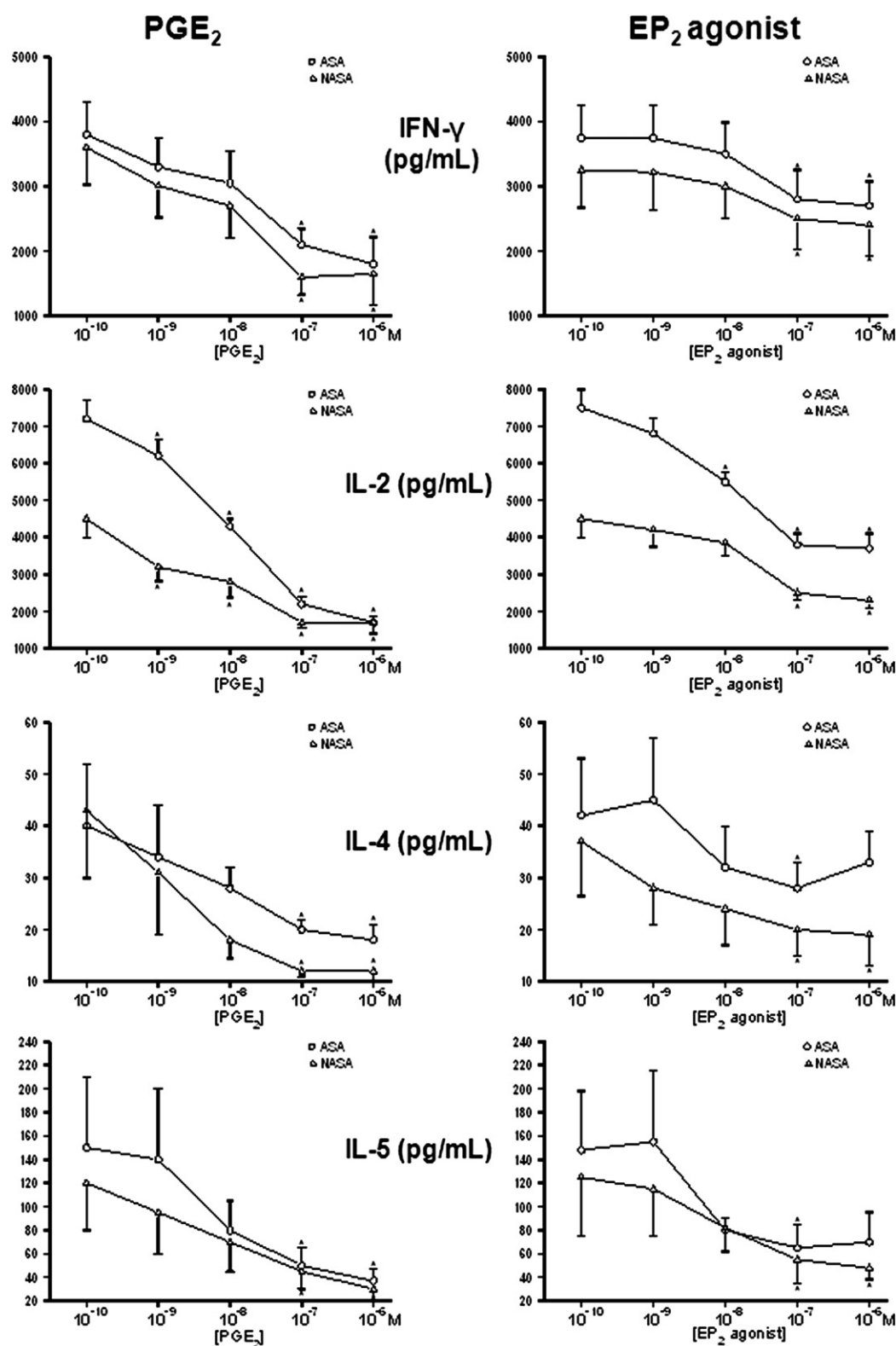


FIG 6. Effects of PGE₂ and EP₂ agonists on cytokine production by CD3/CD28-stimulated PBMCs from patients with ASA and patients with NASA (n = 10 for each group). Data are expressed as means $\pm$ SEMs. **P* < .05 (vs the effect of each agent at 10⁻¹⁰ mol/L concentration).

and those with NASA were not marked. Other studies have demonstrated inhibition by PGE₂ acting through the EP₂ receptor on T-cell activation,^{28,31} dendritic cell activation,³² eosinophil trafficking,³³ and human lung mast cell migration and activation.^{34,35}

On the other hand, and again in accord with other studies,^{19,20} we uncovered no evidence that patients with ASA are deficient in PGE₂ production compared with patients with NASA, at least as judged by comparison of median global concentrations of PGE₂ in the airways undergoing lavage. Overall, these observations support our hypothesis that PGE₂ signaling through the EP₂ receptor is critically deficient in patients with ASA, causing acute escalation of symptoms and CysLT synthesis when these patients encounter COX-1 inhibitors that inhibit PGE₂ synthesis.⁴ The mechanism of this reduced EP₂ receptor expression on inflammatory leukocytes within the respiratory mucosa of patients with ASA remains to be explored. Theoretically, it could reflect selective recruitment of low EP₂-expressing cell numbers, downregulation *in situ*, or both. In addition, although we did not find global reduction of PGE₂ synthesis in patients with ASA, studies on explanted tissue lines^{17,18,36} suggest that production might be locally deficient in the tissues, and this could conceivably affect local expression of PGE₂ receptors, although it is harder to see why this would specifically affect EP₂. Genetic susceptibility might also play a role.²³ Paradoxically, reduced EP₂ expression was least evident on eosinophils, which are thought to be the principal source of leukotrienes in patients with ASA,³⁷ suggesting that PGE₂-induced regulation of leukotriene synthesis might operate indirectly, for example through regulation of cytokine production by adjacent T cells. Finally, we did not observe a significantly increased mean BAL fluid concentration of LTE₄ in the patients with ASA compared with that seen in patients with NASA and HCs. Although it is somewhat dogmatic that patients with ASA overproduce CysLTs in the steady state, there is in fact little evidence to support this assertion in which they have been measured in local airways samples, either breath condensate²⁰ or BAL fluid,³⁸ and our data are consistent with this.

In common with our own previous study on patients with aspirin-sensitive rhinosinusitis,⁹ although the data were presented somewhat differently, we found an overall increase in the total number of leukocytes expressing CysLT₁ in the respiratory mucosa of patients with ASA. We also confirmed findings in previous studies by ourselves^{9,24,39} and others³⁷ that these patients have an increased neutrophil and eosinophil mucosal infiltrate. As mentioned, this might be one reason why patients with ASA overproduce CysLTs as a group because eosinophils appear to be a principal source,³⁷ although mast cells might contribute.⁴ On the other hand, we found reduced mucosal mast cell numbers in patients with ASA. We have previously shown⁴⁰ that endobronchial lysine aspirin challenge of these patients acutely reduces local mucosal mast cell numbers, suggesting acute degranulation, and it is intriguing to speculate that this process might be ongoing even in the absence of exposure to COX-1 inhibitors. In addition, CysLT₂ receptor stimulation has recently been reported to downmodulate CysLT-dependent mitogenic responses of mast cells *in vitro*,⁴¹ and therefore the paucity of this expression in patients with ASA *in vivo* might be of functional significance.

Despite the reduced expression of EP₂ receptors on inflammatory leukocytes in the bronchial mucosa of patients with ASA, there was increased global expression of both EP₁ and EP₂ in the bronchial epithelium and submucosa in all asthmatic patients compared with that seen in HCs. Submucosal EP₃ expression was

also increased in patients with ASA. We observed a similar phenomenon in the inflamed nasal epithelial mucosa.^{22,24} We speculate that EP receptor upregulation might be a nonspecific response to structural tissue injury. There is other evidence that PGE₂ protects epithelial cells from injury and promotes mucosal repair and that its receptors are upregulated on epithelial cells in an inflammatory milieu.^{42,43} Global submucosal CysLT₁ expression was also increased in the asthmatic patients, principally reflecting the increased leukocytic infiltrate.

Finally, the lack of correlation in the asthmatic patients between bronchoconstrictor responses to inhaled LTD₄ and global bronchial mucosal expression of both of its known receptors, CysLT₁ and CysLT₂, adds weight to the growing body of evidence that these and perhaps other effects of CysLTs are mediated through 1 or more receptors that have yet to be characterized.⁴⁴ We also did not observe significant correlations between LTD₄ PC₂₀ values and immunoreactivity for either of these receptors on bronchial smooth muscle cells. It should be borne in mind that immunohistochemistry is a semiquantitative technique and cannot be expected to assess actual numbers of the relevant receptor molecules expressed or their efficacy in signaling. Furthermore, PC₂₀ values might reflect mechanisms other than smooth muscle contraction, such as mucosal edema. Nevertheless, the data do not support the hypothesis that LTD₄-induced bronchoconstriction is mediated through CysLT₁ or CysLT₂ receptors.

In summary, patients with ASA and aspirin-sensitive rhinosinusitis might reflect critical deficiency in PGE₂/EP₂ signaling. EP₂ agonists, which have already been commercially developed, might obviate this deficit and promote epithelial repair in asthma generally.

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Clinical implications: PGE₂ and EP₂ agonists might benefit asthmatic patients, particularly patients with ASA, by inhibiting production of cytokines, leukotrienes, and possibly other inflammatory mediators.

REFERENCES

- Widal MF, Abrami P, Lenmoyez J. Anaphylaxie et idiosyncrasie. *Presse Med* 1922; 30:189-92.
- Samter M, Beers RF. Intolerance to aspirin. Clinical studies and consideration of its pathogenesis. *Ann Intern Med* 1968;68:975-83.
- Bochenek G, Nizankowska E, Szczeklik A. Atopy trait in hypersensitivity to non-steroidal anti-inflammatory drugs. *Allergy* 1996;51:16-23.
- Farooque SP, Lee TH. Aspirin-sensitive respiratory disease. *Annu Rev Physiol* 2009;71:465-87.
- Quiralte J, Blanco C, Castillo R, Delgado J, Carrillo T. Intolerance to non-steroidal anti-inflammatory drugs: results of controlled drug challenges in 98 patients. *J Allergy Clin Immunol* 1996;98:678-85.
- Picado C, Castillo JA, Monsterrat JM, Augusti-Vidal A. Aspirin intolerance as a precipitating factor of life-threatening attacks of asthma requiring mechanical ventilation. *Eur Respir J* 1989;2:127-9.
- Marquette CH, Sanulnier F, Leroy O, Wallaert B, Chopin C, Demarcq JM, et al. Long-term prognosis for near-fatal asthma. A 6-year follow-up study of 145 asthmatic patients who underwent mechanical ventilation for near-fatal attack of asthma. *Am Rev Respir Dis* 1992;146:76-81.
- Yoshimine F, Hasegawa T, Suzuki E, Terada M, Koya T, Kondoh A, et al. Contribution of aspirin-intolerant asthma to near fatal asthma based on a questionnaire survey in Niigata Prefecture, Japan. *Respirology* 2005;10:477-84.

9. Sousa AR, Parikh A, Scadding G, Corrigan CJ, Lee TH. Leukotriene-receptor expression on nasal mucosal inflammatory cells in aspirin-sensitive rhinosinusitis. *N Engl J Med* 2002;347:1493-9.
10. Celik G, Bavbek S, Misirligil Z, Melli M. Release of CysLTs with aspirin stimulation and the effect of prostaglandin E(2) on this release from peripheral blood leucocytes in aspirin-induced asthmatic patients. *Clin Exp Allergy* 2001;31:1615-22.
11. Sestini P, Armetti L, Gambaro G, Pieroni MG, Refini RM, Sala A, et al. Inhaled PGE2 prevents aspirin-induced bronchoconstriction and urinary LTE4 excretion in aspirin-sensitive asthma. *Am J Respir Crit Care Med* 1996;153:572-5.
12. Szczeklik A, Mastalerz L, Nizankowska E, Cmiel A. Protective and bronchodilator effects of prostaglandin E and salbutamol in aspirin-induced asthma. *Am J Respir Crit Care Med* 1996;153:567-71.
13. Wang XS, Wu AY, Leung PS, Lau HY. PGE suppresses excessive anti-IgE induced cysteinyl leukotrienes production in mast cells of patients with aspirin exacerbated respiratory disease. *Allergy* 2007;62:620-7.
14. Holgate ST, Peters-Golden M, Panettieri RA, Henderson WR Jr. Roles of cysteinyl leukotriene in airway inflammation, smooth muscle function, and remodeling. *J Allergy Clin Immunol* 2003;111(suppl):S18-34.
15. Kolodnick JE, Peters-Golden M, Larios J, Toews GB, Thannickal VJ, Moore BB. Prostaglandin E2 inhibits fibroblast to myofibroblast transition via E prostanoicd receptor 2 signaling and cyclic adenosine monophosphate elevation. *Am J Respir Cell Mol Biol* 2003;29:537-44.
16. Vancheri C, Mastruzzo C, Sortino MA, Crimi N. The lung as a privileged site for the beneficial actions of PGE2. *Trends Immunol* 2004;25:40-6.
17. Kowalski ML, Pawliczak R, Wozniak J, Siuda K, Poniatowska M, Iwaszkiewicz J, et al. Differential metabolism of arachidonic acid in nasal polyp epithelial cells cultured from aspirin-sensitive and aspirin-tolerant patients. *Am J Respir Crit Care Med* 2000;161:391-8.
18. Pierchalska M, Szabó Z, Sanak M, Soja J, Szczeklik A. Deficient prostaglandin E2 production by bronchial fibroblasts of asthmatic patients, with special reference to aspirin-induced asthma. *J Allergy Clin Immunol* 2003;111:1041-8.
19. Mastalerz L, Sanak M, Gawlewicz-Mroccka A, Gielicz A, Cmiel A, Szczeklik A. Prostaglandin E2 systemic production in patients with asthma with and without aspirin hypersensitivity. *Thorax* 2008;63:27-34.
20. Sanak M, Gielicz A, Bochenek G, Kaszuba M, Nizankowska-Mogilnicka E, Szczeklik A. Targeted eicosanoid lipidomics of exhaled breath condensate provide a distinct pattern in the aspirin-intolerant asthma phenotype. *J Allergy Clin Immunol* 2011;127:1141-7.
21. Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. *Physiol Rev* 1999;79:1193-226.
22. Ying S, Meng Q, Scadding G, Parikh A, Corrigan CJ, Lee TH. Aspirin-sensitive rhinosinusitis is associated with reduced E-prostanoid 2 receptor expression on nasal mucosal inflammatory cells. *J Allergy Clin Immunol* 2006;117:312-8.
23. Jinnai N, Sakagami T, Sekigawa T, Kakiyama M, Nakajima T, Yoshida K, et al. Polymorphisms in the prostaglandin E2 receptor subtype 2 gene confer susceptibility to aspirin-intolerant asthma: a candidate gene approach. *Hum Mol Genet* 2004;13:3203-17.
24. Corrigan C, Mallett K, Ying S, Roberts D, Parikh A, Scadding G, et al. Expression of the cysteinyl leukotriene receptor cysLT1 and cysLT2 in aspirin-sensitive and aspirin-tolerant chronic rhinosinusitis. *J Allergy Clin Immunol* 2005;115:316-22.
25. Corrigan CJ, Wang W, Meng Q, Fang CL, Lv Z, Fan YQ, et al. T-helper cell type 2 (Th2) memory T cell-potentiating cytokine IL-25 has the potential to promote angiogenesis in asthma. *Proc Natl Acad Sci U S A* 2011;108:1579-84.
26. Ying S, O'Connor B, Ratoff J, Meng Q, Fang CL, Cousins D, et al. Expression and cellular provenance of thymic stromal lymphopoietin and chemokines in patients with severe asthma and chronic obstructive pulmonary disease. *J Immunol* 2008;181:2790-8.
27. Staples KJ, Bergmann M, Tomita K, Houslay MD, McPhee I, Barnes PJ, et al. Adenosine 3',5'-cyclic monophosphate (cAMP)-dependent inhibition of IL-5 from human T lymphocytes is not mediated by the cAMP-dependent protein kinase A. *J Immunol* 2001;167:2074-80.
28. Takahashi HK, Liu K, Wake H, Mori S, Zhang J, Liu R, et al. Prostaglandin E2 inhibits advanced glycation end product-induced adhesion molecule expression, cytokine production, and lymphocyte proliferation in human peripheral blood mononuclear cells. *J Pharmacol Exp Ther* 2009;331:656-70.
29. Chowdhury F, Williams A, Johnson P. Validation and comparison of two multiplex technologies, Luminex and Mesoscale Discovery, for human cytokine profiling. *J Immunol Methods* 2009;340:55-64.
30. Martins JO, Wittlin BM, Anger DB, Martins DO, Sannomiya P, Jancar S. Early phase of allergic airway inflammation in diabetic rats: role of insulin on the signaling pathways and mediators. *Cell Physiol Biochem* 2010;26:739-48.
31. Okano M, Sugata Y, Fujiwara T, Matsumoto R, Nishibori M, Shimizu K, et al. E prostanoicd 2 (EP2)/EP4-mediated suppression of antigen-specific human T-cell responses by prostaglandin E2. *Immunology* 2006;118:343-52.
32. Schmidt LM, Belvisi MG, Bode KA, Bauer J, Schmidt C, Suchy MT, et al. Bronchial epithelial cell-derived prostaglandin E2 dampens the reactivity of dendritic cells. *J Immunol* 2011;186:2095-105.
33. Sturm EM, Schratl P, Schuligoi R, Konya V, Sturm GJ, Lippe IT, et al. Prostaglandin E2 inhibits eosinophil trafficking through E-prostanoid 2 receptors. *J Immunol* 2008;181:7273-83.
34. Kay LJ, Yeo WW, Peachell PT. Prostaglandin E2 activates EP2 receptors to inhibit human lung mast cell degranulation. *Br J Pharmacol* 2006;147:707-13.
35. Duffy SM, Cruse G, Cockerill SL, Brightling CE, Bradding P. Engagement of the EP2 prostanoicd receptor closes the K+ channel KCa3.1 in human lung mast cells and attenuates their migration. *Eur J Immunol* 2008;38:2548-56.
36. Roca-Ferrer J, Garcia-Garcia FJ, Pereda J, Perez-Gonzalez M, Pujols L, Alobid I, et al. Reduced expression of COXs and production of prostaglandin E(2) in patients with nasal polyps with or without aspirin-intolerant asthma. *J Allergy Clin Immunol* 2011;128:66-72.
37. Cowburn AS, Sladek K, Soja J, Adamek L, Nizankowska E, Szczeklik A, et al. Overexpression of leukotriene C4 synthase in bronchial biopsies from patients with aspirin-intolerant asthma. *J Clin Invest* 1998;101:834-46.
38. Szczeklik A, Sladek K, Dworski R, Nizankowska E, Soja J, Sheller J, et al. Bronchial aspirin challenge causes specific eicosanoid response in aspirin-sensitive asthmatics. *Am J Respir Crit Care Med* 1996;154:1608-14.
39. Nasser SM, Pfister R, Christie PE, Sousa AR, Barker J, Schmitz-Schumann M, et al. Inflammatory cell populations in bronchial biopsies from aspirin-sensitive asthmatic subjects. *Am J Respir Crit Care Med* 1996;153:90-6.
40. Nasser S, Christie PE, Pfister R, Sousa AR, Walls A, Schmitz-Schumann M, et al. Effect of endobronchial aspirin challenge on inflammatory cells in bronchial biopsy samples from aspirin-sensitive asthmatic subjects. *Thorax* 1996;51:64-70.
41. Jiang Y, Borrelli LA, Kanaoka Y, Baeska BJ, Boyce JA. CysLT2 receptors interact with CysLT1 receptors and down-modulate cysteinyl leukotriene dependent mitogenic responses of mast cells. *Blood* 2007;110:3263-70.
42. Savla U, Appel HJ, Sporn PH, Waters CM. Prostaglandin E2 regulates wound closure in airway epithelium. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L421-31.
43. Takafuji V, Cosme R, Lublin D, Lynch K, Roche JK. Prostaglandin receptors in intestinal epithelium: selective expression, function, and change with inflammation. *Prostaglandins Leukot Essent Fatty Acids* 2000;63:223-35.
44. Lee TH, Wozczek G, Farooque SP. Leukotriene E4: perspective on the forgotten mediator. *J Allergy Clin Immunol* 2009;124:417-21.