Airway lipoxin A_4/formyl peptide receptor 2–lipoxin receptor levels in pediatric patients with severe asthma

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Background: Lipoxins are biologically active eicosanoids with anti-inflammatory properties. Lipoxin A_4 (LXA_4) signaling blocks asthmatic responses in human and experimental model systems. There is evidence that patients with respiratory diseases, including severe asthma (SA), display defective generation of lipoxin signals despite glucocorticoid therapy.
Objective: We investigated airway levels of formyl peptide receptor 2–lipoxin receptor (FPR2/ALXR), LXA_4, and its counterregulatory compound, leukotriene B_4 (LTB_4), in patients with childhood asthma. We addressed the potential interplay of the LXA_4-FPR2/ALXR axis and glucocorticoids in the resolution of inflammation.
Methods: We examined LXA_4 and LTB_4 concentrations in induced sputum supernatants from children with intermittent asthma (IA), children with SA, and healthy control (HC) children. In addition, we investigated FPR2/ALXR expression in induced sputum cells obtained from the study groups. Finally, we evaluated *in vitro* the molecular interaction between LXA_4 and glucocorticoid receptor–based mechanisms.
Results: We found that children with SA have decreased LXA_4 concentrations in induced sputum supernatants in comparison with children with IA. In contrast to decreases in LXA_4 concentrations, LTB_4 concentrations were increased in children with asthma independent of severity. LXA_4 concentrations negatively correlated with LTB_4 concentrations and with exacerbation numbers in children with SA. FPR2/ALXR expression was reduced in induced sputum cells of children with SA compared with that seen in HC subjects and children with IA. Finally, we describe *in vitro* the existence of crosstalk between LXA_4 and glucocorticoid receptor at the cytosolic level mediated by G protein–coupled FPR2/ALXR in peripheral blood granulocytes isolated from HC subjects, children with IA, and children with SA.
Conclusion: Our findings provide evidence for defective LXA_4 generation and FPR2/ALXR expression that, associated with increased LTB_4, might be involved in a reduction in the ability of inhaled corticosteroids to impair control of airway inflammation in children with SA. (J Allergy Clin Immunol 2016;137:1796-806.)

Key words: *Childhood asthma, inflammation/resolution, induced sputum, lipoxin A_4, formyl peptide receptor 2–lipoxin receptor, glucocorticosteroids*

Bronchial asthma is defined as a chronic inflammatory disease of the airways linked to disease activity.[1] Although airway inflammation, structural changes of the bronchi, and resolution of inflammation are important features of asthma, little is known about the mechanisms responsible for disease severity in children.[2-4]

The natural resolution of inflammation is an active host response driven in part by decreases in levels of proinflammatory mediators.[5] The promotion of resolution is now recognized as a process, with early signaling pathways engaging biosynthetic circuits for the later formation of counterregulatory mediators as result of a "switch" of eicosanoid class from prostaglandin and leukotriene production to resolvin formation.[5] Lead members of this class of proresolving mediators,[6] lipoxins, are lipoxygenase interaction products derived from arachidonic acid as a result of cellular cooperation[7] with well-described anti-inflammatory and proresolution bioactivities.[8] After tissue injury or inflammation, lipoxins modulate both the innate and adaptive immunity, regulating leukocyte trafficking,[7,9] T-lymphocyte activation,[10] and dendritic cell function.[11]

Lipoxins are produced through cell-cell interactions between leukocytes and resident cells,[12] and they act at specific ALXRs expressed on both leukocytes and airway epithelial cells[13-15] to transduce their anti-inflammatory effects, which include inhibition of the formation and *in vivo* actions of leukotrienes, including the neutrophil chemotactic factor leukotriene B_4 (LTB_4), as well as downregulation of cytokines and mediators of allergic airway inflammation.[16] Lipoxin A_4 (LXA_4) and its synthetic stable analogs attenuate TNF-1α–initiated neutrophil responses and trafficking[17] and downregulate proinflammatory gene expression through inhibition of nuclear factor κB pathways

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Abbreviations used

ATS: American Thoracic Society
ERS: European Respiratory Society
FP: Fluticasone propionate
FPR2/ALXR: Formyl peptide receptor 2–lipoxin receptor
GR: Glucocorticoid receptor
GRE: Glucocorticoid response element
HC: Healthy control
IA: Intermittent asthma
ISS: Induced sputum supernatant
LTB4: Leukotriene B_4
LXA_4: Lipoxin A_4
PBG: Peripheral blood granulocyte
p-GR: Phosphorylated glucocorticoid receptor
RT: Room temperature
SA: Severe asthma

in mucosal inflammation.[18] Adults with severe asthma (SA) have decreased peripheral blood and bronchial LXA_4 concentrations,[16,19,20] and low blood concentrations of LXA_4 have been detected in wheezy infants.[21] However, the bioactivity of LXA_4 has not yet been investigated in asthmatic children.

Glucocorticosteroids are the most frequent anti-inflammatory agents used to treat asthma, yet SA in children can be challenging to treat and leads to persistent daily symptoms and exacerbations, contributing to a poor quality of life for the children and their relatives.[22] The reasons for asthma being difficult to treat require detailed investigation. Previously, we demonstrated that children with moderate-to-severe asthma present heterogeneous phenotypes, and although their symptoms are clinically well controlled by inhaled glucocorticosteroid treatment, they are not fully controlled in terms of both bronchial inflammation and disease management.[23,24] Glucocorticosteroids inhibit the synthesis of cytokines and proinflammatory mediators and favor their effects by increasing the expression of specific receptors.[25] In particular, it has been demonstrated that glucocorticosteroids are able to upregulate the formyl peptide receptor 2–lipoxin receptor (FPR2/ALXR),[26] identifying a specific effect of glucocorticosteroids toward the anti-inflammatory receptors. In addition, LXA_4 and its synthetic stable analogs, through G protein–coupled FPR2/ALXR activation signaling, attenuate the induction of proinflammatory gene expression.[8,17,18,27]

In view of the protective actions of lipoxins, the possibility of defective lipoxin counterregulatory signaling in the airways of children with SA can have potentially important pathophysiologic implications for disease severity.

The aim of the present study was to determine whether dysregulated lipoxin biosynthesis and an imbalance in eicosanoid switching during the resolution of inflammation were also present in asthmatic children and to investigate the potential interplay of the LXA_4-FPR2/ALX axis and glucocorticoids in the regulation of airway inflammation.

METHODS

Subjects

Pediatric subjects (age, 6-12 years) were recruited among outpatients attending the Pulmonology/Allergy Clinic of the Italian National Research Council in Palermo. Asthma severity was diagnosed according to European Respiratory Society (ERS)/American Thoracic Society (ATS) guidelines.[28]

Twelve children had intermittent asthma (IA), which was treated with short-acting β_2-agonists as requested during the last 3 months, and 17 children had SA, which was treated with high-dose inhaled corticosteroids (ICSs; ≥500 μg/d fluticasone propionate [FP]) for children less than 12 years of age.[28] The control group consisted of 7 healthy children.

The study was approved by the Institutional Ethics Committee of the Policlinic Hospital of Palermo University and complied with the Helsinki Declaration. Written informed consent was obtained from the parents of the patients enrolled in the study.

Clinical assessment and compliance of patients

Pulmonary function tests were performed, as recommended by the ATS. FEV_1 and forced vital capacity were measured according to ATS guidelines, and the best of 3 technically acceptable and reproducible measurements was retained.[29] Atopic status was assessed based on skin prick test responses[30] to aeroallergens commonly present in the Mediterranean area and total serum IgE measurements. Compliance with treatment was assessed by checking the inhalation technique at each visit. We also measured basal plasma cortisol concentrations at 8 AM by means of electrochemiluminescence twice (visits 1 and 4). Results were expressed in nanomolar concentrations, and adherence to ICSs was considered satisfactory if the cortisol level was less than 100 nmol/L.[31,32] At the beginning of the study, parents were provided with cell phone numbers of physicians who were always on call 24 hours a day for the entire follow-up period to accurately monitor daily symptoms. Parents were instructed to refer to physicians regarding the onset of any symptoms. When symptoms possibly related to exacerbations occurred, on the basis of the physician's evaluation, patients underwent visits on the same or the following day to verify, check, and treat the exacerbations. Moderate and severe exacerbations were defined and differentiated according to the ATS/ERS Task Force on Asthma Control and Exacerbations study.[33] Moderate exacerbations were defined as a 2-day increase in symptoms and signs of asthma requiring an increase in inhaled medications, including ICSs and bronchodilators. Severe exacerbations, which were defined as events requiring urgent action on the part of the patient and physician to prevent a serious outcome, were treated with a short course of oral steroids (1 mg/kg prednisolone for 2-5 days). The number of moderate and severe exacerbations that occurred during the follow-up period was recorded.

Study design

After a 1-month run-in period (started in September), during which FEV_1 values before and after bronchodilation (400 μg of salbutamol) were assessed in eligible children, patients and healthy control (HC) children attended the outpatient clinic (visit 1) and underwent clinical assessment (prebronchodilator and postbronchodilator FEV_1) after 4 (visit 2), 8 (visit 3), and 12 (visit 4) months by the same physician (S.L.G). In patients in whom an upper respiratory tract infection occurred, follow-up visits were postponed 2 weeks. Biomarker levels were measured at visit 1. Total and differential sputum cell counts and LXA_4 and LTB_4 sputum concentrations were measured in 7 HC subjects, 12 children with IA, and 17 children with SA. FPR2/ALX expression was evaluated in group subsets because of limitations in the amount of sputum cells recovered in 5 of 7 HC subjects, 6 of 12 children with IA, and 7 of 17 children with SA.

To better understand the role of LXA_4 in regulation of bronchial inflammation, we evaluated the ability of LXA_4 present in induced sputum supernatants (ISSs) from asthmatic children to direct peripheral blood granulocyte (PBG) migration using a chemotaxis assay. Finally, we investigated the interplay between LXA_4 concentrations and corticosteroids in the resolution of inflammation, assessing the effects of LXA_4 on glucocorticoid receptor (GR)–based mechanisms in PBGs isolated from healthy child donors, children with IA, and children with SA.

Sputum induction and processing

Each subject underwent spirometry before the beginning of the procedure. If FEV_1 was greater than 75% of predicted value at baseline and the child had

not used a short-acting β_2-agonist within the last 6 hours, we performed the procedure without premedication. Patients were exposed to 4% hypertonic saline aerosol solution while monitoring bronchial reactivity, as described elsewhere.[24,34]

Sputum processing was performed according to ERS recommendations.[35] Sputum was collected into previously weighted 50-mL sterile ampules. The volume of induced sputum was previously determined, and an equal volume of dithiothreitol (0.1% in saline; Sigma, St Louis, Mo) was added to the selected sputum. After homogenization, sputum samples were filtered (48-μm nylon cell strainer) and subsequently centrifuged at 800*g* for 10 minutes to separate the supernatants from the cell pellet. The supernatants were then aspirated and frozen at −80°C for subsequent biochemical analysis. Cells obtained from induced sputum were then cytocentrifuged (Cytospin 2; Shandon Instruments, Runcorn, United Kingdom) and stained with Diff-Quick (Merz-Dade, Dudingen, Switzerland). Slides were read blindly by 2 independent investigators, and differential cell counts were expressed as a percentage of 400 cells. Cytospin preparations for immunocytochemistry were prepared on 3-aminopropyltriethoxysilane–coated slides by adding 100 μL of cell suspension (5×10^5 cells/mL) into Shandon II cytocentrifuge cups and centrifugation at 180*g* for 5 minutes. The air-dried slides were fixed in paraformaldehyde-lysine-periodate for 30 minutes and in 15% sucrose in Dulbecco PBS for 30 minutes. The slides were stored at −80°C until use for immunocytochemical staining.

Antibodies and reagents

LXA_4 was purchased from Calbiochem–Merck Chemicals (Darmstadt, Germany). FP was purchased from Sigma-Aldrich. Rabbit polyclonal anti-FPR2/ALX and FPR2/ALXR functional blocking peptide were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif). Rabbit anti-Human phosphorylated glucocorticoid receptor (p-GR) was obtained from Cell Signaling Technology (Danvers, Mass). Finally, anti–β-actin mAb was obtained from Sigma.

Measurement of LXA_4 and LTB_4 concentrations

Determinations of the absolute values of LXA_4 and LTB_4 in supernatants recovered from induced sputum cells of children with IA, children with SA, and HC children were assessed by using commercial ELISA kits (US Biological; Amersham, Little Chalfont, Bucks, United Kingdom). Spiking experiments were performed to assess the eicosanoid yield recovery. Known exogenous compounds were added in induced sputum samples from HC children (n = 2), children with IA (n = 3), and children with SA (n = 3). After ELISA analyses for eicosanoid determinations, recovery of exogenous mediators was 90% for LXA_4 and 92% for LTB_4.

Immunocytochemistry analysis

After thawing, immunoreactivity for FPR2/ALXR was evaluated in induced sputum cells by using the labeled streptavidin-biotin method (Alkaline Phosphatase Rabbit/Mouse/Goat; Universal LSAB + kit; Dako, Glostrup, Denmark), as previously described.[24] Cell identification was based on cell morphology under light microscopy (×400 final magnification), carefully referring to the cell type distribution in corresponding Diff-Quick–stained slides. Rabbit polyclonal anti-FPR2/ALXR (Santa Cruz Biotechnology) was diluted 1:50.

Isolation and culture of PBGs

PBGs were isolated from HC children, children with IA, and children with SA by means of dextran sedimentation and centrifugation over a Ficoll cushion, as previously described.[36] Briefly, heparinized blood was subjected to centrifugation for 15 minutes at room temperature (RT) and 280*g*, platelet-rich plasma was removed, and residual blood was combined with an equal volume of saline and a 0.5 volume of Dextran T-500 (6% wt/vol in saline) followed by thorough mixing and allowed to stand at RT for 30 minutes. The leukocyte-enriched upper phase was centrifuged for 15 minutes at RT and 280*g*. The pelleted cells were then subjected to erythrocyte lysis. Mononuclear cells were separated by means of centrifugation on Ficoll cushions. The pellet containing PBGs was then resuspended in RPMI medium. Purity of the PBGs evaluated by means of microscopic observation after cytocentrifugation and May-Grünwald-Giemsa staining was greater than 95% without contaminating peripheral mononuclear cells and platelets.

PBGs were cultured in complete RPMI medium supplemented with 10% FBS and treated with or without LXA_4 (100 and 200 nmol/L), FPR2/ALXR functional blocking peptide, and FP (10 nmol/L or 100 nmol/L). Treatment with LXA_4 and FP, both alone and in combination, was performed for 30 minutes. Pretreatment with FPR2/ALXR functional blocking peptide was started 10 minutes before LXA_4 addition.

Granulocyte chemotaxis assay

We evaluate the ability of ISSs from children with IA, children with SA, and HC children to induce granulocyte chemotaxis. Chemotaxis assay was performed with a 48-well microchemotaxis chamber (Neuro Probe; Costar, Columbia, Md). PBGs from healthy child donors were loaded into the upper well (5×10^4 PBGs/well), and the sputum supernatant diluted 1:2 was placed in the bottom chamber separated by a filter paper with a pore size of 3 mm. After incubation for 45 minutes at 37°C, cells that had migrated across the filter were dehydrated, fixed, and stained with the Hemacolor Kit (Merck, Darmstadt, Germany). Stained cells from a particular well were then counted in 5 randomly chosen high-power fields.

Total and cytoplasmic/nuclear protein extraction

Freshly isolated PBGs were washed with cold PBS and lysed in a buffer containing 10 mmol/L Tris-HCl (PH 7.4), 50 mmol/L NaCl, 5 mmol/L EDTA, and 1% Nonidet P-40; phosphatase inhibitors consisted of 20 mmol/L β-glycerophosphate, 0.3 mmol/L Na3VO4, and 1 mmol/L Benzamidine (ICN Biochemicals, Aurora, Ohio); and protease inhibitors consisted of complete protease inhibitor cocktail (Roche, Mannheim, Germany). Cell cytoplasmic and nuclear protein fractions were separated with a commercial kit (Pierce, Rockford, Ill). The protein content of supernatants was analyzed with a bicinchoninic acid assay (Pierce).

Western blot analysis

The expression of the phosphorylated form of the GR was evaluated in total protein extracts of PBGs from healthy child donors by using Western blot analyses, as previously described,[23] to identify the effects of LXA_4 treatment on the GR. Anti–p-GR (Cell Signaling, Danvers, Mass) was diluted at 1:500.

TransAM GR assay

GR activation was evaluated by using the TransAM GR kit DNA-binding ELISA (96-well plate) in PBGs of HC children, children with IA, and children with SA. The specific binding of nuclear GR protein to the immobilized oligonucleotide containing the glucocorticoid response element (GRE) consensus sequence (5′-GGTACAnnnTGTTCT-3′) was assessed, and 1 μg of nuclear protein per well was evaluated for each experimental condition. On DNA binding, a primary antibody was used to recognize an accessible epitope on GR proteins. Subsequently, a secondary horseradish peroxidase–conjugated antibody was added to provide a sensitive colorimetric readout quantified by means of spectrophotometry (450 nm) on a Victor Wallac 1240 Multilabel counter (Wallac Oy, Turku, Finland).

Flow cytometry for FPR2/ALXR expression in PBGs

FPR2/ALXR expression was determined in PBGs of HC children by using flow cytometric analyses using indirect label immunofluorescence by a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, Calif), and Cell Quest acquisition with data analysis software (Becton Dickinson), as previously described.[36] PBGs were previously fixed with 4% paraformaldehyde in PBS, washed, and incubated with primary antibodies and rabbit polyclonal anti-FPR2/ALXR for 30 minutes at 4°C. Swine anti-rabbit IgG

TABLE I. Demographic, functional, and cellular characteristics of subjects

	HC children	Children with IA	Children with SA	*P* value C/IA	*P* value C/SA	*P* value IA/SA
No.	7	12	17			
Sex (male/female)	5/2	8/4	11/6			
Age (y)	10 (9-10)	9 (8-10)	10 (8-12)	NS	NS	NS
Total IgE (KU)	41 (17-52)	503 (308-857)	576 (387-770)	<.002	<.001	NS
FEV_1 (%), visit 1	101 (100-103)	96 (94-104)	89 (85-96)	NS	NS	NS
FEV_1 (%), visit 2	105 (100-110)	97 (91-102)	90 (86-98)	NS	NS	NS
FEV_1 (%), visit 3	100 (100-102)	95 (91-100)	87 (78-96)	NS	NS	NS
FEV_1 (%), visit 4	102 (100-106)	98 (89-105)	88 (81-94)	NS	NS	NS
FEV_1/FVC ratio (%), visit 1	100 (98.5-105)	94 (86-103)	84 (75-86)	NS	<.001	<.01
FEV_1/FVC ratio (%), visit 2	103 (102-107)	92 (89-99)	87 (80-90)	.01	<.001	.01
FEV_1/FVC ratio (%), visit 3	101 (100-106)	93 (90-98)	85 (79-88)	NS	<.001	.02
FEV_1/FVC ratio (%), visit 4	104 (100-106.5)	92 (89-99)	86 (79-89)	.01	<.001	.01
Sputum eosinophils, visit 1	0	5.5 (3-8)	4 (3-9)	<.01	<.01	NS
Asthma duration (y)	NA	4 (3-6)	6 (4-6)			NS
Total exacerbations	0	2 (2-3)	3 (2-5)			.01
Severe exacerbations*	0	0 (0-1)	3 (2-3)			<.0001
Cortisol (nmol/L)†	325 (215-458)	200 (178-235)	75 (58-95)	<.001	<.0001	<.004
Cortisol (nmol/L)‡	350 (215-447)	222 (190-256)	71 (56-95)	<.002	<.0001	<.004

Results are expressed as medians (25th-75th percentiles).
FVC, Forced vital capacity; *NS*, not significant.
*Number of severe exacerbations over the 12 months of the study.
†Cortisol levels at study entry.
‡Cortisol levels at the end of the follow-up period (12 months).

fluorescein isothiocyanate $F(ab')_2$ (Dako LSAB, Glostrup, Denmark) was used as a secondary antibody of FPR2/ALXR; fluorescein isothiocyanate–conjugated rabbit IgG_1 was used as an isotype-negative control antibody (Dako LSAB). Granulocytes were gated by using forward and side scatter, and analysis was carried out on 50,000 acquired events for each sample. Cells were considered positive if their fluorescence 1 (FL1) was greater than a gate set to exclude all cells in the FL1 peak from an isotype-matched control antibody. Results of flow cytometry were expressed as the percentage of positive cells.

Statistical analysis

The Kolmogorov-Smirnov normality test was performed to assess data distribution. Clinical demographic and experimental data were generally not normally distributed and analyzed by using the Kruskal-Wallis test, followed by Bonferroni test correction for multiple comparisons. Correlations were calculated according to the Spearman rank test. *In vitro* data were analyzed with ANOVA, followed by Fisher correction.

RESULTS

Demographic and functional characteristics of patients

The demographic and functional characteristics of patients are reported in Table I. Although FEV_1 values were less in children with SA, no significant differences were found among the 3 study groups in terms of FEV_1 values at all visits. We observed a significant FEV_1/forced vital capacity decrease in children with SA in comparison with that seen in HC subjects and in children with SA in comparison with that seen in children with IA at all visits. In children with IA, 24 exacerbations occurred (mean of 2 exacerbations per patient), 22 of which were moderate and 2 of which were severe. In children with SA, 60 exacerbations occurred (mean of 3.5 exacerbations per patient), 32 of which were moderate and 28 of which were severe.

Detection of LXA_4 and LTB_4

LXA_4 and LTB_4 concentrations were determined in ISSs recovered from the 3 groups of subjects. LXA_4 concentrations were significantly increased in children with IA compared with those in HC children (Fig 1, *A*). Children with SA had significantly less LXA_4 than children with IA. LTB_4 concentrations were significantly increased in both children with IA and children with SA compared with those in HC children (Fig 1, *B*).

Total and differential cell counts in induced sputum samples

Eosinophil percentages were significantly higher in children with IA and children with SA than in HC children (Table II). The children with IA and SA also had a significantly increased percentage of neutrophils and a significantly decreased percentage of macrophages when compared with percentages in HC children (Table II). The median viability of the cells obtained from induced sputum samples was 79% (25th-75th percentile, 70% to 85%) in HC children and 70% (25th-75th percentile, 65% to 80%) in asthmatic children.

Expression of FPR2/ALXR in induced sputum cells

We performed immunocytochemistry analyses in induced sputum cells to determine FPR2/ALXR protein expression. The percentage of induced sputum cells positive for FPR2/ALXR (Fig 2) was significantly lower in children with SA than in children with IA. No differences were detected between children with SA and HC children. The percentage of sputum macrophages, eosinophils, and neutrophils positive for FPR2/ALX was significantly lower in children with SA than in children with IA, whereas children with SA and HC children had similar

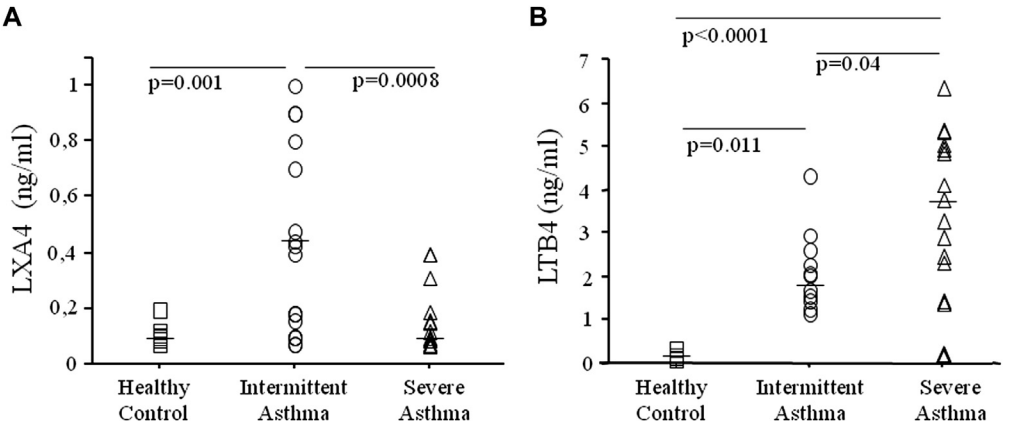

FIG 1. Measurements of LXA_4 and LTB_4 concentrations in ISSs. **A,** Assessment of LXA_4 concentrations in sputum supernatants of HC children, children with IA, and children with SA. **B,** Assessment of LTB_4 concentrations in sputum supernatants of HC children, children with IA, and children with SA. Individual data are shown. *Open squares* represent HC children, *open circles* represent children with IA, and *open triangles* represent children with SA. *Horizontal lines* represent medians.

TABLE II. Total and differential count of cells from induced sputum

				P value		
	HC children, visit 1	Children with IA, visit 1	Children with SA, visit 1	C/IA	C/SA	IA/SA
Total cell counts (10^6 cells/mL)	0.8 (0.5-1.5)	1.5 (0.8-1.9)	1.2 (0.6-1.8)	NS	NS	NS
Squamous cells (%)	3 (2-5)	2 (1-4)	2 (0-3)	NS	NS	NS
Macrophages (%)	91 (90-95)	58 (49.5-70.6)	54.5 (25-63)	†	†	NS
Eosinophils (%)	0	5.5 (3-8)	4 (3-9)	†	†	NS
Neutrophils (%)	9 (5-11)	32 (6-36)	38 (12-61)	*	†	NS
Lymphocytes (%)	1 (0-1.5)	3 (1-3)	2.5 (1.5-4.5)	NS	NS	NS
Epithelial cells (%)	0 (0-2.6)	1.5 (0-2)	1 (0-1.8)	NS	NS	NS

Results are expressed as medians (25th-75th percentiles) of positive cells with respect to total cell counts.
NS, Not significant.
*$P < .005$.
†$P < .0005$.

levels of FPR2/ALXR proteins in differential sputum cell evaluation (Table III).

Correlations

LXA_4 sputum concentrations positively correlated with LTB_4 concentrations in children with IA ($P < .0002$, Rho = 0.94; Fig 3, *A*). In children with SA, we found an inverse correlation between LXA_4 and LTB_4 sputum concentrations ($P = .0003$, Rho = −0.95; Fig 3, *B*). In this group of subjects, the number of severe exacerbations occurring during follow-up significantly and inversely correlated with LXA_4 concentrations detected in ISSs ($P = .04$; Rho = −0.69; Fig 3, *C*). No significant correlations were found between the total number of exacerbations and sputum LXA_4 concentrations.

Effects of sputum LXA_4 on PBG chemotactic activity

ISSs from asthmatic patients were tested for their ability to induce PBG chemotaxis in the presence or absence of exogenous LXA_4 (100 nmol/L [data not shown] or 200 nmol/L). Supernatants recovered from children with SA showed a significantly increased chemotactic activity for granulocytes when compared with supernatants from children with IA and HC children (Fig 4). The addition of exogenous LXA_4 to ISSs from children with IA and children with SA significantly inhibited their chemotactic activity for PBGs.

Effects of LXA_4 treatment on GR phosphorylation

To investigate the relationships between the LXA_4 pathway and corticosteroid-mediated mechanisms, we evaluated the effects of LXA_4 stimulation on GR phosphorylation. Expression of phosphorylated GR protein was evaluated by means of Western blot analyses in PBGs isolated from healthy child donors.

Western blot analyses of protein extracts obtained from PBGs showed that FP (10 nmol/L or 100 nmol/L) and LXA_4 (200 nmol/L) significantly increased the phosphorylation of GR on ser211 in comparison with unstimulated experimental conditions. The combination of 200 nmol/L LXA_4 and 10 nmol/L FP significantly increased GR ser211 phosphorylation when compared with the lower dose of FP. Cell pretreatment with the FPR2/ALXR functional blocking peptide counteracted the effect of LXA_4 on ser211 phosphorylation of the GR (Fig 5).

Effects of LXA_4 treatment on GR activity in PBGs

We next investigated the effects of LXA_4 stimulation on GR activity in PBGs isolated from HC children, children with IA, and children with SA. GR nuclear binding to GRE consensus

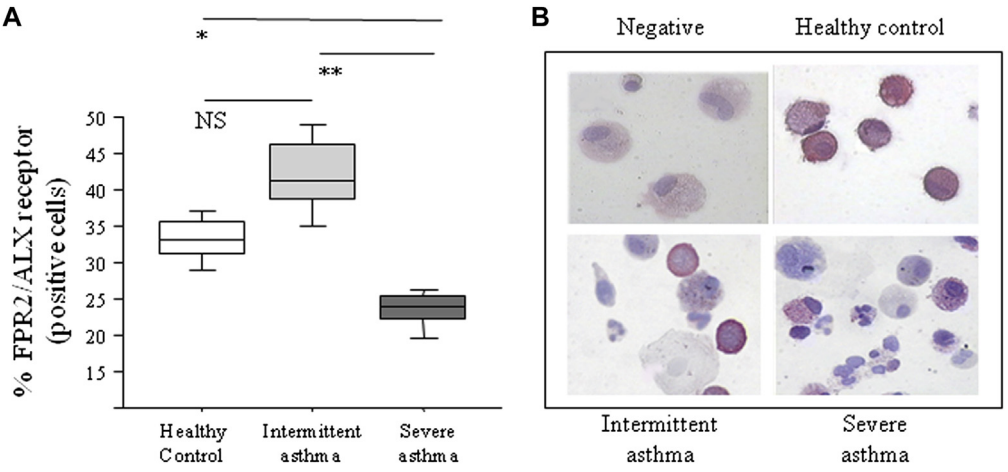

FIG 2. Immunocytochemistry analysis for FPR2/ALX in induced sputum cells from HC children, children with IA, and children with SA. **A,** Percentage of induced sputum cells expressing FPR2/ALXR in HC children, children with IA, and children with SA, respectively. **B,** Representative immunostaining of induced sputum cells for FPR2/ALXR expression (magnification ×40). *NS*, Not significant. *$P < .01$ and **$P < .001$.

TABLE III. Subcellular distribution of FPR2/ALX receptor by different cell types

	HC children, visit 1	Children with IA, visit 1	Children with SA, visit 1	*P* value		
				C/IA	C/SA	IA/SA
Total cells	36 (31-36)	45 (38-46)	24 (21-25)	NS	*	†
Macrophages (%)	32 (26-34)	20 (18-26)	10 (9-12)	NS	†	†
Eosinophils (%)	0 (0-0.5)	13 (12-15)	6 (7-9)	†	*	*
Neutrophils (%)	1 (0-2)	12 (10.5-15.5)	7 (5.5-8.5)	†	*	*
Lymphocytes (%)	0 (0-0.5)	1 (0.5-2)	1 (0.5-2)	NS	NS	NS

NS, Not significant.
*$P < .005$.
†$P < .0005$.

sequences was evaluated by using the TransAM GR assay. We found that FP (10 nmol/L or 100 nmol/L) significantly increased GR activity in PBGs of HC children, children with IA, and children with SA. LXA_4 induced GR activity, although this increment did not reach statistical significance in the 3 study groups. The combined treatment of 10 nmol/L FP and LXA_4 was significantly more effective than 10 nmol/L FP alone in inducing binding of the GR to its specific consensus sequence in PBGs of HC children, children with IA, and children with SA (Fig 6). The effect of FP alone and in combination with LXA_4 on GR activity was significantly less efficient in PBGs of children with SA than in HC children and children with IA.

Effects of FP and LXA_4 treatment on FPR2/ALXR expression in PBGs

Using flow cytometry, we evaluated the effects of FP and LXA_4 stimulation on FPR2/ALXR expression in PBGs isolated from healthy child donors.

Flow cytometric analyses showed that FP (10 nmol/L and 100 nmol/L) significantly increased FPR2/ALXR expression (Fig 7). Combined treatment of FP (10 nmol/L) and LXA_4 (200 nmol/L) was significantly more effective in inducing FPR2/ALX G-coupled receptor expression than lower doses of FP alone (Fig 7).

DISCUSSION

For the first time, the present findings demonstrate that children with SA have diminished LXA_4 concentrations in ISSs, despite high-dose inhaled glucocorticoid treatment, in comparison with those in children with IA. In contrast to decreases in LXA_4 concentrations, LTB_4 concentrations were increased in children with asthma independent of severity. Decreased LXA_4 concentrations, associated with increased LTB_4 concentrations in induced sputum would determine an imbalance in bioactive lipid mediators, favoring airway inflammation as a consequence of a potential persistent recruitment of granulocytes within the airways. In addition to LXA_4 concentrations, LXA_4 receptor expression was also decreased in induced sputum cells of children with SA. Moreover, we describe *in vitro* a functional interplay of LXA_4 and GR-based mechanisms.

Lipoxins are a class of eicosanoids that promote resolution of cytokine-driven acute inflammation,[37] block airway responsiveness, and reduce airway inflammation,[38] leading to a decrease in the number of inflammatory cells, inhibition of granulocyte infiltration in inflammation sites, and lower levels of proinflammatory mediators, such as cysteinyl leukotrienes.[37,38]

LXA_4 concentrations have been found to be decreased in children with SA.[39] In asthmatic children blood LXA_4 concentrations were gradually decreased with the severity of asthma, whereas blood LTB_4 concentrations were increased in asthmatic children from mild to moderate and severe degrees.[40] Here we describe

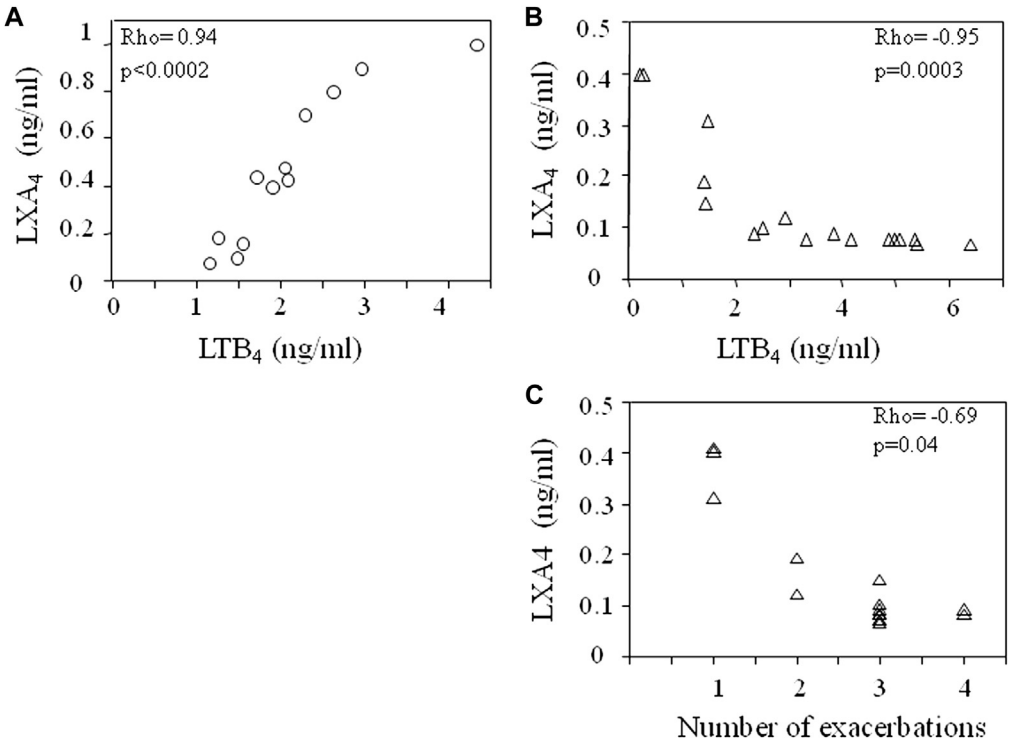

FIG 3. Correlations between sputum LXA_4 concentrations and sputum LTB_4 concentrations in children with IA **(A)** and children with SA **(B)** and between sputum LXA_4 concentrations and severe exacerbations **(C)** occurring in children with SA during 1-year follow-up. *Open circles* represent children with IA, and *open triangles* represent children with SA. Correlations were calculated by using the Spearman test.

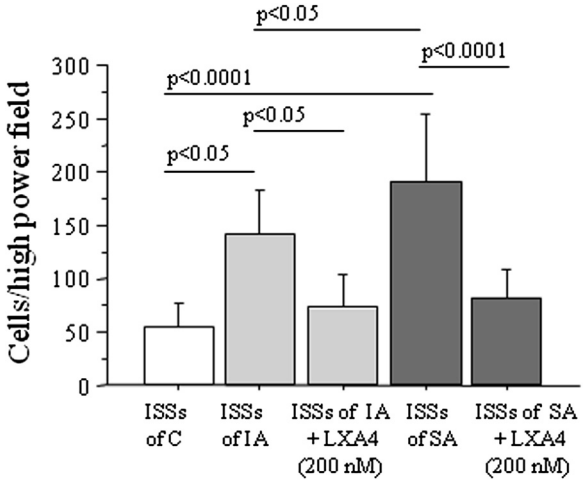

FIG 4. Granulocyte chemotaxis assay. Assessment of PBG chemotactic activity induced by sputum supernatants obtained from HC children, children with IA, and children with SA in the presence or absence of 200 nmol/L LXA_4. Results are expressed as means and SDs.

a significant decrease in LXA_4 concentrations in induced sputum samples of children with SA in comparison with those in children with IA, extending to the respiratory tract the earlier findings of dysregulated blood LXA_4 biosynthesis in children with moderate-to-severe asthma.[40] Unlike concentrations of LXA_4, LTB_4 concentrations were increased in induced sputum samples isolated from children with IA and children with SA in comparison with those from HC children. Although we acknowledge that the role of other resolving compounds in the regulation of asthma-induced inflammation should be further investigated, our findings suggest that an insufficient generation of LXA_4 and overproduction of leukotrienes might be the reason for difficult-to-control asthma in children. In addition, although we found a significant positive correlation between sputum LXA_4 and LTB_4 concentrations in children with IA, in children with SA, sputum concentrations of LXA_4 inversely correlated with LTB_4 concentrations, suggesting a trend toward an imbalance in eicosanoid switching during resolution of inflammation in this group of patients. These findings confirm previous results showing an imbalance between proinflammatory mediators and LXA_4 concentrations in airways of adults with SA.[39]

FPR2/ALXRs are G protein–coupled receptor proteins that bind LXA_4 with high affinity[41,42] and are expressed in leukocytes and structural cells of the lung. During allergic airway inflammation, LXA_4 is generated and FPR2/ALXR signaling decreases the generation of inflammatory mediators, leukocyte trafficking, and the tissue-resident cellular inflammatory response.[17] Significant decreases in FPR2/ALXR expression in induced sputum cells isolated from children with SA in comparison with that seen in children with IA and HC children show coordinate changes in both LXA_4 concentrations and its receptor expression in airways of this group of patients. Although we found heterogeneity in the group of children with SA, our data suggest that despite the ongoing ICS therapy, these patients display an unresolved evolution of inflammation as result of the altered LXA_4 biosynthetic pathway. These findings are further supported by the inverse correlation between LXA_4 concentrations in induced sputum from children with SA and the number of severe

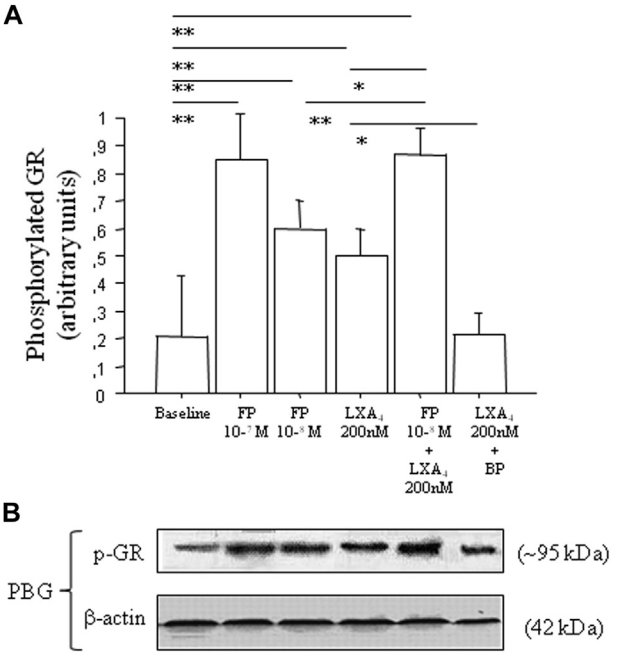

FIG 5. Phosphorylated GR protein expression in PBGs isolated from HC children. **A,** Expression of p-GR protein in PBGs. Signals corresponding to p-GR were semiquantified by using a densitometric scanner and expressed as the ratio of the band intensity of the phosphorylated protein versus the band intensity of the β-actin protein (National Institutes of Health Image/Gel Plotting analysis program). **B,** Representative Western blot analysis of p-GR in PBGs. Results are expressed as means and SDs. $*P < .01$ and $**P < .001$.

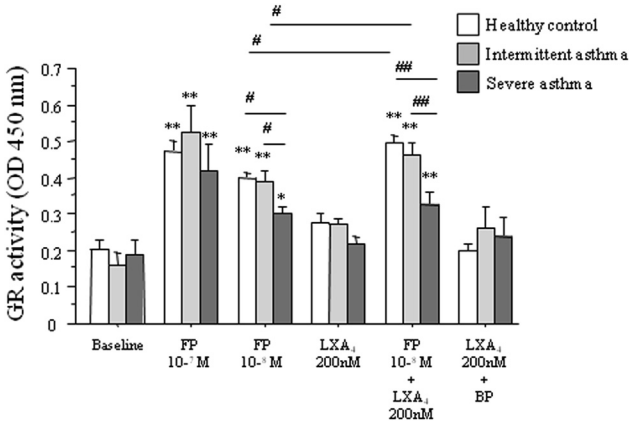

FIG 6. DNA-binding ELISA for GR activity. Assessment of nuclear GR protein binding to GRE sequences in PBGs isolated by HC children (n = 4), children with IA (n = 4), and children with SA (n = 5) in the presence or absence of FP, LXA_4, and LXA_4 blocking peptide. Modulation of GR binding activity under different experimental conditions is shown. Data are expressed as means and SDs. $*P < .01$ and $**P < .001$, significantly different from baseline values. $\#P < .01$ and $\#\#P < .001$ for the indicated comparisons.

exacerbations that occurred during the 1-year follow-up, uncovering a clinical relationship between LXA_4 biosynthesis, severity, and control of childhood asthma. In addition, these data support the relevance of pharmacologic control of the LXA_4-FPR2/ALX axis.

On the basis of these observations, we evaluated the role played by sputum LXA_4 in the regulation of granulocyte chemotactic activity, a process actively involved in the initiation and persistence of airway inflammation in asthma. We found that the chemotactic ability of PBGs induced by coincubation with sputum supernatants of children with SA was higher compared with that seen in children with IA, showing higher LXA_4 concentrations. The addition of exogenous LXA_4 at a nanomolar concentration to the sputum supernatants of children with SA resulted in a significant inhibitory effect of spontaneous granulocyte chemotactic activity. These findings suggest that increased chemotaxis of granulocytes exposed to induced sputum supernatants of children with SA is likely to be associated with both increased levels of neutrophil chemoattractants, such as LTB_4 or IL-8,[24] and diminished LXA_4 concentrations. In addition, the low concentration of resolving LXA_4 might interfere with leukocyte recruitment and trafficking, impairing the effects of chemotactic factors and favoring the persistence of airway inflammation.

The functional role of LXA_4 on chemoattraction of granulocytes is a constructive point to better understand the contribution of this pathway to limit airway inflammation in asthmatic patients, although further investigations on cell infiltration mechanisms need to be performed at the airway tissular level. In support, it has been shown recently that LXA_4 counterregulates eosinophilic granulocyte activation[43] and might favor eosinophil apoptosis through activation of natural killer cells.[44] Altogether, these data identify new important targets involved in active resolution of inflammation in children with SA.

Relationships between corticosteroid-mediated mechanisms and LXA_4 production are not well established in asthmatic patients. Although little information is available about the direct regulation of LXA_4 by corticosteroids and *vice versa*, it has been shown recently that glucocorticoids are able to induce the expression of FPR2/ALXR and to drive the resolution of inflammation,[26,45,46] suggesting a molecular link between glucocorticosteroids and the lipoxin pathway. Downstream signaling after FPR2/ALXR activation involves transient phosphorylation of extracellular-regulated kinases 1 and 2, which are capable of mediating human glucocorticoid receptor S211 phosphorylation in lymphoid cells.[47] GR phosphorylation on serine 211 has been linked to the GR transcriptional activation status and ability to transrepress.[48] Of interest, the induction of proinflammatory gene expression and regulation of the cytokine-chemokine axis are attenuated by LXA_4 and its synthetic stable analogs.[17,18]

In light of the abovementioned observations and to better characterize the interplay between LXA_4 and corticosteroids in the resolution of inflammation affecting the activity of granulocytes expressing FPR2/ALXR, we evaluated the role of LXA_4 on GR-based mechanisms in PBGs isolated from healthy child donors, children with IA, and children with SA. We found that LXA_4 is able to promote phosphorylation of the GR on ser211, an event mediated by FPR2/ALXR, as demonstrated by a reduction in GR phosphorylation after pretreatment of PBGs with the FPR2/ALXR functional blocking peptide.

We further investigated the effects of LXA_4 on GRE transactivation. Although we found that LXA_4 induces GR phosphorylation in a ligand-independent manner, we did not observe ligand-independent GRE activation in the presence of LXA_4 alone in PBGs of HC children. Similar results were obtained in PBGs isolated from children with IA and children with SA. However, combined treatment with FP and LXA_4 was

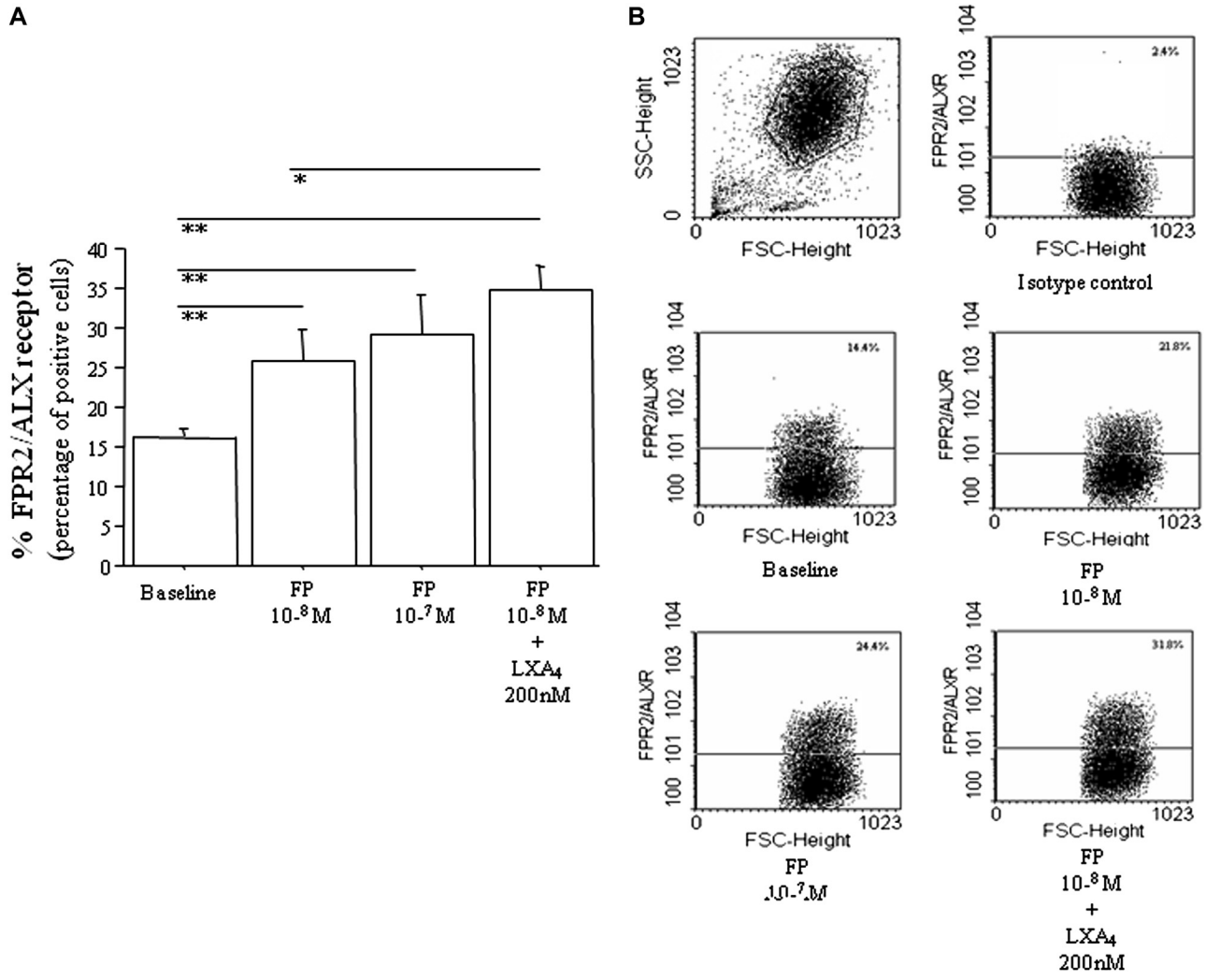

FIG 7. **A** and **B**, Flow cytometric analysis for FPR2/ALXR expression in PBGs of HC children at baseline and in the presence or absence of FP (10 nmol/L or 100 nmol/L), LXA_4 (200 nmol/L), and their combination. *Bars* represent means ± SDs of the percentage of positive cells. $*P < .01$ and $**P < .001$, significantly different from baseline values. Representative flow cytometry of FPR2/ALXR of each experimental condition.

significantly more effective in inducing GR binding to GREs than lower doses of FP alone in PBGs of HC children and children with IA. Interestingly, the effect of FP alone and in combination with LXA_4 on GR activity was significantly less efficient in PBGs of children with SA than in HC children and children with IA. These findings indicate a reduced responsiveness of primary granulocytes of children with SA to the combined treatment of FP with LXA_4, which was probably related to reduced expression of FPR2/ALXR.

Although the principal effects of glucocorticoids are mediated by transcriptional responses, a growing body of evidence suggests that GRs can also act through nongenomic mechanisms involving multiple mechanisms that ultimately impinge on the activity of various kinases strictly related to G coupled–receptor activation signaling.[49] Accordingly, here we extend to primary child granulocytes the earlier findings demonstrating that the steroidal molecule FP is able to induce expression of FPR2/ALXR.[26,46] Moreover, we found that in PBGs combined treatment with FP and LXA_4 was significantly more effective in inducing expression of FPR2/ALX G-coupled receptor than lower doses of FP alone.

Our results indicate the existence of crosstalk between LXA_4 and the GR at the cytosolic level mediated by the G protein–coupled FPR2/ALXR. In addition, induction of GR transactivation, as results from combined use of LXA_4 and FP, suggests a potential beneficial molecular anti-inflammatory interaction between LXA_4 and corticosteroids.

Although we acknowledge that the biological and clinical relevance of these findings needs to be further supported by larger studies, our results clearly indicate that SA in children is characterized by decreased bronchial LXA_4 concentrations associated with decreased ALXR availability in sputum cells. Impairment of LXA_4 biosynthesis, which is associated with increased LTB_4 production, might be involved in reduced effectiveness of the ongoing inhaled glucocorticoid therapy, as shown by the interplay of the LXA_4-FPR2/ALX axis and GR-based activation in PBGs.

Our findings suggest potential consideration of new therapeutic strategies directed toward combination of resolving compounds and glucocorticosteroids to sustain counterregulatory pathways.

Key messages

- **Severe childhood asthma is associated with decreased bronchial LXA_4 concentrations and FPR2/ALXR availability in airway cells.**
- **Crosstalk between LXA_4 and the GR at the cytosolic level is mediated by FPR2/ALX G-coupled receptor, and LXA_4 potentially induces GR transactivation mechanisms.**
- **The defective interplay of the LXA_4-FPR2/ALX axis and GR-based mechanisms might be involved in the reduced effectiveness of glucocorticosteroid therapy.**

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