

# Sputum indoleamine-2, 3-dioxygenase activity is increased in asthmatic airways by using inhaled corticosteroids

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**Background:** Indoleamine-2, 3-dioxygenase (IDO), a tryptophan-degrading enzyme, plays a key role in the regulation of T-lymphocyte function. IDO inhibits eosinophilic inflammation in a murine asthma model, but little is known about its role in asthmatic patients or the effects of corticosteroids on this key regulatory enzyme.

**Objective:** We studied IDO activity and the effect of inhaled corticosteroids (ICSs) in patients with asthma and how this correlated with eosinophilic inflammation.

**Methods:** After a 1-week run-in period on no therapy, 34 asthmatic patients were treated with only short-acting  $\beta_2$ -agonists as required or an ICS or an ICS in combination with a long-acting  $\beta_2$ -agonist, which were required for asthma control, and the treatment was continued for a further 4 weeks. Each patient underwent sputum induction at the end of the run-in and treatment periods. Sputum supernatant specimens were analyzed for IDO activity and kynurenine concentrations by using HPLC.

**Results:** All patients with mild intermittent and mild-to-moderate persistent asthma had low baseline IDO activity in induced sputum compared with that seen in age-matched nonasthmatic subjects. The IDO activity was markedly enhanced by either ICS ( $P = .03$ ) or ICS/long-acting  $\beta_2$ -agonist ( $P < .0001$ ) treatment, and this increase negatively correlated with sputum eosinophils but was positively associated with an increase in IL-10-positive macrophages.

**Conclusion:** ICSs might exert their anti-inflammatory activity in asthmatic airways, at least in part, through the upregulation of IDO activity associated with increased IL-10 secretion from macrophages.

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**Key words:** Asthma; indoleamine-2, 3-dioxygenase; kynurenine; inhaled corticosteroid; IL-10

## Abbreviations used

DTT: Dithiothreitol

ICS: Inhaled corticosteroid

IDO: Indoleamine-2, 3-dioxygenase

LABA: Long-acting  $\beta_2$ -agonist

Indoleamine-2, 3-dioxygenase (IDO) is an intracellular enzyme involved in tryptophan catabolism and mainly expressed by dendritic cells and macrophages. It mediates immunosuppressive effects on T cells by actively depleting tryptophan in specific local tissue microenvironments<sup>1-3</sup> and by generating downstream tryptophan metabolites released into the surrounding milieu. Kynurenine, the first stable catabolite downstream of IDO, inhibits T cell-mediated inflammation by suppressing proliferation.<sup>4</sup> IDO expression increases when inflammation occurs as a consequence of normal tissue function to protect tissues from collateral damage and facilitate tissue healing.<sup>5</sup> High enzymatic activity of IDO in the lung epithelial cells appears necessary to inhibit the development of eosinophilic airway inflammation induced by ovalbumin sensitization in a murine model of asthma.<sup>6</sup> However, the association between the expression of IDO activity and airway inflammation in human asthma has not yet been investigated.

Corticosteroids are the most effective and widely used anti-inflammatory agents for asthma, and inhaled corticosteroids (ICSs) are recommended therapy for all patient with persistent asthma in current treatment guidelines.<sup>7</sup> These drugs act by complexing to an intracytoplasmic receptor and directly increase or repress gene transcription or interact with other transcription factors to suppress multiple inflammatory genes.<sup>8</sup> Corticosteroids potently inhibit T-cell activation and the production of  $T_H2$  cytokines *in vitro*<sup>9</sup> and have been shown to reduce the expression of  $T_H2$  cytokines in the airway mucosa in asthmatic patients *in vivo*.<sup>10,11</sup> However, the effect of ICSs on IDO activity in human asthmatic airways is not yet known.

Cytokines released from activated  $T_H2$  cells in asthma have differential effects on IDO activity. IL-4 and IL-13 suppress and the anti-inflammatory cytokine IL-10 enhances IDO activity.<sup>1</sup> Alveolar macrophages are a major source of IL-10 in the airways, and alveolar macrophages release less IL-10 *in vitro* from steroid-naïve asthmatic patients than from healthy subjects. By contrast, alveolar macrophages from steroid-treated asthmatic patients exhibited an enhanced capability to produce IL-10.<sup>12,13</sup> Similarly, *in vivo* corticosteroid therapy inhibits  $T_H2$  cytokine secretion while enhancing IL-10 production.<sup>12</sup> This led us to hypothesize that corticosteroids might potentiate IDO activity through enhancing IL-10 release from alveolar macrophages. If future therapy aimed at boosting IDO activity is to be developed for asthma, it will likely be used in conjunction with corticosteroids to

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**TABLE I.** Demographic and clinical characteristics of patients

|                                         | Nonasthmatic control subjects (n = 5) | Asthmatic patients                 |                               |                                    | P value            |                    |                    |
|-----------------------------------------|---------------------------------------|------------------------------------|-------------------------------|------------------------------------|--------------------|--------------------|--------------------|
|                                         |                                       | Group 1: steroid untreated (n = 4) | Group 2: ICS treated (n = 11) | Group 3: ICS/LABA treated (n = 17) | Group 1 vs group 2 | Group 2 vs group 3 | Group 1 vs group 3 |
| Age (y)                                 | 32.2 ± 5.0                            | 31.7 ± 4.1                         | 42.4 ± 2.7                    | 47.7 ± 1.9                         | .07                | .11                | .002               |
| Sex (M/F)                               | 4/1                                   | 2/2                                | 0/11                          | 7/10                               | —                  | —                  | —                  |
| FEV <sub>1</sub> (% predicted)          | 99.8 ± 2.8                            | 93.9 ± 5.4                         | 72.5 ± 4.3                    | 74.9 ± 3.9                         | .02                | .69                | .04                |
| Postbronchodilator FEV <sub>1</sub> (L) | 3.5 ± 0.3                             | 2.8 ± 0.2                          | 1.97 ± 0.07                   | 2.1 ± 0.1                          | .0004              | .62                | .01                |
| Reversibility (%)                       | 0.97 ± 0.1                            | 6.7 ± 3.6                          | 20.0 ± 4.1                    | 12.4 ± 2.9                         | .11                | .14                | .39                |
| PC <sub>20</sub> methacholine (mg/mL)   | 89.9 ± 9.5                            | 2.6 ± 0.9                          | 1.4 ± 0.4                     | 1.19 ± 0.2                         | .22                | .62                | .06                |
| Atopy, n                                | 0                                     | 4                                  | 8                             | 13                                 | —                  | —                  | —                  |
| ICS dose (μg/d)                         | 0                                     | 0                                  | 600 ± 56                      | 484 ± 41.7                         | —                  | .12                | —                  |

The measurements were done at the screening visit, and values are presented as means ± SEMs. P values for comparison among groups were derived from the unpaired *t* test.

enhance asthma control. The present study was designed to investigate IDO activity in induced sputum of patients with mild intermittent and mild-to-moderate persistent asthma and to determine whether this could be modulated by ICS treatment.

## METHODS

### Subjects

Thirty-four nonsmoking stable asthmatic patients, defined according to American Thoracic Society criteria, participated in the study (Table I). None had received therapy with oral corticosteroids in the 3 months before the study. Subjects had a baseline FEV<sub>1</sub> of 60% of predicted value or greater and demonstrated a reversibility of FEV<sub>1</sub> after therapy with nebulized albuterol (2.5 mg) of 12% or greater and a PC<sub>20</sub> of 4 mg/mL or less. Allergic status was defined by the presence of a positive skin prick test response to at least one of 4 common aeroallergens (grass pollen, cat dander, *Dermatophagoides pteronyssinus*, and *Aspergillus fumigatus*). Exclusion criteria were any asthma exacerbation or respiratory tract infection within 4 weeks before the study. The patients with exacerbations or those who showed loss of asthma control during the study were excluded and received appropriate treatment. Therefore there was no placebo treatment in this study because this was thought to be unethical. Written informed consent was obtained from each patient, and the study was approved by the Ethics Committee of Siriraj Hospital.

The asthmatic subjects were distributed into 3 groups as follows: 6 subjects who had experienced mild intermittent asthma and were allowed to use only short-acting β<sub>2</sub>-agonists as needed to control their asthma symptoms during the 4-week treatment period; 11 subjects who had mild-to-moderate persistent asthma controlled by inhaled steroids (≤800 μg/d budesonide or ≤500 μg/d beclomethasone dipropionate); and 17 subjects with mild-to-moderate persistent asthma controlled by inhaled steroids (≤640 μg/d budesonide or ≤1000 μg/d fluticasone propionate) in combination with long-acting β<sub>2</sub>-agonists (LABAs).

The nonasthmatic control subjects lacked a medical history of allergic disease, including asthma, allergic rhinitis, and eczema and had no respiratory tract infections within 4 weeks of the start of the study and negative skin prick test and bronchial provocation test results. The nonasthmatic control subjects did not receive any treatment throughout the study and underwent spirometry, methacholine challenge testing, and sputum induction at the initial visit and at the 4-week follow-up period.

### Study design

This study was aimed at investigating the expression of IDO activity in induced sputum from steroid-naïve patients with mild intermittent asthma and steroid-treated patients with mild-to-moderate persistent asthma compared with that from age-matched nonasthmatic control subjects. The study was conducted in a single-blind manner, including a 1-week run-in period and a 4-week treatment period, for a total of 4 visits. At the screening visit, patients completed a questionnaire about their asthma histories and were requested to

stop their current asthma medications other than ICS and ICS/LABA inhalers. Additionally, skin prick tests, spirometry, and methacholine airway responsiveness measurements (PC<sub>20</sub>) were performed, as shown in Table I. One week after the screening visit, the patients were assessed for asthma control status, and their previous ICS or ICS/LABA inhalers were replaced with inhaled short-acting β<sub>2</sub>-agonists used as rescue medication. Subjects returned 1 week later (week 0, visit 3) for baseline measurements. A subsequent visit was made in 4 weeks' time (week 4, visit 4). At visits 1, 3, and 4, spirometric results were measured, and methacholine airway responsiveness was assessed. Sputum induction was performed at weeks 0 and 4. At week 0, all patients were switched to an ICS (500 μg/d beclomethasone dipropionate or 400-800 μg/d budesonide) or a combination ICS/LABA (250-1000 μg/d fluticasone and 50 μg/d salmeterol or 320-640 μg/d budesonide and 9-18 μg/d formoterol) for 4 weeks at the doses that had been previously required for asthma control. The research assistants performed all measurements in a blind manner. The primary end point was the change in levels of sputum IDO activity after active treatment, with changes in sputum eosinophil numbers, kynurenine concentrations, and IL-10–positive sputum macrophage numbers being the secondary end points. Sputum parameters measured at visits 3 to 4 were used for the calculation of pretreatment/posttreatment changes. Measurements for sputum IDO activity, kynurenine concentration, sputum eosinophil number, and sputum IL-10–positive macrophage number were made before and after active treatment.

### Lung function measurements and methacholine challenge

FEV<sub>1</sub> and forced vital capacity were measured by using a dry wedge spirometer (Vitalograph, Buckingham, United Kingdom). Values are expressed as the percentage of predicted normal values. Baseline values were measured after 15 minutes of rest and were taken as the highest of 3 readings. Single readings only were taken at other times. The methacholine PC<sub>20</sub> was determined by using the linear interpolation of the concentration-FEV<sub>1</sub> response curve. Lung function measurement and methacholine challenge were performed at the screening visit and the end of the run-in and treatment periods.

### Sputum induction and processing

Sputum induction was performed as previously described.<sup>14</sup> The supernatants were kept frozen at –70°C until further analysis. For immunocytochemistry, cytospin preparations were fixed with 4% paraformaldehyde (BDH Ltd, Poole, United Kingdom) and stored at –20°C. Total cell counts were recorded with a hemocytometer by using Kimura stain. Cell viability was determined by means of trypan blue exclusion before cytospin preparation was undertaken. The slides were stained with May-Grunwald-Giemsa stain. Differential cell counts were made by a blinded observer. Three hundred inflammatory cells were counted on 2 slides for each sample in a blind manner. Differential cell counts are expressed as the percentage of inflammatory cells. Samples with cell viability of greater than 70% and less than 30% squamous cell

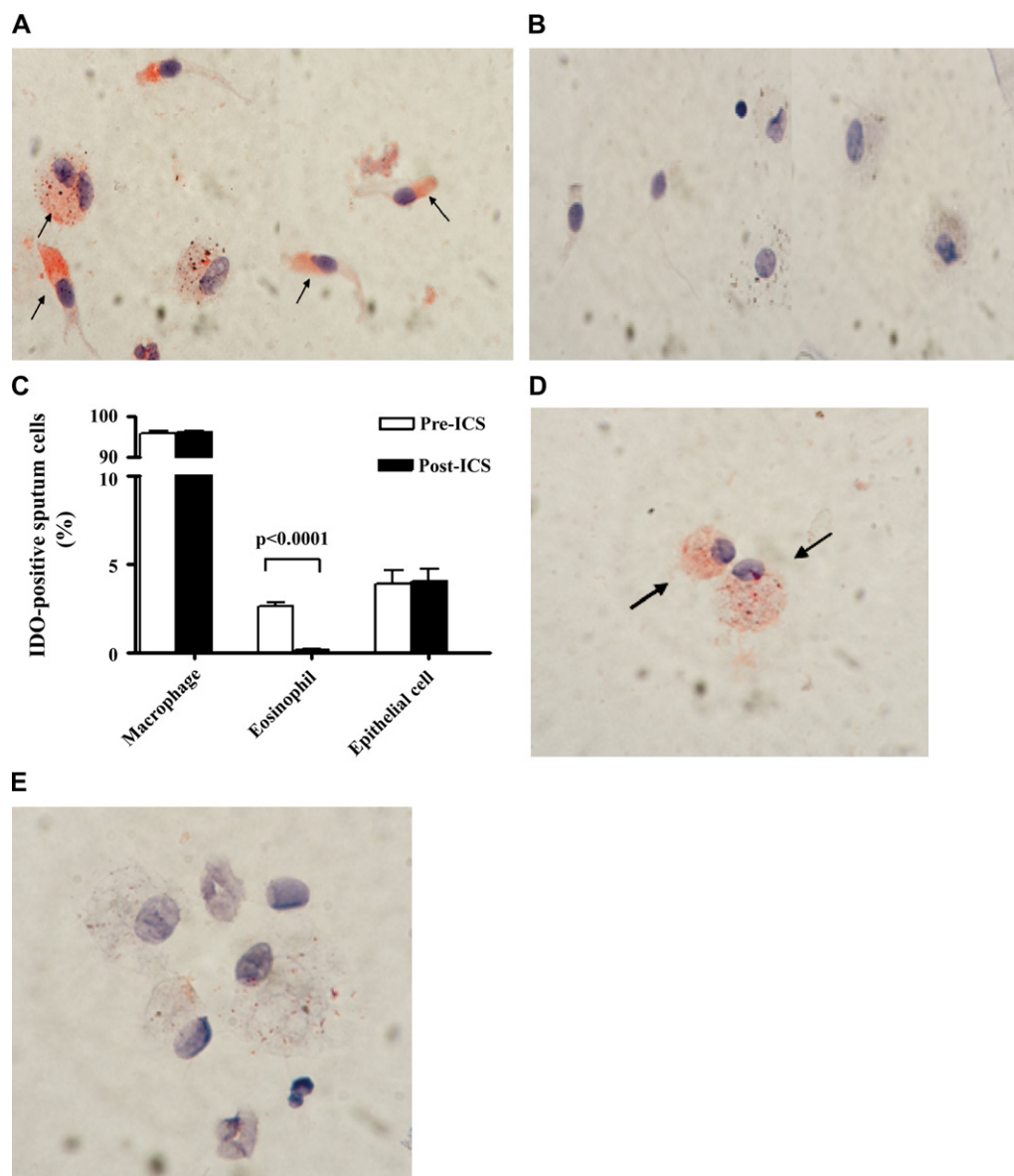


**FIG 1.** IDO and IL-10 expression in sputum cells from ICS-treated asthmatic patients: **A**, IDO-expressing macrophages and epithelial cells (indicated by arrows); **B**, negative control; **C**, mean percentage value  $\pm$  SEM of specific cell types that are IDO positive before and after ICS treatment ( $n = 11$ ); **D**, IL-10-positive macrophages (indicated by arrows); **E**, negative control. Original magnification  $\times 100$ .

contamination were considered adequate. Four sputum samples from 2 patients with mild intermittent asthma were withdrawn from analysis because of inadequate samples.

### Alkaline phosphatase immunostaining

Indirect staining of sputum cells was performed by using the alkaline phosphatase–antialkaline phosphatase method with a commercial kit (Vectastain Laboratories, Burlingame, Calif), as previously described.<sup>15</sup> Cells were permeabilized for 10 minutes with 0.5% Nonidet P-40 (Sigma Chemicals, St Louis, Mo) and blocked in 20% normal swine nonimmune serum (Vector Laboratories) for 30 minutes at room temperature. IDO and IL-10 were detected with monoclonal anti-mouse antibodies against IDO (1:100; Chemicon International Inc, Temecula, Calif) and IL-10 (1:100; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif). After incubating with the secondary biotinylated goat anti-mouse antibody, the immunoreaction was detected by using the

alkaline phosphatase–antialkaline phosphatase system to produce red staining. Slides were counterstained with hematoxylin for cellular identification and examined under light microscopy. Anti-IDO–positive and anti-IL-10–positive cells, which were identified by using red immunoreactive signals on sputum cytospin preparations, were counted by an experienced observer blind to the clinical characteristics of the subjects. IDO-positive cells were counted among 300 cells of each cell population and expressed as a percentage. IL-10–positive cells were counted among 300 macrophages.

### IDO enzymatic activity

Sputum supernatants were assayed for kynurenine and tryptophan concentrations to quantify the enzymatic activity of IDO *in vivo*. Kynurenine and tryptophan were detected according to the method of Yong and Lau,<sup>16</sup> with minor modifications using HPLC (Thermoseparation products with UV/VIS Detectors, Fremont, Calif). Sputum samples were prepared by extracting

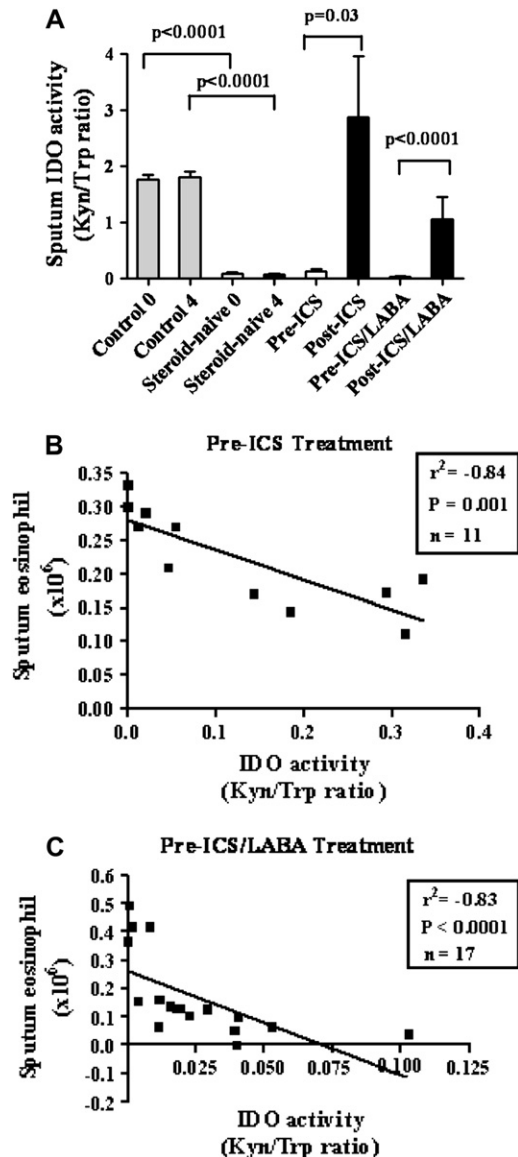


**FIG 2.** The expression of IDO activity in sputum among nonasthmatic control subjects, steroid-naïve subjects, and asthmatic individuals in response to ICS or ICS/LABA treatment (before and after treatment; **A**) is shown. The results are presented as the mean  $\pm$  SEM. Relationship between IDO activity and sputum eosinophilia before ICS (**B**) and ICS/LABA (**C**) treatment are shown. Squares represent individual asthmatic patients.

600  $\mu$ L of sputum supernatant with 1300  $\mu$ L of methanol. Precipitated proteins were removed by means of centrifugation, and the supernatant was dried with a vacuum. The resulting samples were resuspended in 100  $\mu$ L of deionized water and injected into the column. The absorbance of column effluent was monitored at 230 nm for both tryptophan and kynurenine. The peaks of kynurenine and tryptophan were identified by means of comparison with the retention time of previously determined the standard compounds, and quantification was based on the peak areas of the compounds. Kynurenine and tryptophan levels were measured by calculating the area under the curve with the Chromquest 4.1 SP 2 Version 4.1 analysis program (Thermo Electron, Fremont, Calif). The kynurenine/tryptophan ratio was calculated as an estimate of the IDO enzymatic activity.

Because sputum supernatant specimens contained 0.05% dithiothreitol (DTT) at the final concentration, we measured kynurenine and tryptophan in the control buffers in the absence and presence of DTT to determine whether it

had potential effects on the kynurenine assay. We found that DTT did not affect tryptophan and kynurenine assays.

## ELISA for IL-10

The IL-10 concentration in the sputum supernatant was assessed by using an ELISA (R&D, Minneapolis, Minn). This assay used the quantitative sandwich enzyme immunoassay technique.

## Statistical analysis

Data are expressed as means  $\pm$  SEM. Changes in kynurenine/tryptophan ratios (IDO activity) and sputum cell numbers after active treatment were compared by using the Mann-Whitney *U* test. The *r* value was determined for the correlation of kynurenine/tryptophan ratio with eosinophil numbers, IL-10-positive cells, FEV<sub>1</sub>, and PC<sub>20</sub> within the groups by using the Spearman rank correlation test. All statistical tests were 2-sided, and significance was accepted at a level of 95% and a *P* value of less than .05 with GraphPad Prism software (GraphPad Software, Inc, San Diego, Calif).

## RESULTS

### Baseline expression of IDO activity in induced sputum cells

We first investigated whether IDO is expressed in sputum cells obtained from asthmatic subjects before ICS treatment by using immunocytochemistry staining. We detected immunoreactivity of IDO (Fig 1, A) that is localized mainly to sputum macrophages and, to a lesser extent, in sputum eosinophils and epithelial cells (mean  $\pm$  SEM: 95.8%  $\pm$  0.46%, 2.6%  $\pm$  0.22%, and 3.8%  $\pm$  0.78%, respectively; Fig 1, C).

The expression of constitutively functional IDO activity, measured based on the ratio of kynurenine to tryptophan in sputum supernatants from steroid-naïve asthmatic patients, was extremely low compared with that observed in nonasthmatic control subjects (mean  $\pm$  SEM: 0.07  $\pm$  0.03 vs 1.76  $\pm$  0.07, respectively;  $P < .0001$ ; Fig 2, A). In addition, patients with steroid-naïve asthma still had a significantly low expression of IDO activity after 4 weeks when compared with nonasthmatic control subjects (mean  $\pm$  SEM: 0.07  $\pm$  0.01 vs 1.8  $\pm$  0.08, respectively;  $P < .0001$ ; Fig 2, A). Similarly, low baseline levels of IDO enzymatic activity in sputum supernatants were detected in ICS-treated and ICS/LABA-treated groups (mean  $\pm$  SEM: 0.128  $\pm$  0.04 and 0.02  $\pm$  0.01, respectively; Fig 2, A). Consistently, the baseline kynurenine concentrations in sputum supernatants of steroid-naïve, ICS-treated, and ICS/LABA-treated asthmatic patients were markedly lower than those in supernatants of nonasthmatic control subjects (mean  $\pm$  SEM: 19.6  $\pm$  8.1, 29.5  $\pm$  7.9, and 7.6  $\pm$  1.5 vs 211.9  $\pm$  35.2  $\mu$ mol/L, respectively; Table II). We also found that low levels of sputum IDO activity detected in asthmatic individuals before ICS and ICS/LABA treatment were significantly correlated with the extent of sputum eosinophilia ( $P = .001$ ,  $r^2 = -0.84$  and  $P < .0001$ ,  $r^2 = -0.83$ , respectively; Fig 2, B and C).

### Effect of ICSs on expression of IDO enzymatic activity in induced sputum

ICS treatment caused a 22-fold increase of IDO activity in sputum supernatants from asthmatic patients compared with its basal activity (2.87  $\pm$  1.1 vs 0.128  $\pm$  0.04,  $P = .03$ ; Fig 2, A). In addition, combination therapy with ICS/LABA resulted in a 53-fold increase of the IDO activity in sputum supernatants from

**TABLE II.** Total and specific sputum cell counts at baseline and after ICS and ICS/LABA treatment

|                                        | Steroid naive |              | ICS treated   |                               | ICS/LABA treated |                               |
|----------------------------------------|---------------|--------------|---------------|-------------------------------|------------------|-------------------------------|
|                                        | Week 0        | Week 4       | Before        | After                         | Before           | After                         |
| Prebronchodilator FEV <sub>1</sub> (L) | 2.86 ± 0.2    | 2.87 ± 0.2   | 1.88 ± 0.09   | 1.99 ± 0.1                    | 1.89 ± 0.13      | 2.0 ± 0.12                    |
| Total cells (10 <sup>6</sup> /mL)      | 4.4 ± 1.4     | 3.8 ± 0.8    | 4.7 ± 0.2     | 5.1 ± 0.7                     | 4.7 ± 0.4        | 5.5 ± 0.5                     |
| Macrophages (%)                        | 76.2 ± 2.9    | 72.7 ± 7.1   | 67.3 ± 4.7    | 66.4 ± 8.7                    | 76.1 ± 4.1       | 82.3 ± 4.6                    |
| Macrophages (10 <sup>6</sup> /mL)      | 3.3 ± 0.96    | 2.7 ± 0.6    | 3.1 ± 0.2     | 3.4 ± 0.8                     | 3.6 ± 0.4        | 4.3 ± 0.3                     |
| Neutrophils (%)                        | 17.0 ± 4.3    | 21.7 ± 5.2   | 27.4 ± 4.6    | 33.1 ± 8.6                    | 16.7 ± 4.2       | 16.5 ± 4.7                    |
| Neutrophils (10 <sup>6</sup> /mL)      | 0.7 ± 0.3     | 0.9 ± 0.3    | 1.3 ± 0.2     | 1.7 ± 0.6                     | 0.8 ± 0.2        | 1.2 ± 0.5                     |
| Eosinophils (%)                        | 4.75 ± 0.5    | 5.2 ± 2.5    | 4.73 ± 0.4    | 0.9 ± 0.4, <i>P</i> = .0001   | 3.3 ± 0.5        | 1.2 ± 0.2, <i>P</i> = .001    |
| Eosinophils (10 <sup>6</sup> /mL)      | 0.18 ± 0.03   | 0.25 ± 0.17  | 0.2 ± 0.02    | 0.05 ± 0.02, <i>P</i> = .0003 | 0.17 ± 0.03      | 0.06 ± 0.01, <i>P</i> = .0068 |
| Lymphocytes (%)                        | 0.0 ± 0.0     | 0.25 ± 0.2   | 0.4 ± 0.2     | 0.3 ± 0.2                     | 0.06 ± 0.05      | 0.0 ± 0.0                     |
| Lymphocytes (10 <sup>6</sup> /mL)      | 0.0 ± 0.0     | 0.01 ± 0.01  | 0.01 ± 0.01   | 0.005 ± 0.0                   | 0.002 ± 0.0      | 0.0 ± 0.0                     |
| Kynurenine (μmol/L)                    | 19.6 ± 8.1    | 18.2 ± 4.5   | 29.5 ± 7.9    | 88.3 ± 26.1, <i>P</i> = .02   | 7.6 ± 1.5        | 54.2 ± 8.8, <i>P</i> < .0001  |
| Tryptophan (μmol/L)                    | 282.8 ± 48.05 | 259.4 ± 28.8 | 902.7 ± 387.5 | 441 ± 287.8, <i>P</i> = .03   | 988.2 ± 388.8    | 138 ± 33.2, <i>P</i> = .04    |
| IDO activity(kynurenine/tryptophan)    | 0.07 ± 0.03   | 0.07 ± 0.01  | 0.128 ± 0.04  | 2.87 ± 1.1, <i>P</i> = .03    | 0.02 ± 0.01      | 1.06 ± 0.4, <i>P</i> < .0001  |
| IL-10–positive macrophages (%)         | 18.0 ± 2.5    | 19.0 ± 2.9   | 19.6 ± 1.9    | 34.1 ± 2.5, <i>P</i> = .0001  | 17.6 ± 1.3       | 25.9 ± 1.8, <i>P</i> = .002   |
| IL-10 (pg/mL)                          | 6.17 ± 0.3    | 6.19 ± 0.16  | 6.4 ± 0.5     | 39.4 ± 2.5, <i>P</i> < .0001  | 6.6 ± 0.36       | 42.1 ± 1.9, <i>P</i> < .0001  |

Two-sided *P* values for within-treatment differences were derived from the Mann-Whitney *U* test.

its basal activity ( $1.06 \pm 0.4$  vs  $0.02 \pm 0.01$ ,  $P < .0001$ ; Fig 2, A), although this was insignificant when compared with that of ICS treatment ( $P = .34$ ). The increased IDO activity in sputum supernatants from ICS- and ICS/LABA-treated asthmatic patients significantly increased kynurenine concentrations from baseline ( $88.3 \pm 26.1$  vs  $29.5 \pm 7.9$  μmol/L [ $P = .02$ ] and  $54.2 \pm 8.8$  vs  $7.6 \pm 1.5$  μmol/L [ $P < .0001$ ], respectively; Table II). In addition, sputum IDO activity after ICS treatment positively correlated with the percentage of IDO-positive macrophages ( $P = .002$ ,  $r^2 = 0.82$ ). However, ICS treatment did not cause a significant increase in the number of IDO-positive macrophages compared with that before ICS treatment ( $96.2\% \pm 0.32\%$  vs  $95.9\% \pm 0.46\%$ , respectively;  $P = .39$ ), as well as the number of IDO-positive epithelial cells ( $4.0\% \pm 0.7\%$  vs  $3.8\% \pm 0.78\%$ ,  $P = .8$ ; Fig 1, C). In contrast, ICS treatment caused a significant reduction in the number of IDO-positive eosinophils ( $0.12\% \pm 0.06\%$  vs  $2.6\% \pm 0.22\%$ ,  $P < .0001$ ; Fig 1, C).

During the run-in period off treatment, all steroid-naïve and ICS-treated and ICS/LABA-treated asthmatic patients had eosinophilic airway inflammation, as demonstrated by differential sputum eosinophil counts ( $4.75\% \pm 0.5\%$ ,  $4.73\% \pm 0.4\%$ , and  $3.3\% \pm 0.5\%$ , respectively; Fig 3, A) and absolute eosinophil counts ( $0.18 \pm 0.03 \times 10^6$ ,  $0.2 \pm 0.02 \times 10^6$ , and  $0.17 \pm 0.03 \times 10^6$  cells, respectively; Fig 3, B). In contrast, eosinophilic airway inflammation observed in patients with mild-to-moderate persistent asthma was attenuated by treatment with an ICS or an ICS in combination with a LABA. Both sputum eosinophil differential counts and absolute numbers were markedly reduced after 4 weeks of treatment with an ICS in the absence or presence of a LABA ( $4.73\% \pm 0.4\%$  vs  $0.9\% \pm 0.4\%$  [ $P = .0001$ ] and  $3.3\% \pm 0.5\%$  vs  $1.2\% \pm 0.2\%$  [ $P = .001$ ], respectively;  $0.2 \pm 0.02$  vs  $0.05 \pm 0.02 \times 10^6$  [ $P = .0003$ ] and  $0.17 \pm 0.03$  vs  $0.06 \pm 0.01 \times 10^6$  [ $P = .0068$ ], respectively; Fig 3, A and B). Furthermore, after ICS and ICS/LABA treatment, eosinophil counts were significantly correlated with the levels of IDO enzymatic activity in induced sputum ( $P < .0001$ ,  $r^2 = -0.88$  and  $P < .0001$ ,  $r^2 = -0.9$ , respectively; Fig 3, C and D). The inverse correlation between sputum eosinophil percentages and IDO activity was also observed after ICS and ICS/LABA treatment ( $P = .001$ ,  $r^2 = -0.86$  and

$P < .0001$ ,  $r^2 = -0.82$ , respectively; Fig 3, E and F). In addition, we could demonstrate a positive correlation between changes in IDO activity and prebronchodilator FEV<sub>1</sub> and between changes in IDO activity and PC<sub>20</sub> in those patients who were treated with an ICS ( $P = .023$ ,  $r^2 = 0.67$  and  $P = .016$ ,  $r^2 = 0.7$ , respectively; Fig 3, G and H).

### Correlation between upregulation of IDO activity and increased IL-10 production

Previous studies have suggested that corticosteroids could enhance IL-10 expression in alveolar macrophages from asthmatic patients<sup>13,17</sup> and IL-10 could enhance IDO activity,<sup>1</sup> and ICS-upregulated IDO activity in our observation did not result from the upregulation of IDO expression in sputum macrophages (Fig 1, C). This raised the possibility that ICS-upregulated IDO activity might be associated with IL-10. The immunocytochemistry data showed that IL-10 was predominantly expressed in sputum macrophages (Fig 1, D). ICS- or ICS/LABA-treated asthmatic patients exhibited a significant increase in IL-10 production, as demonstrated by the number of IL-10–positive macrophages ( $19.6\% \pm 1.9\%$  vs  $34.1\% \pm 2.5\%$  [ $P = .0001$ ] and  $17.6\% \pm 1.3\%$  vs  $25.9\% \pm 1.8\%$  [ $P = .002$ ], respectively; Table II and Fig 4, A) and IL-10 levels in sputum supernatants ( $6.4 \pm 0.5$  vs  $39.4 \pm 2.5$  pg/mL [ $P < .0001$ ] and  $6.6 \pm 0.36$  vs  $42.1 \pm 1.9$  pg/mL [ $P < .0001$ ], Table II). The changes in the number of IL-10–positive cells was positively correlated with changes in IDO activity during 4 weeks of treatment with the ICS ( $P = .003$ ,  $r^2 = 0.8$ ; Fig 4, B) and the ICS and LABA in combination ( $P = .002$ ,  $r^2 = 0.68$ ; Fig 4, C).

### DISCUSSION

The present study has demonstrated for the first time that baseline IDO enzymatic activity in induced sputum from asthmatic patients is very low compared with that seen in nonasthmatic control subjects and is inversely related to sputum eosinophilia. The low IDO activity is increased by ICS and combination ICS/LABA treatment. This increase in IDO activity might be mediated, at least in part, by enhancing IL-10 production

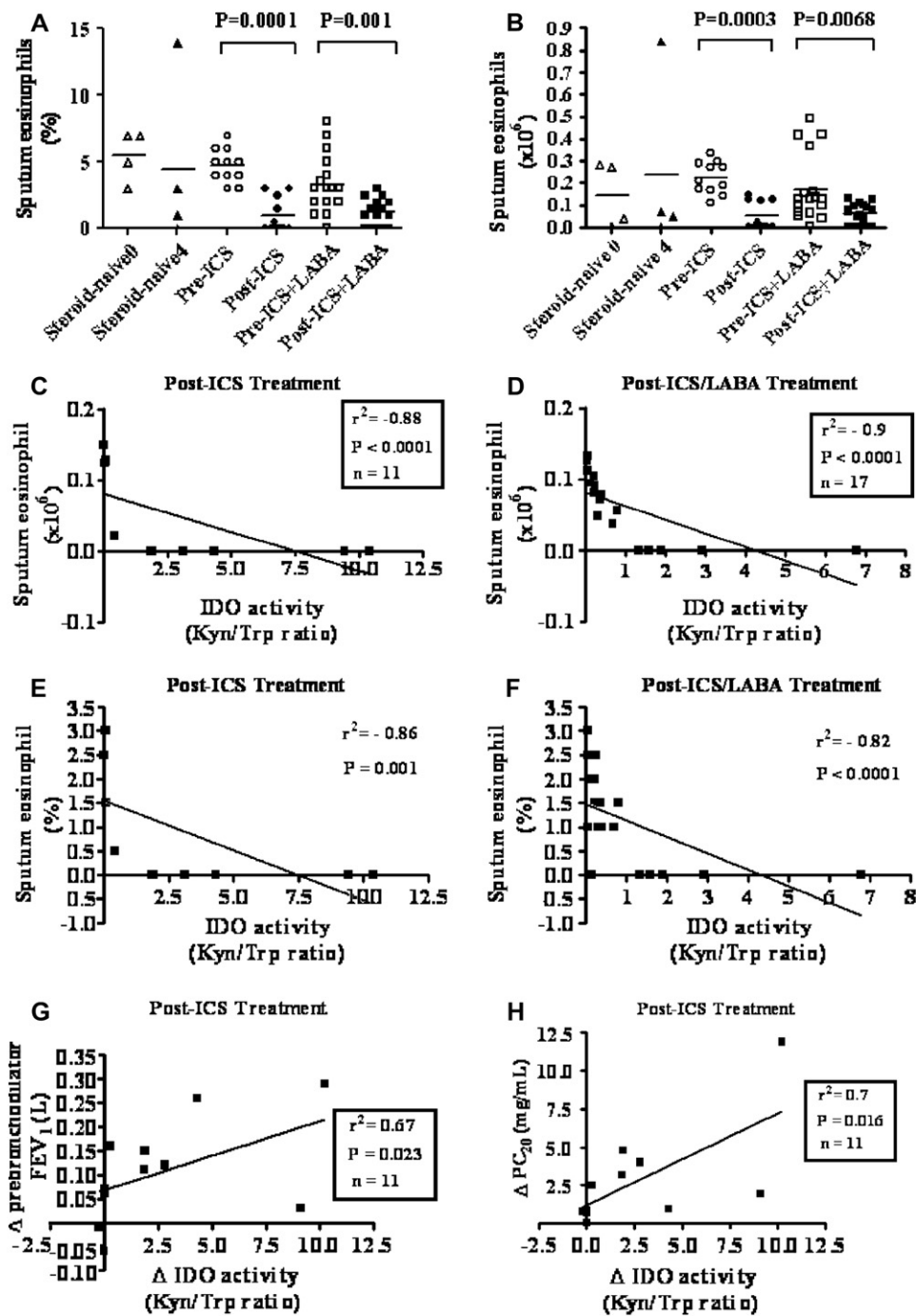


**FIG 3.** A and B, Sputum eosinophils in asthmatic individuals during pretreatment and posttreatment with ICS and ICS/LABA, including steroid-naïve subjects. C-F, Relationship between IDO activity and eosinophil numbers (Fig 3, C and D) and percentages (Fig 3, E and F) during post-ICS and post-ICS/LABA treatment. G and H, The correlation between changes in IDO activity and  $FEV_1$  (Fig 3, G) and  $PC_{20}$  (Fig 3, H) after ICS treatment, as well as after ICS/LABA treatment (not shown;  $n = 17$ ;  $P = .028$ , and  $r^2 = 0.53$  for  $FEV_1$ ;  $P = .009$  and  $r^2 = 0.61$  for  $PC_{20}$ ).

by sputum macrophages because changes in IDO activity were positively correlated with the number of IL-10-expressing macrophages in induced sputum.

Previous studies have shown that IDO activity is less in atopic than in nonatopic individuals.<sup>18</sup> However, low constitutive IDO activity in the airways of asthmatic patients has not

previously been described. IDO might inhibit  $T_H2$ -mediated lung inflammation in several ways: (1) depletion of tryptophan availability in the microenvironment, thereby reducing T-cell function<sup>2</sup>; (2) promotion of the generation of various toxic tryptophan metabolites, which induce  $T_H$  cell death<sup>4,19</sup>; (3) generation of other compounds (eg, formylkynurenine) through a reaction

that removes oxygen radicals at inflammatory sites<sup>20</sup>; and (4) inhibition of the generation of 5-hydroxytryptamine, a bronchoconstrictor, in the case of T<sub>H</sub>2-mediated lung inflammation.<sup>21</sup> These activities of IDO are likely to collaborate in suppressing T<sub>H</sub>2-mediated experimental asthma and to attenuate T<sub>H</sub>1-mediated pulmonary inflammation. Indeed, in this study we have confirmed that the high level of IDO enzymatic activity observed in sputum supernatants after ICS administration was correlated with a reduction in eosinophilic airway inflammation. Because IDO expression in sputum cells was mainly detected in macrophages, we speculate that sputum macrophages might be the site of induction of IDO enzymatic activity after ICS treatment. Hayashi et al,<sup>6</sup> however, observed the enzymatic activity of IDO in the lung epithelial cells. It is therefore possible that airway epithelial cells might also contribute to the upregulation of sputum IDO activity after ICS treatment.

The anti-inflammatory effects of ICSs are mediated through inhibition of transcription of multiple genes involved in inflammation, such as proinflammatory cytokines, and through upregulation of anti-inflammatory cytokines, such as IL-10.<sup>8,22</sup> ICSs might have a direct effect on induction of IL-10 release by macrophages and regulatory T cells. Increased plasma concentrations of IL-10 are associated with enhanced systemic IDO activity in asymptomatic atopy.<sup>23</sup> We demonstrated a positive correlation between the number of IL-10–positive macrophages and changes in the levels of IDO activity in asthmatic sputum in response to ICS treatment. However, it was not possible to demonstrate the direct effects of IL-10 from sputum macrophages on IDO activity in this *in vivo* study. Further *in vitro* studies with macrophages or monocytes from asthmatic patients are now needed to study the relationship between IL-10 and IDO activity. However, ICSs might modulate IDO through a direct effect on enzyme activity in macrophages, and this will also require further *in vitro* studies.

Although IDO is an intracellular enzyme, measurements of kynurenine in sputum supernatants, as demonstrated in our study, could reflect IDO function for 2 reasons. The first is that tryptophan breakdown has been demonstrated to suppress T-cell proliferation by critically reducing the availability of tryptophan in local microenvironments.<sup>24</sup> The second reason is that kynurenine, the first stable downstream metabolite of tryptophan catabolism, acts to suppress immune reactivity, probably through direct interactions with effector T cells and eosinophils. These 2 mechanisms are not mutually exclusive, and each might have a role in the immunobiology of IDO.<sup>25</sup> This can be inferred by our observation that the absolute amount of kynurenine was significantly increased in association with a marked decrease in tryptophan levels in sputum supernatants after ICS treatment (Table II).

We found that IDO is constitutively expressed in macrophages, eosinophils, and airway epithelial cells, which is consistent with previous observations.<sup>2,6,26</sup> IDO expressed in eosinophils is suggested to maintain eosinophilic inflammation in asthma, possibly by potentiating the apoptotic effect on T<sub>H</sub>1 cells, which in turn results in T<sub>H</sub>2 polarization.<sup>26</sup> Therefore we could not exclude the association between eosinophilic inflammation and IDO-expressing eosinophils, although the number of IDO-expressing eosinophils was observed in the present study to be rather low. In contrast to eosinophils, IDO-expressing macrophages are previously demonstrated to mediate immunosuppressive effects on T cells. In addition, IDO activity could be enhanced through the autocrine effect of IL-10 on macrophages. The present study showed that IDO activity in asthmatic sputum before ICS

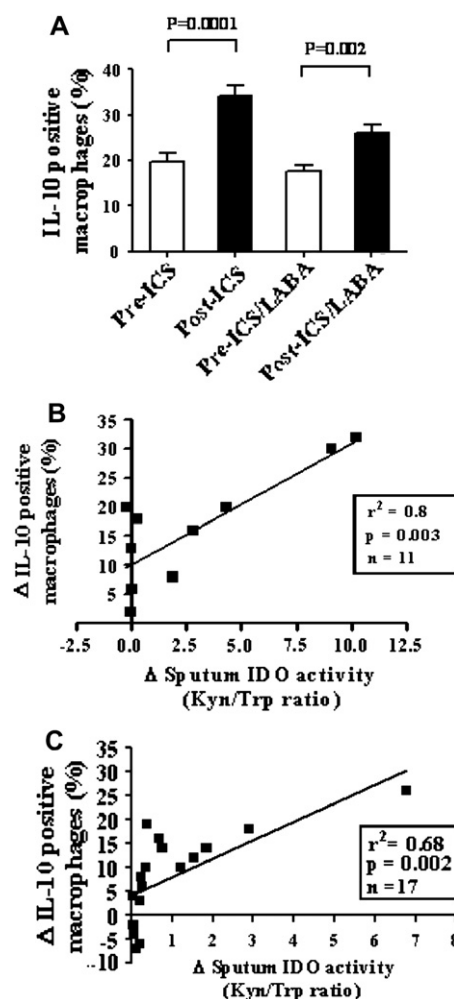


**FIG 4.** The number of sputum macrophages expressing IL-10 in asthmatic individuals in response to ICS and ICS/LABA treatment (A) is shown. The data are presented as means  $\pm$  SEM. The correlation between changes (posttreatment/pre-treatment) in IDO activity and IL-10–positive macrophage numbers in sputum after ICS (B) and ICS/LABA (C) treatment is also shown. Squares represent asthmatic individuals.

treatment was extremely low and increased after ICS treatment, whereas there was no significant change in the number of IDO-positive macrophages between pre-ICS and post-ICS treatment. This observation led us to hypothesize that IDO activity might be enhanced by IL-10 released from macrophages.

A reduction in the capacity of macrophages to produce IL-10 might influence the progression of airway inflammation in asthma. ICS therapy in patients with mild asthma has been shown to increase IL-10 secretion from alveolar macrophages.<sup>13</sup> Our data suggest that ICSs modified both IDO activity and the number of IL-10–producing macrophages in asthmatic sputum, and this mechanism might account for the immunomodulatory effect of corticosteroids because IDO activity is associated with increased expression of IL-10 and reduced eosinophilia.

Despite its novel observation, our study still has a number of limitations. Our exclusion criteria of the present study including persistent asthmatic patients who had exacerbations during the treatment period does not allow us to have a proper control group because that is thought to be unethical. Therefore a randomized placebo-controlled study will be required to confirm our findings.

In addition, because of different ICSs and LABAs used, we do not know whether individual ICSs enhance IDO activity to a similar degree and in a dose-dependent manner.

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**Clinical implications: Boosting IDO activity in asthma might constitute a novel target for treatment strategy in conjunction with corticosteroids to enhance asthma control.**

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