

# Dysregulated synthesis of protectin D1 in eosinophils from patients with severe asthma

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**Background:** Protectin D1 (PD1) is an anti-inflammatory and proresolving lipid mediator biosynthesized from the omega-3 fatty acid docosahexaenoic acid (DHA). Exogenous PD1 conferred protection against eosinophilic inflammation in animals with experimental asthma, although its endogenous cellular source and functions in human airways are of interest. **Objective:** We sought to investigate the synthesizing capacity of PD1 in eosinophils from healthy subjects and patients with severe asthma and its direct effects on eosinophil functions. **Methods:** Human eosinophil-derived metabolites of arachidonic acid and DHA were analyzed with liquid chromatography–tandem mass spectrometry–based lipidomic analysis. The biological activities of PD1 on the function of human eosinophils, including chemotaxis, adhesion molecule expressions, degranulation, superoxide anion generation, or survival, were examined.

**Results:** We identified PD1 as one of the main anti-inflammatory and proresolving molecules synthesized in human eosinophils. PD1, in nanomolar concentrations, suppressed the chemotaxis induced by CCL11/eotaxin-1 or 5-oxo-eicosatetraenoic acid and modulated the expression of the adhesion molecules CD11b and L-selectin, although it had no effects on the degranulation, superoxide anion generation, or survival of the eosinophils. Compared with the cells harvested from healthy subjects, we observed a prominent decrease in the biosynthesis of PD1 by eosinophils from patients with severe asthma, even in presence of DHA.

**Conclusion:** These observations are a first indication that activated human eosinophils represent a major source of PD1,

which can act as a self-resolving machinery in eosinophilic inflammation, whereas the production of PD1 is impaired in patients with severe asthma. (*J Allergy Clin Immunol* 2012;■■■:■■■-■■■.)

**Key words:** Asthma, CCL11, docosahexaenoic acid, 15-lipoxygenase, 5-oxo-eicosatetraenoic acid, IL-5, lipid mediator, platelet-activating factor

There is growing evidence that both the amplification and resolution phases of acute inflammation are active processes and that inflammation is delicately balanced between positive and negative feedback systems. For example, neutrophils, innate immune cells essential in the host defense and tissue destruction that occurs in various diseases, produce leukotriene (LT) B<sub>4</sub>,<sup>1</sup> which acts as a strong autochemoattractant,<sup>2</sup> and amplify inflammation, whereas the interaction between neutrophils and platelets/airway epithelial cells generates lipoxin (LX) A<sub>4</sub> (5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid),<sup>3,4</sup> which, by inhibiting the trafficking of neutrophils to the tissue and by enhancing the removal of apoptotic neutrophils by phagocytic macrophages, contributes to the self-termination of inflammation.<sup>5</sup>

Eosinophils are another kind of multifunctional leukocyte implicated in the host defense against protozoan infection, as well as in the pathogenesis of allergic and immune diseases, such as asthma, eosinophilic pneumonia, and vasculitis.<sup>6–8</sup> Eosinophilic inflammation is usually suppressed by corticosteroids, although it often recurs during the tapering or after the discontinuation of treatment. In patients with refractory asthma, tissue eosinophilia persists despite treatment with high doses of inhaled corticosteroids. Like neutrophils, eosinophils are capable of synthesizing LTB<sub>4</sub>, LTC<sub>4</sub>, platelet-activating factor (PAF), prostaglandin (PG) E<sub>2</sub>, and PGD<sub>2</sub>,<sup>9–12</sup> which act as a positive feedback machinery in eosinophilic inflammation. However, it is unclear whether this cell type possesses an inhibitory self-regulatory system and, if it exists, whether it is dysfunctional in patients with diseases associated with persistent eosinophilic inflammation, such as severe asthma.

The consumption of fish oil, which is rich in omega-3 polyunsaturated fatty acid, such as docosahexaenoic acid (DHA; C22:6), has been associated with a decreased risk of asthma.<sup>13,14</sup> Furthermore, a dietary supplementation with DHA and other omega-3 fatty acids helps control the symptoms of asthma and other eosinophilic airway diseases.<sup>15,16</sup> Although unconfirmed, one of the possible mechanisms by which DHA promotes the resolution of eosinophilic airway inflammation is through the production of lipid mediators, such as neuroprotectin D1/protectin D1 (NPD1/PD1, 10R,17S-dihydroxy-11E,13E,15Z-DHA) and resolvins,

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**Abbreviations used**

|                               |                               |
|-------------------------------|-------------------------------|
| DHA:                          | Docosahexaenoic acid          |
| FITC:                         | Fluorescein isothiocyanate    |
| HBSS:                         | Hank's buffered salt solution |
| HETE:                         | Hydroxyeicosatetraenoic acid  |
| LC:                           | Liquid chromatography         |
| LT:                           | Leukotriene                   |
| LX:                           | Lipoxin                       |
| MS/MS:                        | Tandem mass spectrometry      |
| O <sub>2</sub> <sup>-</sup> : | Superoxide anion              |
| 5-oxo-EETE:                   | 5-Oxo-eicosatetraenoic acid   |
| PAF:                          | Platelet-activating factor    |
| PD1:                          | Protectin D1                  |
| PG:                           | Prostaglandin                 |
| SOD:                          | Superoxide dismutase          |

which are present in inflammatory exudates and neuronal tissues.<sup>17-19</sup> PD1 and resolvins not only act as potent anti-inflammatory lipid mediators limiting polymorphonuclear cell influx but also promote resolution of the inflammation by stimulating the clearance of apoptotic cells and inflammatory debris by monocytes.<sup>5</sup> It is noteworthy that the exogenous administration of PD1 attenuates the eosinophilic inflammation that takes place in murine lungs sensitized and challenged with an allergen without modifying the synthesis of IL-5 *in vivo*,<sup>20</sup> suggesting that the activity of PD1 might inhibit directly the chemotaxis, survival, or both of eosinophils.

In our lipidomic analysis in search of anti-inflammatory and proresolving molecules produced by human peripheral blood eosinophils, we have identified PD1 as one of the main anti-inflammatory lipid mediators produced by eosinophils. We tested its activities as a negative self-regulator of eosinophils and compared its biosynthesis in healthy subjects versus that in patients presenting with severe asthma.

**METHODS****Purification of human eosinophils from peripheral blood**

We used CD16 negative and positive selection method<sup>21</sup> to isolate eosinophils and neutrophils from the peripheral blood of healthy volunteers and patients presenting with severe asthma. Severe asthma was defined according to the American Thoracic Society workshop consensus definition of severe/refractory asthma.<sup>22</sup> This study was approved by the Institutional Review Boards of Keio University School of Medicine and the University of Tokyo, and all patients provided written informed consent to participate.

**Targeted liquid chromatography–tandem mass spectrometry–based lipidomics of eosinophils**

Liquid chromatography (LC)–tandem mass spectrometry (MS/MS) was performed as previously described.<sup>23,24</sup> Human eosinophils were isolated from healthy subjects and patients with severe asthma, suspended in Hank's buffered salt solution (HBSS;  $1.0 \times 10^6$  cells/mL), incubated with or without 1 to 10  $\mu$ mol/L DHA, and stimulated at 37°C for 30 minutes with 2  $\mu$ mol/L Ca<sup>2+</sup> ionophore. The incubation was stopped with 2 volumes of ice-cold methanol. The methanol extract was then diluted with 10 volumes of water, acidified with HCl to a pH of 3.5, and applied to Sep-Pak C18 cartridges (Waters, Milford, Mass) for solid-phase extraction. A deuterated internal standard, consisting of 1 ng of LTB<sub>4</sub>-6, 7, 14, 15-d<sub>4</sub> (Enzo Life Sciences, Farmingdale, NY), was added to the cell supernatants before extraction. We used a triple quadrupole linear ion trap mass spectrometer (QTRAP5500; AB SCIEX) equipped with a 1.7- $\mu$ m,  $1.0 \times 150$ -mm Acquity UPLC BEH C18 column

(Waters). The MS/MS analyses were performed in negative ion mode, and the eicosanoids and docosanoids were identified and quantified by using multiple reaction monitoring. Calibration curves between 1 and 1000 pg and the LC retention times for each compound were constructed with synthetic standards. LTC<sub>4</sub> and LTB<sub>4</sub> levels were measured by using ELISA (Cayman Chemical, Ann Arbor, Mich).

**PD1 preparation**

PD1 was prepared as described elsewhere.<sup>23</sup> Briefly, PD1 was synthesized by means of incubation of DHA (Sigma, St Louis, Mo) and soybean lipoxygenase (Type IB, Sigma) and purified by means of HPLC, and the synthesis was confirmed by using UV chromophore and LC-MS/MS analysis.

**Quantitative RT-PCR**

Total RNA was extracted from human eosinophils or neutrophils isolated from healthy subjects or severely asthmatic patients with an RNeasy Mini Kit (Qiagen, Valencia, Calif). The amounts of transcripts for *ALOX15*, *ALOX15B*, *LTC4S*, *LTA4H*, *ALOX5*, *ALOX5AP*, and *PGHS2* (the genes for 15-lipoxygenase-1 and 15-lipoxygenase-2, LTC<sub>4</sub> synthase, LTA<sub>4</sub> hydrolase, 5-lipoxygenase, 5-lipoxygenase-associated protein, and PGH synthase-2 [COX-2], respectively) were measured by means of reverse transcription with a High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, Calif), followed by quantitative PCR amplification using the TaqMan method with an ABI PRISM 7500 (Applied Biosystems). The comparative threshold cycle method was validated and used to interpret the results. Premixed PCR primers and TaqMan probes for human *ALOX15*, *ALOX15B*, *LTC4S*, *LTA4H*, *ALOX5*, *ALOX5AP*, *PGHS2*, and glyceraldehyde3-phosphate dehydrogenase (*GAPDH*) were obtained from Applied Biosystems. The PCR conditions were 1 cycle at 95°C for 20 seconds and 50 cycles at 95°C for 3 seconds and at 60°C for 30 seconds.

**Assay of eosinophilic chemotaxis**

The chemotactic activity of eosinophils was studied by using the previously described Boyden chamber method.<sup>21</sup> In brief, 300  $\mu$ L of 10 nmol/L human recombinant CCL11/eotaxin-1 (R&D Systems, Minneapolis, Minn) or 100 nmol/L 5-oxo-eicosatetraenoic acid (5-oxo-EETE, Cayman Chemical) was added to the lower compartment of a 96-well chemotaxis plate separated by a 3- $\mu$ m pore size filter (NeuroProbe, Gaithersburg, Md). We pretreated  $1.0 \times 10^6$  eosinophils/mL for 30 minutes in the presence or absence of 0.01 to 100 nmol/L PD1 and placed them in the upper compartment. After incubation, the eosinophil peroxidase activity in the lower chamber was measured by (1) incubating the plate for 10 minutes with a substrate solution composed of 0.5 mmol/L *o*-phenylenediamine, 10 mmol/L H<sub>2</sub>O<sub>2</sub>, and 0.1% Triton X-100 in Tris buffer (pH 8.0) followed by the addition of 4 mol/L H<sub>2</sub>SO<sub>4</sub> to stop the reaction and (2) measuring the absorbance at 490 nm.

**Flow cytometric analysis of the expression of adhesion molecule**

Eosinophils at  $1.0 \times 10^6$  cells/mL preincubated with or without PD1 (0.01–100 nmol/L) at 37°C in 5% CO<sub>2</sub> for 30 minutes were stimulated with 100 nmol/L CCL11 for 60 minutes or 100 nmol/L 5-oxo-EETE for 15 minutes. The cells were labeled with fluorescein isothiocyanate (FITC)–conjugated anti-CD11b mAb (Beckman Coulter, Fullerton, Calif) and phycoerythrin-conjugated anti-L-selectin mAb (Beckman Coulter) simultaneously for 30 minutes at 4°C. The expressions of CD11b and L-selectin were analyzed with an EPICS XL SYSTEMS flow cytometer (Beckman Coulter).<sup>25</sup> All measurements were corrected for the value derived from the corresponding control isotype antibody.

**Measurement of superoxide anion production**

The eosinophilic production of superoxide anion (O<sub>2</sub><sup>-</sup>) was measured as the superoxide dismutase (SOD)–inhibitable decrease in cytochrome C

levels, as described elsewhere.<sup>26,27</sup> We initially added 0.2 mg/mL SOD in HBSS supplemented with 0.1% gelatin to SOD control wells and HBSS/0.1% gelatin to all wells to a final volume of 100  $\mu$ L. After incubation with 0.01 to 100 nmol/L PD1,  $1.25 \times 10^6$  eosinophils/mL in HBSS supplemented with 0.1% gelatin mixed 4:1 with cytochrome C (12 mg/mL in HBSS supplemented with 0.1% gelatin) were incubated with 100 nmol/L 5-oxo-EETE, 1  $\mu$ mol/L PAF, or both. The absorbance of the cell suspensions in the wells was measured at 550 nm in a Synergy 4 microplate reader (Bio-Tek, Tokyo, Japan), followed by repeated measurements during the next 120 minutes. The production of  $O_2^-$  was calculated at an extinction coefficient of  $21.1 \text{ mM}^{-1} \text{ cm}^{-1}$  and expressed as nanomoles of cytochrome C decreased per  $1.0 \times 10^6$  cells minus the SOD control. By using trypan blue exclusion, the eosinophil viability after activation was greater than 95%.

### Degranulation assay

Purified eosinophils at  $1.0 \times 10^6$  cells/mL in HBSS supplemented with 0.1% gelatin were preincubated with 0.01 to 100 nmol/L PD1 at 37°C in 5%  $CO_2$  for 30 minutes. The cells were stimulated for 4 hours with or without 1  $\mu$ mol/L PAF, 100 nmol/L 5-oxo-EETE, or both.<sup>28</sup> After centrifugation, the concentration of eosinophil-derived neurotoxin protein in the cell-free supernatant was measured according to the instructions of MBL International (Nagoya, Japan), the ELISA's manufacturer.<sup>29</sup>

### Survival assay

Purified eosinophils were resuspended to  $1.0 \times 10^6$  cells/mL in RPMI 1640 supplemented with 10% FBS, 100 IU/mL of penicillin, and 100 mg/mL streptomycin. After pretreatment for 30 minutes with 0.01 to 100 nmol/L PD1 at 37°C in 5%  $CO_2$ , the cells were seeded for 24 to 48 hours in the presence or absence of 1 ng/mL IL-5. The extent of apoptosis in the eosinophil population was measured with a TACS Annexin V-FITC apoptosis detection kit (R&D Systems), as previously described.<sup>30</sup> The eosinophils were incubated at room temperature for 15 minutes with FITC-conjugated Annexin V and propidium iodide and analyzed with an EPICS XL SYSTEMS flow cytometer.

### Statistical analysis

Data are presented as means  $\pm$  SEMs. Differences in lipid mediator biosynthesis and mRNA expression were examined by using the Student *t* test. Dose-response relationships of DHA on PD1 biosynthesis and PD1 on eosinophil activities were analyzed with repeated-measures ANOVA, followed by the Bonferroni/Dunn procedure as a *post hoc* test. The data were analyzed with GraphPad Prism 4.0c software (GraphPad Software, San Diego, Calif). A *P* value of less than .05 was considered significant.

## RESULTS

### Lipidomic analysis of human peripheral blood eosinophils

For more information on lipidomic analysis of human peripheral blood eosinophils, see Fig 1. We have developed an LC-MS/MS system that can measure simultaneously more than 250 lipid metabolites derived from arachidonic acid, eicosapentanoic acid, and DHA. By using a CD16<sup>+</sup> selection method, human eosinophils isolated from the peripheral blood of nonatopic healthy subjects were stimulated with 2  $\mu$ mol/L of the  $Ca^{2+}$  ionophore A23187. Under these conditions, in 3 experiments the most abundant mediators of COX and 5-lipoxygenase were thromboxane B<sub>2</sub> ( $23.0 \pm 0.6 \text{ ng}/10^6$  eosinophils [mean  $\pm$  SEM]) and LTC<sub>4</sub> ( $99.1 \pm 11.3 \text{ ng}/10^6$  eosinophils), respectively. In addition to LTC<sub>4</sub>, these cells produced considerable amounts of LTB<sub>4</sub> ( $6.7 \pm 0.4 \text{ ng}/10^6$  eosinophils) and 5-oxo-EETE ( $8.7 \pm 1.7 \text{ ng}/10^6$  eosinophils), both of which are mediators of 5-lipoxygenase and potent activators of eosinophils. 15-Hydroxyeicosatetraenoic acid (15-HETE;  $21.6 \pm$

$5.8 \text{ ng}/10^6$  eosinophils) was the main metabolite among the metabolites of 15-lipoxygenase, whereas modest amounts of the metabolite 17-hydroxy DHA ( $1.6 \pm 0.4 \text{ ng}/10^6$  eosinophils) were detected. In contrast, less than  $0.1 \text{ ng}/10^6$  eosinophils of anti-inflammatory and proresolving lipid mediators, such as LXA<sub>4</sub> or LXB<sub>4</sub>, from arachidonic acid were produced, and only trace amounts of PD1 or resolvin D series from DHA were detectable.

A prominent shift in the lipidomic profile occurred through supplementation of the culture medium with 10  $\mu$ mol/L DHA before stimulation with A23187. The biosynthesis of 4-, 7-, 10-, 13-, 14-, and 17-hydroxy DHA metabolites was significantly increased, whereas the amounts of arachidonic acid-derived mediators were unchanged (5-HETE and 5-oxo-EETE) or decreased (LTB<sub>4</sub>, LTC<sub>4</sub>, and thromboxane B<sub>2</sub>). A large production of PD1 ( $10.9 \pm 4.2 \text{ ng}/10^6$  eosinophils) was notably induced in the presence of DHA, although the productions of the other DHA-derived mediators, resolvin D1 or D2, remained less than  $1 \text{ ng}/10^6$  eosinophils. Therefore PD1 was the dominant anti-inflammatory and proresolving molecule biosynthesized in human eosinophils harvested from healthy subjects.

### PD1 biosynthesis in eosinophils and neutrophils

The biosynthesis of PD1 in eosinophils was dependent on the presence of both A23187 and DHA (Fig 2, A). A clear dose-response relationship was observed between the amount of PD1 and the concentrations of DHA at 1 to 10  $\mu$ mol/L (Fig 2, B). When the eosinophils were stimulated with more physiologic molecules, such as 1  $\mu$ mol/L PAF and 1  $\mu$ mol/L 5-oxo-EETE, in the presence of DHA (10  $\mu$ mol/L), a moderate production of PD1 ( $0.87 \pm 0.24 \text{ ng}/10^6$  eosinophils; *n* = 3 experiments) was observed.

We found little synthesis of PD1 in the polymorphonuclear cell fraction of peripheral blood, consisting mostly of neutrophils, even in the presence of 2  $\mu$ mol/L A23187 and 10  $\mu$ mol/L DHA (Fig 2, A). To clarify the differences between eosinophils and neutrophils in the production of PD1, we examined the mRNA expression of the enzymes essential in the synthesis of PD1 and other lipid mediators. Compared with the eosinophils, the neutrophils expressed higher levels of *PGHS2*, *ALOX5*, and *ALOX5AP* mRNA. However, they lacked the mRNA expression of *ALOX15* and *ALOX15B*, which are necessary for the production of PD1, as well as that of *LTC4S* (Fig 3). In contrast, the eosinophils expressed both *ALOX15* and *ALOX15B*, explaining the high PD1-synthesizing power of this cell type.

### Effects of PD1 on 5-oxo-EETE- and CCL11-induced eosinophil chemotaxis

We then examined the biological effects of PD1 on the eosinophils. We first determined its effects on cellular chemotaxis by using a Boyden chamber assay and observed no direct effect on mobility in concentrations of up to 100 nmol/L (data not shown). After pretreatment of the eosinophils with PD1 in concentrations between 10 and 100 nmol/L, their chemotaxis on exposure to 100 nmol/L of the potent eosinophil attractant 5-oxo-EETE was significantly inhibited (*P* < .05; Fig 4, A). To exclude a specific activity conferred by PD1 on the 5-oxo-EETE receptor, we performed further experiments using 10 nmol/L CCL11/eotaxin-1, a CCR3 receptor ligand, as the chemoattractant. We observed, as with 5-oxo-EETE, a prominent inhibition by PD1 of the eosinophil

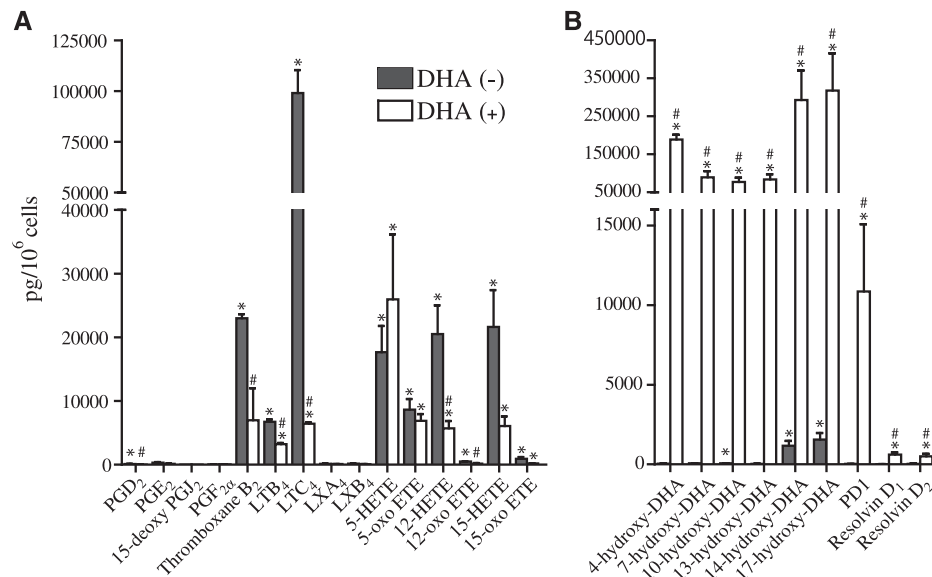


**FIG 1.** Lipidomic analysis of eicosanoids (**A**) and docosanoids (**B**) biosynthesized by activated eosinophils. Peripheral blood eosinophils purified from healthy subjects were stimulated with 2  $\mu\text{mol/L}$   $\text{Ca}^{2+}$  ionophore in the presence (*open bars*) or absence (*solid bars*) of 10  $\mu\text{mol/L}$  DHA. The results are means  $\pm$  SEMs of 3 experiments. \* $P < .05$  compared with unstimulated cells. # $P < .05$  compared with the levels in the absence of DHA.

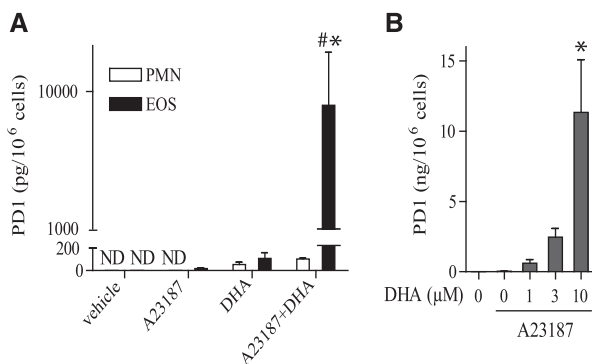


**FIG 2.** **A**, PD1 biosynthesis in polymorphonuclear leukocytes (PMN; *open bars*) and eosinophils (EOS; *solid bars*) isolated from peripheral blood of healthy subjects stimulated with 2  $\mu\text{mol/L}$  A23187, 10  $\mu\text{mol/L}$  DHA, or both. The results are means  $\pm$  SEMs of 3 experiments. ND, Not detected. \* $P < .05$  compared with vehicle-treated cells. # $P < .05$  compared with PMNs. **B**, PD1 biosynthesis in eosinophils in the presence or absence of 1 to 10  $\mu\text{mol/L}$  DHA. The results are means  $\pm$  SEMs of 3 experiments. \* $P < .01$  versus the nonstimulated cells.

chemotaxis to CCL11 in concentrations between 10 and 100 nmol/L ( $P < .05$ ; Fig 4, B).

### Effects of PD1 on the expression of CD11b and L-selectin on eosinophils

We further studied the effects of PD1 on eosinophilic function by examining whether it modulates the expression of adhesion molecules on the cell surface. PD1 levels, in a concentration-dependent manner between 0.01 and 100 nmol/L, markedly decreased (1) the expression of CD11b (a subunit of  $\beta_2$ -integrin, Mac-1) and (2) CD62L(L-selectin) shedding in eosinophils induced by 5-oxo-EETE or CCL11 (Fig 5).

### Effects of PD1 on the production of $\text{O}_2^-$ , degranulation, and eosinophil survival

The production of  $\text{O}_2^-$  and degranulation was measured by using a SOD-inhibitable cytochrome C reduction assay and release of eosinophil-derived neurotoxin, respectively, in eosinophils stimulated with 100 nmol/L 5-oxo-EETE with or without 1  $\mu\text{mol/L}$  PAF. Over 120 minutes of observation, PD1 at concentrations of up to 100 nmol/L neither inhibited the generation of  $\text{O}_2^-$  in stimulated eosinophils (Fig 6, A) nor had an effect on the degranulation of eosinophils (Fig 6, B).

We then studied the modulation of apoptosis by PD1 in eosinophils in the presence or absence of 1 ng/mL IL-5. After 48 hours of culture, 20.7%  $\pm$  6.3% of eosinophils were Annexin V positive in the presence versus 63.1%  $\pm$  7.9% in the absence of IL-5. In either condition, PD1 in concentrations between 0.01 and 100 nmol/L had no significant effect on the survival of eosinophils (Fig 6, C).

### Biosynthesis of PD1 in eosinophils from patients with severe asthma

After confirmation that PD1 is a self-produced negative regulator of eosinophils, we examined whether this negative feedback system is impaired in patients with severe asthma. Demographic data of the studied subjects are presented in Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org). Two patients were treated with oral corticosteroids, 1 patient was treated with an anti-IgE antibody, and all patients received a leukotriene receptor antagonist.

Compared with cells derived from healthy subjects, the biosynthesis of PD1 in the eosinophils of the patients with severe asthma was markedly decreased (Fig 7, A). There was also a significant decrease in their eosinophils in the levels of 15-HETE, a molecule synthesized through 15-lipoxygenase, by using arachidonic acid as the substrate. In contrast, the levels of 5-HETE synthesized from the same arachidonic acid

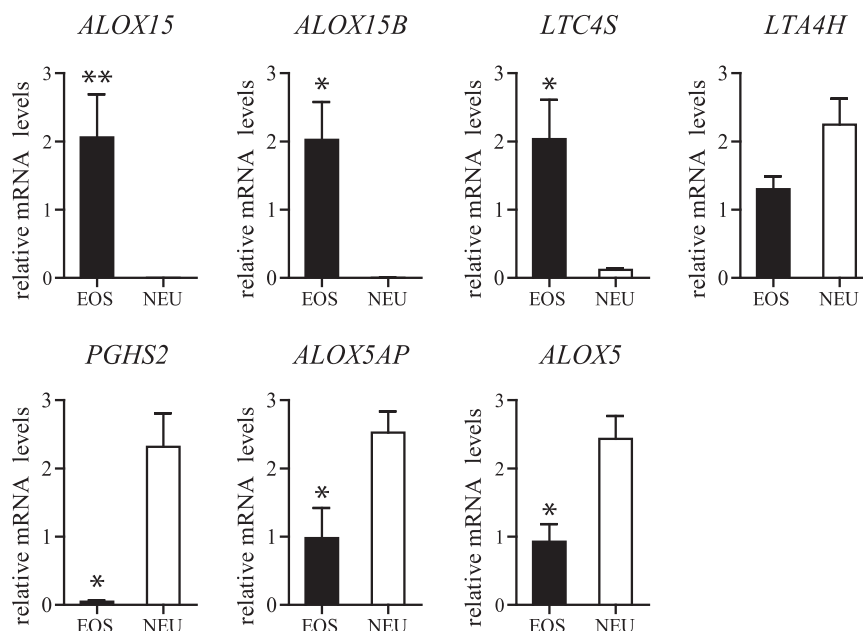


**FIG 3.** Gene expression of major synthetic enzymes for lipid mediators in eosinophils (EOS; solid bars) and neutrophils (NEU; open bars) isolated from healthy subjects. The results are means  $\pm$  SEMs of 3 or 4 experiments. \* $P < .05$  and \*\* $P < .01$  versus the levels in neutrophils. *ALOX5*, 5-Lipoxygenase; *ALOX5AP*, 5-lipoxygenase-associated protein; *ALOX15*, 15-lipoxygenase-1; *ALOX15B*, 15-lipoxygenase-2; *LTA4H*,  $LTA_4$  hydrolase; *LTC4S*,  $LTC_4$  synthase; *PGHS2*, PGH synthase-2.

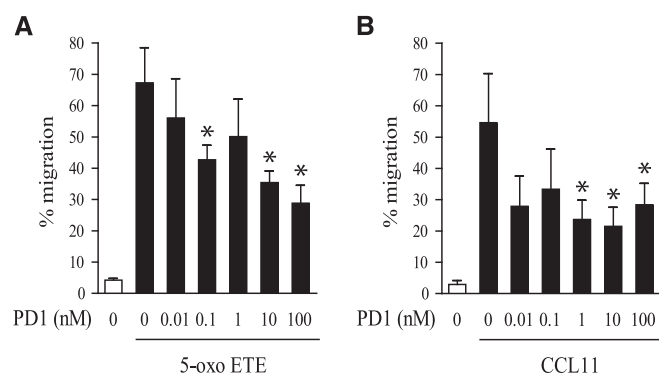


**FIG 4.** Effects of PD1 on the chemotaxis of eosinophils. Eosinophils were pretreated with PD1, and the chemotaxis to 5-oxo-ETE (100 nmol/L, **A**) or CCL11 (10 nmol/L, **B**) was examined. Chemotactic activity (percentage migration) is expressed as the ratio of eosinophil peroxidase activity in the migrated cells to the activity in the total loaded cells. The results are means  $\pm$  SEMs of 4 to 5 experiments. \* $P < .05$  compared with cells stimulated in the absence of PD1.

substrate, although with 5- instead of 15-lipoxygenase, were similar in the eosinophils of healthy subjects and patients with severe asthma. We also studied the gene expression of *ALOX15* in the eosinophils isolated from patients with severe asthma and found no significant differences in their *ALOX15* mRNA levels compared with those seen in the cells isolated from healthy subjects (Fig 7, B). The levels of 4-hydroxy DHA, a metabolite of DHA, were also decreased in the eosinophils harvested from patients with severe asthma (Fig 7, A).

## DISCUSSION

This study is the first to show that PD1 is the dominant anti-inflammatory, proresolving, DHA-derived lipid mediator

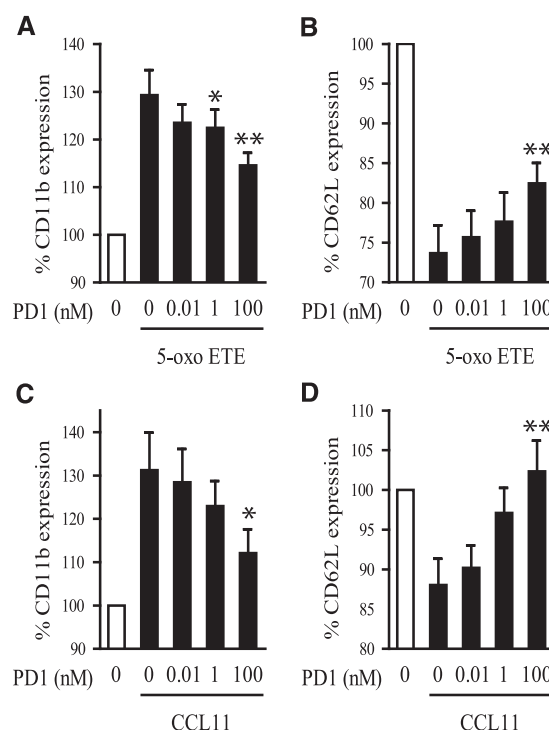


**FIG 5.** Modulation of the expression of CD11b and CD62L (L-selectin) shedding in eosinophils by PD1. The levels of CD11b and L-selectin expression were measured in eosinophils cultured in the presence or absence of 100 nmol/L 5-oxo-ETE (**A** and **B**) and 100 nmol/L CCL11 (**C** and **D**). \* $P < .05$  and \*\* $P < .01$  versus vehicle-treated cells ( $n = 4-5$ ).

biosynthesized in human eosinophils. In addition, PD1 itself negatively regulates the functions of eosinophils by inhibiting their chemotaxis and expression of adhesion molecules. We also

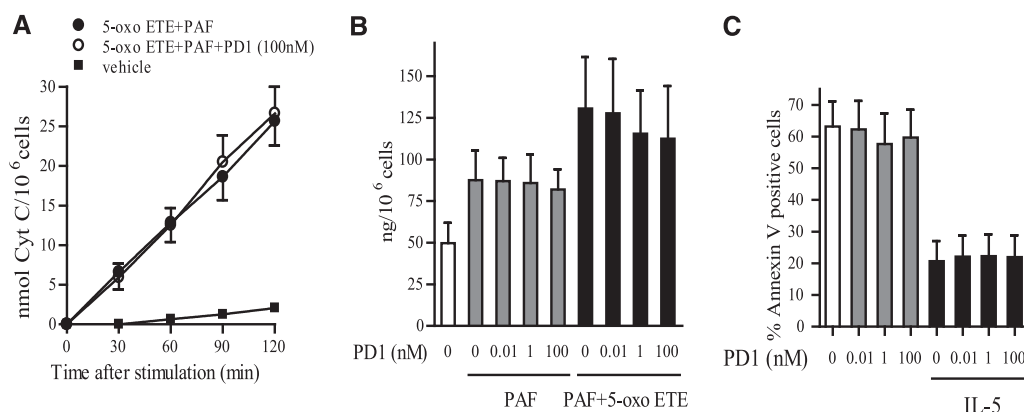


**FIG 6.** Effects of PD1 on the production of  $O_2^-$  (A) and degranulation (B) of eosinophils stimulated with 1  $\mu$ mol/L PAF in the presence or absence of 100 nmol/L 5-oxo-ETE and apoptosis/survival (C). The  $O_2^-$  production and degranulation was measured as the SOD-inhibitable decrease in cytochrome C (Cyt C) levels and the release of eosinophil-derived neurotoxin, respectively. Eosinophil apoptosis and IL-5-dependent survival was evaluated with flow cytometric analysis. The results are means  $\pm$  SEMs of 3 to 5 experiments.

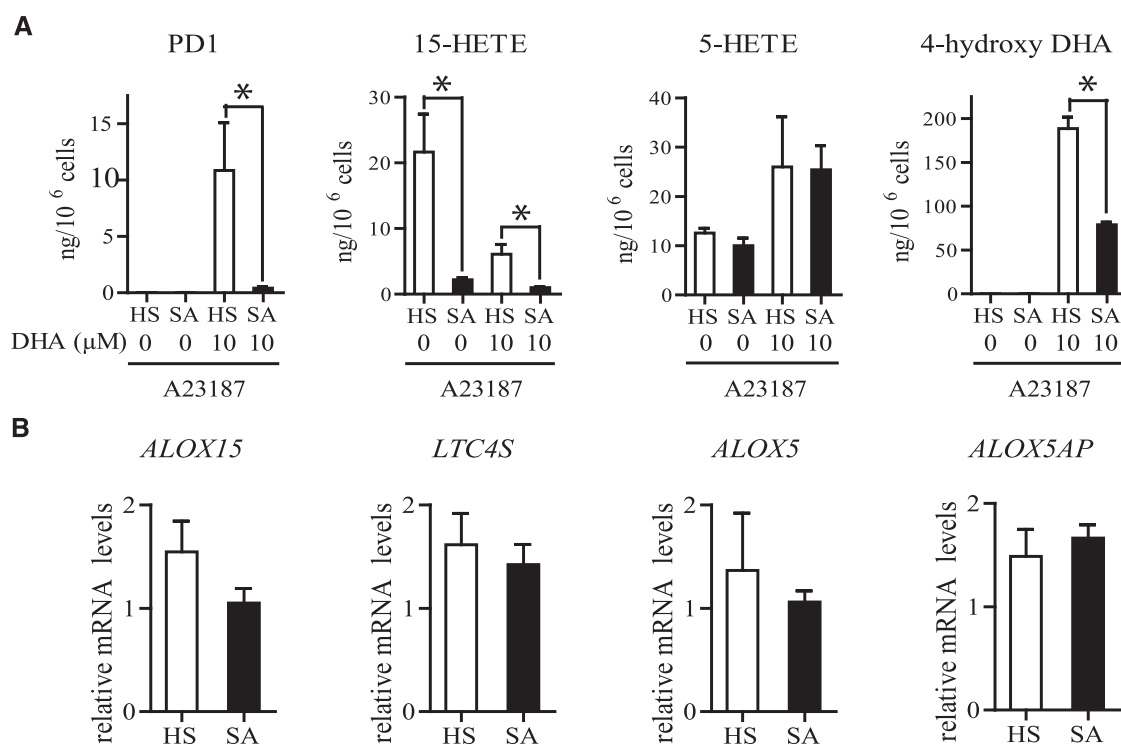


**FIG 7.** A, Amounts of 15-lipoxygenase mediators (PD1 and 15-HETE) and 5-lipoxygenase metabolites (5-HETE and 4-hydroxy DHA) released from eosinophils derived from healthy subjects (HS; n = 4) or patients with severe asthma (SA; n = 4). \* $P < .01$  compared with healthy subjects. B, Gene expression of 15-lipoxygenase (ALOX15), leukotriene  $C_4$  synthase (LTC4S), 5-lipoxygenase (ALOX5), and 5-lipoxygenase-associated protein (ALOX5AP) in eosinophils.

showed an impairment of this negative feedback system mediated by PD1 in the eosinophils of patients with severe asthma.

Several studies have suggested that DHA or DHA-derived lipid mediators suppress the development of eosinophilic inflammation *in vivo*. Exposure to aerosolized DHA decreases the accumulation of eosinophils in the lungs of sensitized mice in response to exposure to the allergen ovalbumin.<sup>31</sup> DHA-derived PD1 administered intravenously also inhibits the eosinophilic inflammation of the

airways and bronchial hyperresponsiveness in mice.<sup>20</sup> It might directly inhibit the migration of eosinophils, as suggested in a report of the attenuated chemotactic and chemokinetic activity to  $LTB_4$  of murine peritoneal eosinophils pretreated with DHA *in vivo*.<sup>32</sup> Our study has now confirmed that PD1 inhibits directly the migration and adhesion of human eosinophils, although not their survival. PD1 suppressed both CCL11- and 5-oxo-ETE-induced responses in eosinophils, indicating that the effects of PD1 are

not mediated by the blockade of a specific chemoattractant receptor but by the activation of intracellular stop signaling.

The biosynthesis of PD1 in the airways of healthy human subjects has been documented in condensates of exhaled breath.<sup>20</sup> Allergic sensitization of Fat-1 transgenic mice, which contain endogenous omega-3 polyunsaturated fatty acids abundantly in the lung, further increases the synthesis of PD1 in the lung tissue.<sup>33</sup> Because 15-lipoxygenases are the crucial enzymes that convert DHA to PD1,<sup>19,34</sup> specific cell types that express these enzymes, such as airway epithelial cells, eosinophils, and alveolar macrophages, should be the source of PD1 in the lungs. Neutrophils resident in the tissue or under chronic inflammation might also exhibit 15-lipoxygenase activity,<sup>35-37</sup> although freshly isolated neutrophils lack 15-lipoxygenase (Fig 2). Our observations showed unequivocally that stimulated eosinophils can produce large amounts of PD1 in the presence of its substrate, DHA.

The exact mechanism of how the biosynthesis of PD1 and other 15-lipoxygenase metabolites is regulated *in vivo* remains undetermined yet. Previous reports showed that calcium influx maximally activates 15-lipoxygenase in granulocytes,<sup>38,39</sup> which is consistent with the data of our experiments using  $\text{Ca}^{2+}$  ionophore. In contrast, the production of 15-HETE or PD1 in eosinophils was unchanged, with transient  $\text{Ca}^{2+}$  influx caused by combined stimulation of CCL11/PAF or CCL11/PAF/IL-5, although these stimuli significantly enhanced the synthesis of LTC<sub>4</sub> (see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Relatively large amounts of PD1 can be synthesized in unstimulated human eosinophils ( $12.7 \pm 5.2$  ng/mL PD1) in the presence of 10  $\mu\text{mol/L}$  DHA for a longer period (60 minutes, see Fig E1). Such a constitutive production of PD1 might be important for the PD1-mediated resolution of the inflammation *in vivo*.<sup>23</sup>

We found a surprisingly marked decrease in the biosynthesis of PD1 in the eosinophils harvested from patients with severe asthma, even in the presence of abundant substrate, an observation concordant with a previous report of a decrease in PD1 levels to less than the detection limit in exhaled breath condensates of asthmatic patients during exacerbation of the disease.<sup>20</sup> The decreased PD1 biosynthesis in the eosinophils from patients with severe asthma might be explained by (1) a decreased expression/activity of synthetic enzymes, such as 15-lipoxygenases, or (2) a decreased availability of DHA in the eosinophils. We found that the production of 15-HETE, a 15-lipoxygenase-dependent metabolite of arachidonic acid (a different substrate), was also decreased in the eosinophils from patients with severe asthma, supporting an impairment of 15-lipoxygenase activity. In contrast, the levels of 5-HETE, a 5-lipoxygenase-dependent metabolite of arachidonic acid, were similar in patients and healthy subjects, suggesting a selective dysregulation of the 15-lipoxygenase pathway. Our data are consistent with previous reports showing selective decrease among patients with severe asthma in the synthesis of LXA<sub>4</sub>, which is dependent on 15- and 5-lipoxygenase activities, and reduced expression of 15-lipoxygenase in the airways.<sup>40-42</sup>

Further scrutiny of the results of our lipidomic analysis indicated that the production of 4-hydroxy DHA, a DHA metabolite, in the eosinophils of patients with severe asthma was also decreased, although to a lesser extent ( $58\% \pm 2\%$ ) than that of 15-lipoxygenase-mediated DHA mediators, such as PD1 ( $96\% \pm 1\%$ ) or 17-hydroxy DHA ( $83\% \pm 2\%$ ). Therefore we hypothesize that the availability of exogenous precursors and the activity of synthetic enzyme are both decreased in the eosinophils of

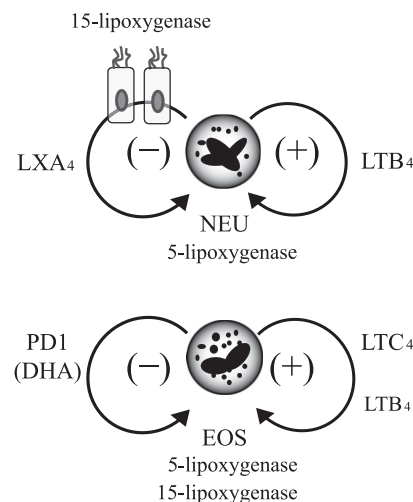


**FIG 8.** Hypothetical self-regulatory mechanism by lipid mediators of human neutrophils (NEU; top) and eosinophils (EOS; bottom). LTB<sub>4</sub> and LTC<sub>4</sub> produced by neutrophils, eosinophils, or both activate these cells through BLT1 and cysteinyl leukotriene 1/2 receptors. In contrast, LXA<sub>4</sub> biosynthesized in the presence of neutrophils and epithelial cells that express 15-lipoxygenase inhibits the neutrophil functions through its receptor, ALX. In eosinophils a similar self-regulatory mechanism is mediated by PD1 and its unknown receptor in the presence of DHA.

asthmatic patients, resulting in a profoundly decreased biosynthesis of PD1.

The abnormal substrate availability and activity of 15-lipoxygenase in the eosinophils of patients with severe asthma remains to be explained. Systemic corticosteroids might modulate the expression or activity (or both) of lipoxygenases. The 5-lipoxygenase activity of neutrophils isolated from patients treated with systemic corticosteroids is decreased without changing its expression levels,<sup>43</sup> an observation concordant with our finding of similar *ALOX15* gene expression in healthy subjects and asthmatic patients despite significant differences in enzymatic activities. In another study dexamethasone inhibited the production of LXA<sub>4</sub>, but not LTB<sub>4</sub>, in alveolar macrophages isolated from patients with severe asthma.<sup>44</sup> The leukotriene receptor antagonist or antagonists that all patients were receiving might also have changed the profile of lipid mediator synthesis. Further study with a larger number of samples from patients with mild-to-severe asthma will be required in the future.

In conclusion, this study shows that PD1 is one of the primary mediators of self-termination of eosinophilic inflammation (Fig 8). PD1 also inhibits the transmigration of neutrophils and promotes the clearance of apoptotic neutrophils,<sup>2</sup> although neutrophils cannot biosynthesize PD1 because of their lack of 15-lipoxygenase activity. We previously found that murine eosinophils recruited in the late phase of inflammation play an important role in a 12/15-lipoxygenase-dependent manner in the resolution of tissue neutrophilia.<sup>23,24</sup> Therefore the defects in the synthetic pathway of PD1 observed in the eosinophils harvested from patients with severe asthma might exacerbate not only eosinophilic but also neutrophilic inflammation. In these patients supplementation with DHA alone would be ineffective to suppress the inflammation because of the impaired conversion to PD1, whereas the administration of PD1 or of a molecule that can restore its synthetic pathway might represent a new and effective treatment of severe asthma.

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**Clinical implications: PD1 or its analogs that enhance the resolution of allergic inflammation might be useful to treat patients with severe asthma, in whom PD1 biosynthesis is severely impaired.**

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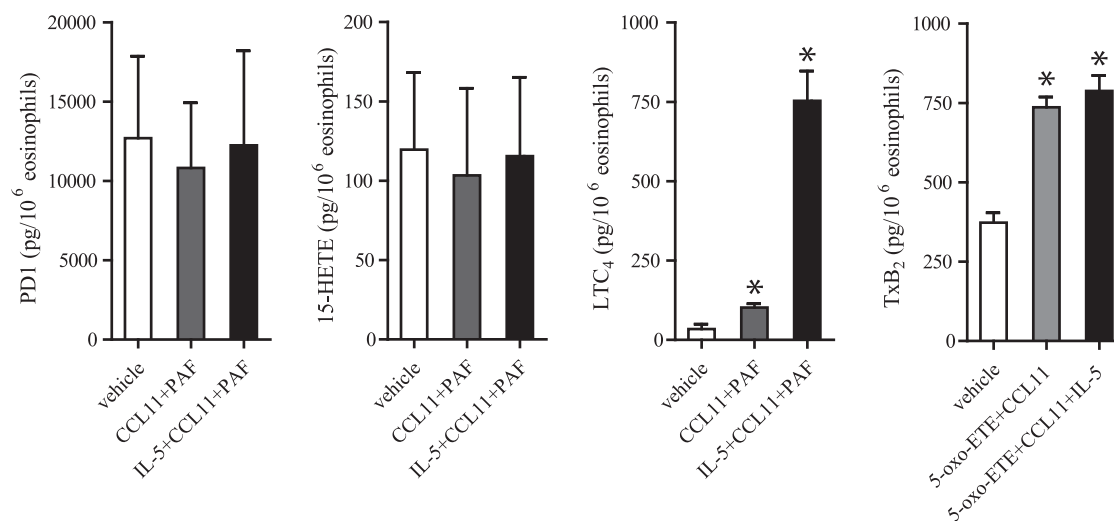


**FIG E1.** Lipid mediator biosynthesis in human eosinophils stimulated at 37°C for 60 minutes with 1  $\mu$ mol/L PAF, 10 nmol/L CCL11, 10 ng/mL IL-5, or their combination. The levels of PD1 (in the presence of 10  $\mu$ mol/L DHA) and 15-HETE were measured by using the LC-MS/MS method, and the levels of LTC<sub>4</sub> and thromboxane B<sub>2</sub> (TxB<sub>2</sub>) were measured by using ELISA. The results are means  $\pm$  SEMs of 3 to 8 experiments. \* $P$  < .05 compared with unstimulated cells.

**TABLE E1.** Demographic data of studied subjects

|                                                            | Healthy subjects | Patients with severe asthma |
|------------------------------------------------------------|------------------|-----------------------------|
| No.                                                        | 4                | 4                           |
| Age (y)                                                    | 36 ± 3           | 68 ± 6                      |
| Sex (female/male)                                          | 0/4              | 3/1                         |
| Peripheral blood eosinophils (%)                           | ND               | 5.1 ± 0.3                   |
| Total serum IgE levels (IU/mL)                             | ND               | 207 ± 72                    |
| FEV <sub>1</sub> (% predicted)                             | ND               | 64.5 ± 1.6                  |
| Treatments                                                 |                  |                             |
| Daily dose of inhaled corticosteroids (mg of budesonide/d) | —                | 1600 ± 0                    |
| No. of patients taking oral corticosteroids                | —                | 2/4                         |
| No. of patients taking leukotriene receptor antagonists    | —                | 4/4                         |
| No. of patients taking long-acting β-agonists              | —                | 3/4                         |
| No. of patients receiving anti-IgE antibody                | —                | 1/4                         |

Values are presented as mean ± SEMs where shown.

ND, Not determined.