

## ORIGINAL ARTICLE

## Decreased indoleamine 2,3-dioxygenase activity and IL-10/IL-17A ratio in patients with COPD

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**ABSTRACT**

**Rationale** Indoleamine 2,3-dioxygenase (IDO) induces generation of regulatory T cells but suppresses Th17 cells and therefore might attenuate neutrophilic inflammation. The role of IDO in neutrophilic airway diseases such as chronic obstructive pulmonary disease (COPD) remains unknown. We evaluated IDO activity and expression and interleukin (IL)-10 and IL-17A levels in sputum from patients with COPD.

**Methods** IDO activity and cytokine concentrations in sputum supernatants from patients with COPD of varying severity and in smoking and non-smoking control subjects were determined by high-performance liquid chromatography and ELISA, respectively.

**Results** Patients with COPD had reduced sputum IDO activity and expression and IL-10 levels, with increased IL-17A, IL-6 and CXCL8 concentrations and sputum neutrophils. These changes were significantly correlated with disease severity. IDO activity was decreased, but to a lesser extent, in normal smokers compared with non-smoking controls.

**Conclusions** Patients with COPD have a progressive reduction in IDO activity with reversal of the balance between IL-10 and IL-17A, resulting in chronic airway neutrophilic inflammation.

**INTRODUCTION**

Chronic obstructive pulmonary disease (COPD) is a chronic airway inflammatory disorder which involves an abnormal inflammatory response to noxious particles and gases. Cigarette smoking activates innate immune cells such as epithelial cells and macrophages.<sup>1</sup> Activated dendritic cells induce adaptive immune cells including the two lineages of CD4 T helper (Th) cells (interferon (IFN)- $\gamma$ -secreting Th1 cells and interleukin (IL)-17-secreting Th17 cells), CD8 cytotoxic T cells (Tc1) and B cells.<sup>1</sup> This leads to the formation of lymphoid follicles with chronic airway inflammation which is characterised by a predominance of CD8 T cells at all airway levels including the lung parenchyma.<sup>2–3</sup> There is also an increase in CD68 cells (monocytes/macrophages) in the bronchial subepithelium and alveoli<sup>4</sup> and an increased number of neutrophils in the airways and sputum. Previous studies have emphasised the role of several inflammatory cells in the pathogenesis of COPD—mainly alveolar macrophages, neutrophils and T lymphocytes.<sup>5</sup> However, although proinflammatory CD4 T cell activation is involved in amplifying and perpetuating the chronic inflammatory state of

**Key messages****What is the key question?**

- The key question is whether indoleamine 2, 3-dioxygenase (IDO) plays an important role in neutrophilic airway inflammation in COPD.

**What is the bottom line?**

- In this study we demonstrated that IDO activity was progressively reduced in patients with COPD of increasing clinical severity which resulted in the induction of IL-17A and concomitant loss of IL-10 secretion.

**Why read on?**

- In addition to in vivo data we also conducted in vitro study to confirm the effect of reduced IDO activity on the balance between IL-17A and IL-10 expression in monocyte-derived macrophages. The results may lead to a novel approach to treating inflammation in COPD by increasing IDO activity.

stable COPD, the role of regulatory T (Treg) cells and CD4 Th17 cells remains uncertain.

There is an interplay between Treg and Th17 cells in regulating the development of autoimmunity and chronic inflammation.<sup>6</sup> These lineages are both derived from naïve CD4 T cells, which undergo polarisation, and there is mutual suppression between these subtypes in vitro and presumably in vivo.<sup>7</sup> The differentiation of Treg cells may be linked to the differentiation of Th17 cells depending on the overall polarising cytokine environment. The imbalance between Th17 and Treg cells may contribute to the chronic inflammatory response with reduced resolution of airway inflammation in COPD.

IL-17 is predominantly expressed by Th17 cells and has a potential proinflammatory role in COPD by stimulating the production of neutrophil chemotactic mediators such as CXCL1 (GRO- $\alpha$ ), CXCL8 (IL-8) and IL-6 from airway epithelial cells.<sup>8</sup> In addition, IL-17 is upregulated in the bronchial mucosa of patients with COPD.<sup>9–10</sup> However, there are data that question the role of IL-17 in COPD as its expression is not well correlated with airway neutrophilia.<sup>9–10</sup>

Indoleamine 2,3-dioxygenase (IDO), the main inducible and rate-limiting tryptophan degrading enzyme, has recently been shown to act as an important T cell immunomodulator and inducer of tolerance.<sup>11</sup> It is predominantly expressed in

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macrophages and dendritic cells.<sup>11</sup> The IDO pathway has a potential role in the control of airway inflammation in asthma, and this may be mediated via the anti-inflammatory cytokine IL-10<sup>12</sup> which is a potent activator of IDO.<sup>13</sup> IDO inhibits T cell responses in various inflammatory disorders including autoimmune diseases, asthma, allograft rejection and viral infections. It regulates the balance of Th17 to Treg cells through the Treg differentiation inducible effects of kynurenine,<sup>14 15</sup> thereby possibly suppressing Th17-mediated inflammation. However, it is currently unknown whether COPD is associated with reduced IDO activity and how this affects the balance between Th17 and Treg cells. In addition to its anti-inflammatory effects, IDO could suppress oxidative stress by using superoxide anions as a substrate and a cofactor in its catalytic process.<sup>16</sup> Furthermore, tryptophan catabolites act as potent radical scavengers.<sup>17</sup> This led us to hypothesise that patients with increasing severity of COPD may have a progressive reduction in sputum IDO activity that may affect the balance between IL-10 and IL-17A. We therefore measured sputum IDO activity, IL-10 and IL-17A levels in patients with COPD of different severity compared with age-matched non-smoking and smoking controls.

We demonstrate here that patients with COPD have a reduction in IDO activity which is associated with increased IL-17A and decreased IL-10 concentrations and that this is correlated with disease severity. In addition, sputum IL-17A concentrations were positively correlated with IL-6 and CXCL8 concentrations at all COPD severity stages, whereas they were negatively associated with forced expiratory volume in 1 s/forced vital capacity (FEV<sub>1</sub>/FVC%).

## METHODS

### Patients

Patients with COPD who were current or former smokers with a smoking history of  $\geq 10$  pack-years were recruited.

We included male and female subjects aged 40–80 years with baseline post-bronchodilator FEV<sub>1</sub>/FVC < 0.7. COPD severity was graded according to the Global Initiative for Obstructive Lung Disease (GOLD).<sup>18</sup> Patients were excluded if they had a history of asthma or a respiratory infection or a COPD exacerbation within 8 weeks prior to screening and sputum induction. Normal smoker controls had the same demographics and a similar smoking history but were free from significant diseases as determined by history and physical examination and had baseline post-bronchodilator FEV<sub>1</sub> > 80% predicted normal value and FEV<sub>1</sub>/FVC > 0.7. Non-smoking controls were age-matched to normal smokers but with no smoking history. The study was approved by the ethics review committees of Siriraj Hospital and all subjects gave written informed consent.

### Study design

This study was aimed at investigating the levels of sputum IDO activity in patients with COPD of different severity. All patients entering the run-in phase stopped inhaled corticosteroids (ICS), theophylline and long-acting bronchodilators (LABA) and received inhaled short-acting  $\beta_2$  agonists used as rescue medication for 2 weeks prior to sputum induction. At the end of the run-in period, patients were eligible for the study if they had complied with the run-in treatment and had no COPD exacerbations. Seven patients with GOLD stage III COPD dropped out in the run-in period when ICS, theophylline and LABA or long-acting muscarinic antagonists had been withdrawn.

### Statistical analysis

Data are presented as means  $\pm$  SEM. Demographic data were described by number and percentage of patients for categorical variables. Comparisons of IDO activity between patients with COPD who were current or ex-smokers and between cigarette smoke extract (CSE)-untreated and CSE-treated macrophages

**Table 1** Demographic data and clinical characteristics of study subjects

Characteristic	Non-smoking controls (N=7)	Normal smoker controls (N=12)	Patients with COPD		
			GOLD I (N=10)	GOLD II (N=20)	GOLD III (N=14)
Age (years)	62.7 $\pm$ 1.77	53.2 $\pm$ 4.09	74.7 $\pm$ 3.28*,**	68.9 $\pm$ 1.82*,**	67.8 $\pm$ 2.54*,**
Sex (% male)	42.8	100	80	85	92.85
Smoking (pack-years)	0	25.8 $\pm$ 2.11	39.0 $\pm$ 6.04*	43.4 $\pm$ 7.39	27.9 $\pm$ 5.8
Ex-smoker (n)	0	3	8	16	13
ICS/LABA (n)	0	0	6	11	6
FEV <sub>1</sub> /FVC (%)	81.3 $\pm$ 0.87	78.8 $\pm$ 1.21	59.06 $\pm$ 3.09*,**	54.94 $\pm$ 2.22*,**	38.46 $\pm$ 2.69*,**
Postbronchodilator FEV <sub>1</sub> (% predicted)	97.45 $\pm$ 3.3	102.1 $\pm$ 4.15	101.4 $\pm$ 5.47	68.68 $\pm$ 1.88*,**	40.94 $\pm$ 2.13*,**
Prebronchodilator FEV <sub>1</sub> (l)	1.92 $\pm$ 0.15	2.62 $\pm$ 0.08	1.70 $\pm$ 0.16*,**	1.35 $\pm$ 0.07*,**	0.92 $\pm$ 0.06*,**
FEV <sub>1</sub> reversibility (%)	0.62 $\pm$ 0.81	2.72 $\pm$ 0.89	7.31 $\pm$ 2.01	7.71 $\pm$ 1.75	7.9 $\pm$ 2.71*
Total cell count ( $\times 10^6$ )	0.80 $\pm$ 0.06	1.01 $\pm$ 0.13	1.26 $\pm$ 0.22	1.54 $\pm$ 0.15*,**	1.43 $\pm$ 0.31**
Macrophage number ( $\times 10^6$ )	0.34 $\pm$ 0.088	0.32 $\pm$ 0.042**	0.30 $\pm$ 0.05**	0.28 $\pm$ 0.053**	0.198 $\pm$ 0.022*,**
Macrophages (%)	43.46 $\pm$ 11.36	34.64 $\pm$ 4.57**	25.82 $\pm$ 3.4**	21.13 $\pm$ 4.22*,**	17.16 $\pm$ 2.01*,**
Neutrophil number ( $\times 10^6$ )	0.44 $\pm$ 0.10	0.66 $\pm$ 0.12**	0.9 $\pm$ 0.18**	1.2 $\pm$ 0.15*,**	1.20 $\pm$ 0.3*,**
Neutrophils (%)	54.4 $\pm$ 11.56	63.56 $\pm$ 4.42**	69.74 $\pm$ 4.46**	76.26 $\pm$ 4.23*,**	80.72 $\pm$ 2.0*,**
Eosinophil number ( $\times 10^6$ )	0.004 $\pm$ 0.002	0.02 $\pm$ 0.005**	0.024 $\pm$ 0.008**	0.02 $\pm$ 0.005	0.02 $\pm$ 0.008**
Eosinophils (%)	0.57 $\pm$ 0.27	2.22 $\pm$ 0.49**	1.76 $\pm$ 0.53**	1.38 $\pm$ 0.25	2.04 $\pm$ 0.62**
Lymphocyte number ( $\times 10^6$ )	0.0043 $\pm$ 0.002	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.001 $\pm$ 0.0008	0.0 $\pm$ 0.0
Lymphocytes (%)	0.56 $\pm$ 0.24	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.089 $\pm$ 0.05	0.0 $\pm$ 0.0
Epithelial cell number ( $\times 10^6$ )	0.007 $\pm$ 0.003	0.0 $\pm$ 0.0	0.017 $\pm$ 0.01	0.007 $\pm$ 0.002	0.0007 $\pm$ 0.0007
Epithelial cells (%)	1.004 $\pm$ 0.54	0.0 $\pm$ 0.0	1.84 $\pm$ 1.22	0.51 $\pm$ 0.16	0.05 $\pm$ 0.05

Data presented as means  $\pm$  SEM unless otherwise indicated.

\* $p < 0.05$  vs smoking controls.

\*\* $p < 0.05$  vs non-smoking controls.

COPD, chronic obstructive pulmonary disease; GOLD, Global Initiative for Obstructive Lung Disease.

were performed by the Mann–Whitney U test. The  $r_s$  correlation coefficient was determined for the correlation of the kynurenine/tryptophan (Kyn/Trp) ratio, IL-10 and IL-17A with FEV<sub>1</sub>/FVC% using the Spearman rank correlation test. Multiple linear regression analyses were performed for independent variables (medication, number of pack-years). The median was calculated if the distribution of the variables was not normal. Statistical analysis for multiple comparisons was performed using one-way ANOVA and the Welch test with the Dunnett T3 correction<sup>19</sup> for equal variances not assumed or Bonferroni corrections for equal variances assumed. All statistical tests were two-sided, and significance was accepted at the level of 95% and  $p < 0.05$  using PASW statistics 18 (SPSS, IBM, Somers, New York, USA).

Additional details of the study method are provided in the online supplement.

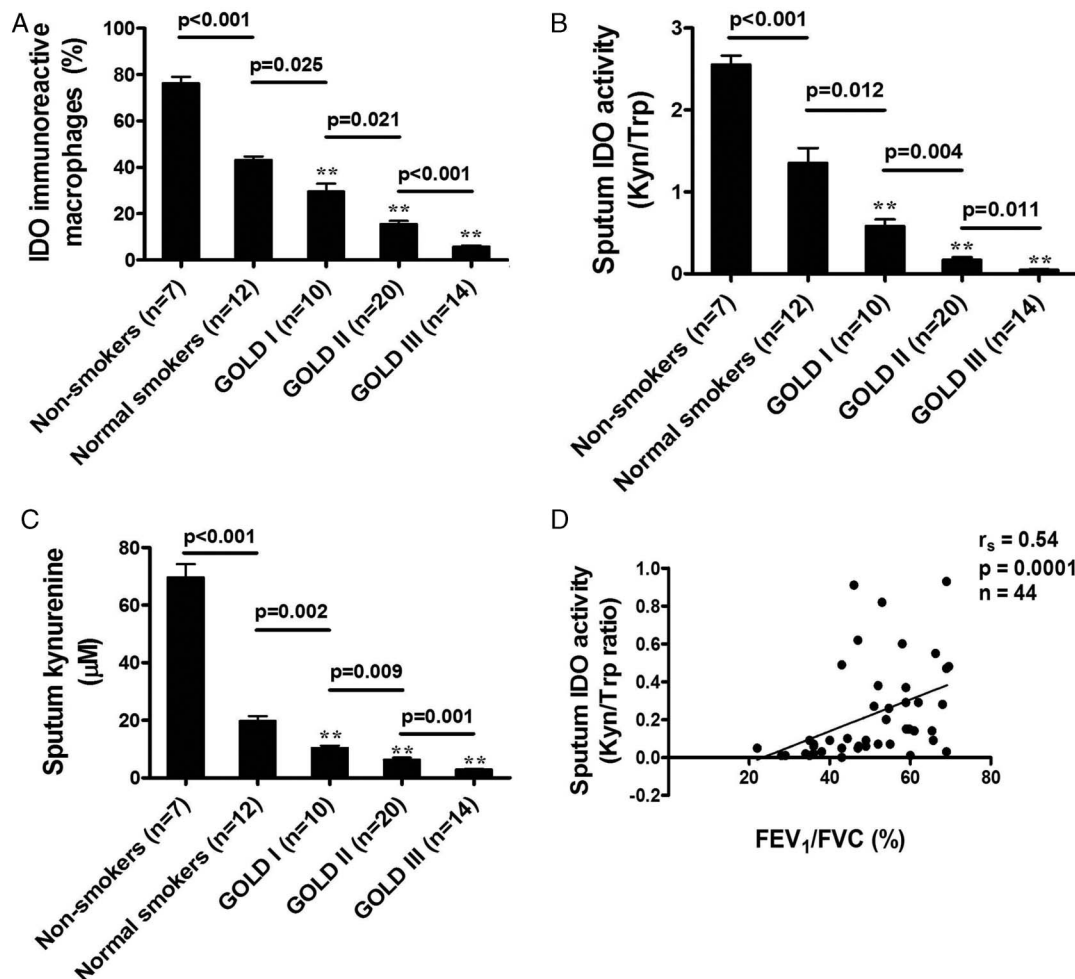
## RESULTS

### Subject characteristics

We studied 63 subjects: 44 with COPD (10 GOLD stage I, 20 GOLD stage II, 14 GOLD stage III), 12 smoking controls without COPD (9 current smokers) and 7 never smokers with normal lung function (table 1). The percentage of sputum neutrophils increased with GOLD grades.

### IDO expression and activity

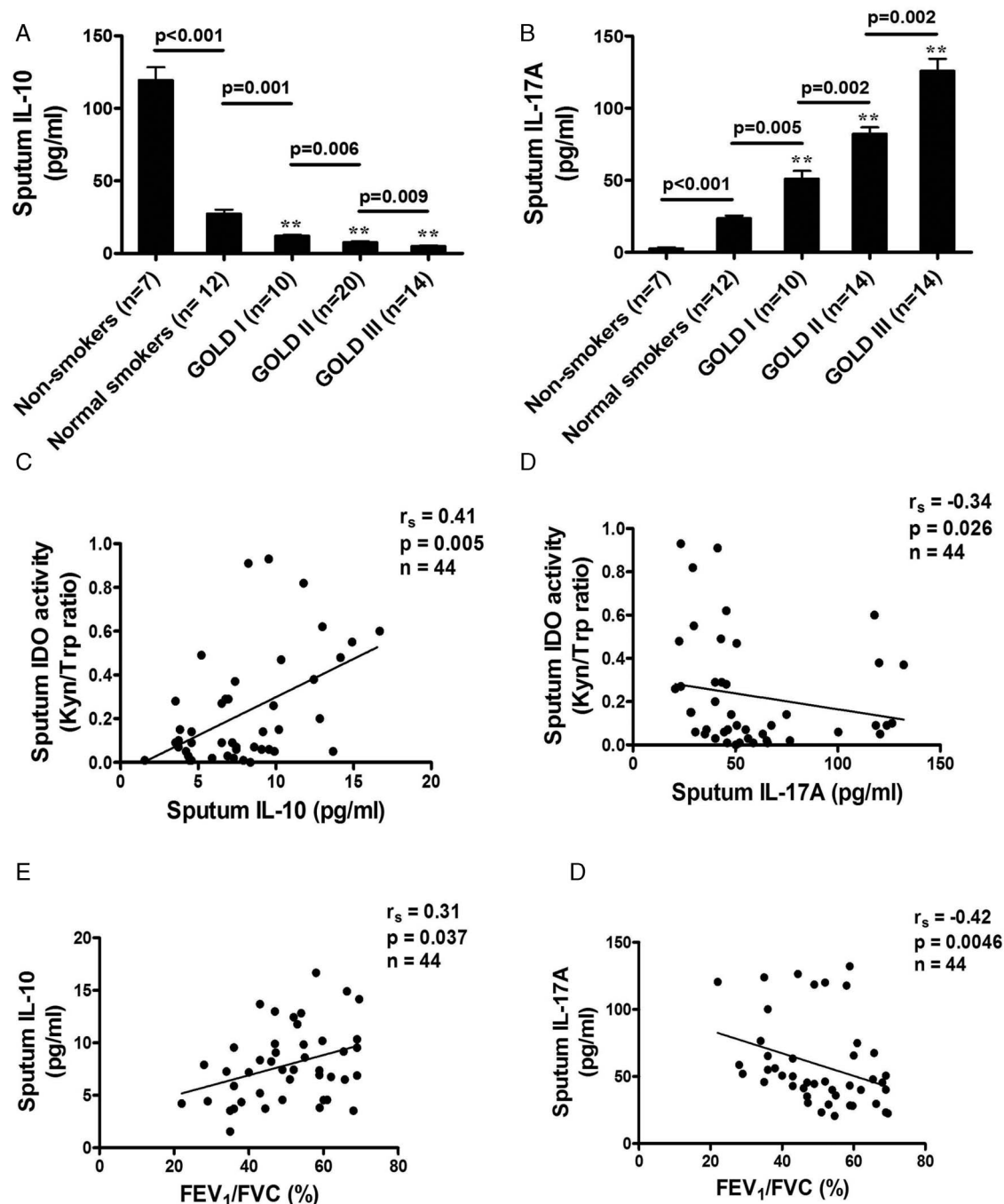
IDO expression and bioactivity (expressed as the Kyn/Trp ratio) measured in sputum samples were both decreased in patients with COPD compared with non-smoking controls, and this was correlated with disease severity (figure 1A,B). These differences reached significance in samples from smoking normal controls (IDO immunoreactive cells  $43.2 \pm 1.5\%$ ,  $p < 0.001$ ; Kyn/Trp ratio  $1.36 \pm 0.18$ ,  $p < 0.001$ ), GOLD stage I COPD (IDO immunopositive cells  $29.5 \pm 3.4\%$ ,  $p < 0.001$ ; Kyn/Trp ratio  $0.58 \pm 0.07$ ,  $p < 0.001$ ), GOLD stage II COPD (IDO immunopositive cells  $15.6 \pm 1.3\%$ ,  $p < 0.001$ ; Kyn/Trp ratio  $0.17 \pm 0.03$ ,  $p < 0.001$ ) and GOLD stage III COPD (IDO immunopositive cells  $5.8 \pm 0.5\%$ ,  $p < 0.001$ ; Kyn/Trp ratio  $0.048 \pm 0.009$ ,  $p < 0.001$ ) compared with those from non-smoking controls (IDO immunopositive cells  $76.28 \pm 2.7\%$ ; Kyn/Trp ratio  $2.55 \pm 0.1$ ). There was a graded reduction in IDO expression and bioactivity in patients with increasing clinical severity of COPD: for IDO expression (smoking controls vs GOLD stage I,  $p = 0.025$ ; GOLD stage I vs GOLD stage II,  $p = 0.021$ ; GOLD stage II vs GOLD stage III,  $p < 0.001$ ); for IDO activity (smoking controls vs GOLD stage I,  $p = 0.012$ ; GOLD stage I vs GOLD stage II,  $p = 0.004$ ; GOLD stage II vs GOLD stage III,  $p = 0.011$ ). Concentrations of kynurenine were also significantly reduced in sputum samples from patients with increasing



**Figure 1** Comparison of (A) sputum indoleamine 2,3-dioxygenase (IDO) expression, (B) IDO activity and (C) kynurenine concentrations in patients with chronic obstructive pulmonary disease of different Global Initiative for Obstructive Lung Disease (GOLD) stages, normal smokers and non-smokers. (D) A positive correlation is shown between IDO activity and forced expiratory volume in 1 s/forced vital capacity (FEV<sub>1</sub>/FVC%). Results expressed as means  $\pm$  SEM. \* $p < 0.05$  vs non-smokers; \*\* $p < 0.001$  vs non-smokers. Kyn/Trp ratio, kynurenine/tryptophan ratio.

severity of COPD compared with samples from non-smoking controls (figure 1C). The differences were significant between samples from non-smoking controls ( $69.6 \pm 4.5 \mu\text{M}$ ) and those from normal smokers ( $19.7 \pm 1.7 \mu\text{M}$ ,  $p < 0.001$ ), GOLD stage I COPD ( $10.4 \pm 0.78 \mu\text{M}$ ,  $p < 0.001$ ), GOLD stage II COPD ( $6.3 \pm 0.72 \mu\text{M}$ ,  $p < 0.001$ ) or GOLD stage III COPD ( $2.9 \pm 0.26 \mu\text{M}$ ,  $p < 0.001$ ). The graded reduction in kynurenine levels also corresponded with the increasing severity of COPD

(smoking controls vs GOLD stage I,  $p = 0.002$ ; GOLD stage I vs GOLD stage II,  $p = 0.009$ ; and GOLD stage II vs GOLD stage III,  $p = 0.001$ ). In addition, IDO activity was significantly correlated with the degree of airflow obstruction ( $\text{FEV}_1/\text{FVC} \%$ ) ( $r_s = 0.54$ ,  $p = 0.0001$ ; figure 1D) and the number of pack-years (coefficient of variance 0.003,  $p = 0.022$ ). There was no difference in IDO activity between patients with COPD who were treated with ICS/LABA (coefficient of variance 0.085,  $p = 0.51$ ).



**Figure 2** Comparison of sputum concentrations of (A) interleukin (IL)-10 and (B) IL-17A in patients with chronic obstructive pulmonary disease of different Global Initiative for Obstructive Lung Disease (GOLD) stages, normal smokers and non-smoking controls. (C, D) Relationship between sputum indoleamine 2,3-dioxygenase (IDO) and IL-10 and IL-17A in patients of all GOLD stages showing the association of lower sputum IDO concentrations in patients with lower IL-10 levels and in patients with higher IL-17A levels. (E, F) Relationship between forced expiratory volume in 1 s/forced vital capacity ( $\text{FEV}_1/\text{FVC} \%$ ) and IL-10 and IL-17A in patients of all GOLD stages showing an association of lower  $\text{FEV}_1/\text{FVC} \%$  in patients with lower IL-10 levels and in patients with higher IL-17A levels. Results expressed as means±SEM. \*\* $p < 0.001$  vs non-smokers.



However, there was no significant difference in IDO activity between patients with COPD who were current smokers or ex-smokers (current smokers  $0.31 \pm 0.09$  vs ex-smokers  $0.22 \pm 0.04$ ,  $p=0.4$ ).

### IL-10

Concentrations of IL-10 were lower in sputum samples from patients with more severe COPD than in samples from non-smoking controls. A significant reduction was found in sputum samples from smoking control subjects ( $27.5 \pm 2.7$  pg/ml) compared with samples from non-smoking controls ( $119.5 \pm 8.9$  pg/ml,  $p<0.001$ ) and from patients with GOLD stage I COPD ( $12.1 \pm 0.84$  pg/ml,  $p<0.001$ ; figure 2A). A further significant reduction was detected in samples from patients with GOLD stage III disease ( $5.0 \pm 0.49$  pg/ml) compared with samples from non-smoking controls ( $p<0.001$ ) and those with GOLD stage II disease ( $7.83 \pm 0.59$  pg/ml,  $p<0.001$ ). Patients with COPD of greater clinical severity exhibited a greater reduction in sputum IL-10 levels (smoking controls vs GOLD stage I,  $p=0.001$ ; GOLD stage I vs GOLD stage II,  $p=0.006$ ; GOLD stage II vs GOLD stage III,  $p=0.009$ ). In addition, sputum IL-10 levels were significantly correlated with IDO activity ( $r_s=0.41$ ,  $p=0.005$ ) and the degree of airflow obstruction ( $r_s=0.31$ ,  $p=0.037$ ; figure 2C,E).

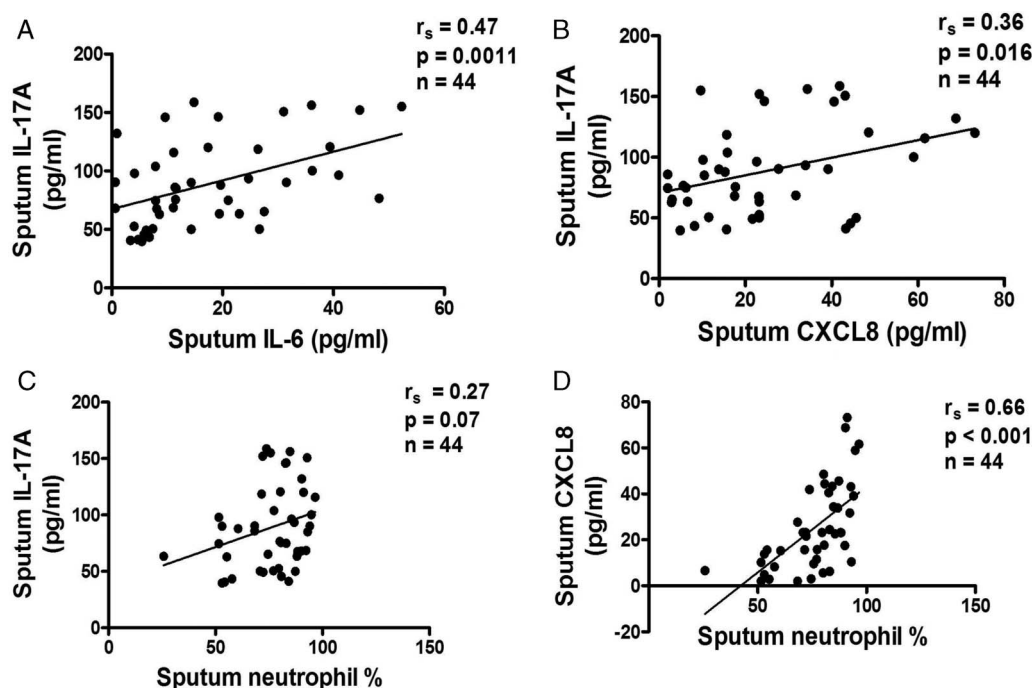
### IL-17A

Patients with COPD of greater severity had an increase in sputum IL-17A concentrations (smoking controls vs GOLD stage I,  $p=0.005$ ; GOLD stage I vs GOLD stage II,  $p=0.002$ ; GOLD stage II vs GOLD stage III,  $p=0.002$ ). A significant increase was found in sputum samples from smoking controls ( $23.6 \pm 1.8$  pg/ml) compared with samples from non-smoking controls ( $2.6 \pm 0.7$  pg/ml,  $p<0.001$ ) and patients with GOLD

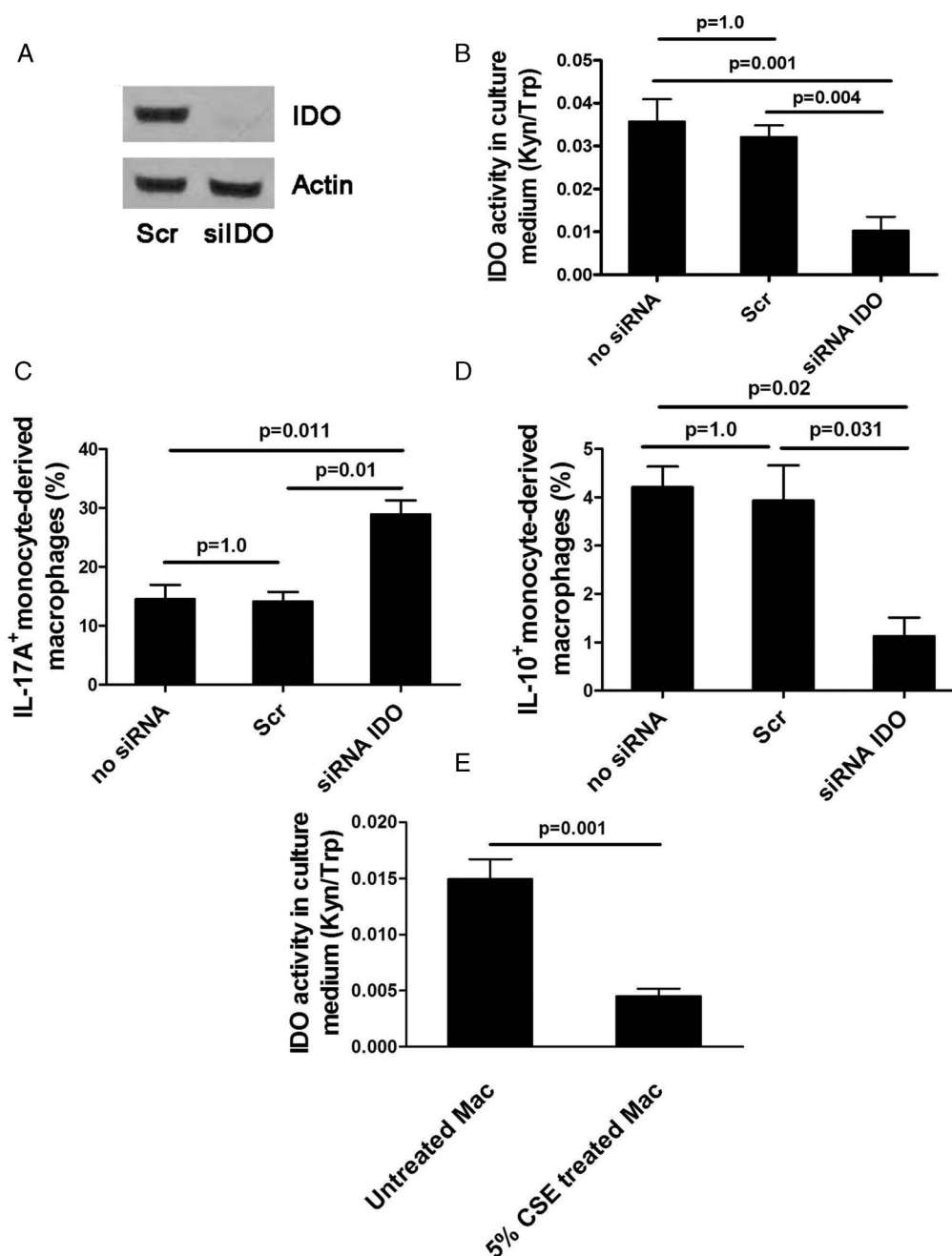
stage I COPD ( $51.1 \pm 5.4$  pg/ml,  $p<0.001$ ; figure 2B). A further significant increase was detected in samples from patients with GOLD stage II COPD ( $82.2 \pm 4.4$  pg/ml) compared with samples from non-smoking controls ( $p<0.001$ ) and from patients with GOLD stage III COPD ( $125.9 \pm 8.3$  pg/ml,  $p<0.001$ ). Sputum IL-17A levels in COPD patients were inversely correlated with IDO activity ( $r_s=-0.34$ ,  $p=0.026$ ; figure 2D) and the degree of airflow obstruction ( $r_s=-0.42$ ,  $p=0.0046$ ; figure 2F). The upregulation of IL-17A levels was correlated with increased sputum IL-6 concentrations ( $r_s=0.47$ ,  $p=0.0011$ ) and CXCL8 concentrations ( $r_s=0.36$ ,  $p=0.016$ ) in patients with COPD of all severity stages (figure 3A,B) but not with percentage sputum neutrophils ( $r_s=0.27$ ,  $p=0.07$ ; figure 3C), whereas increased CXCL8 concentrations were strongly correlated with percentage sputum neutrophils ( $r_s=0.66$ ,  $p<0.001$ ; figure 3D).

### In vitro experiments

We conducted an in vitro study to investigate the effect of reduced IDO activity on the balance between IL-17A and IL-10 expression in monocyte-derived macrophages by knockdown of IDO with siRNA. Treatment of macrophages with siRNA IDO resulted in IDO knockdown both its mRNA and protein expression and bioactivity (Kyn/Trp ratio  $0.01 \pm 0.003$ ,  $p=0.004$ ) compared with scrambled siRNA ( $0.03 \pm 0.002$ ; figure 4A,B), with 77% reduction in IDO mRNA expression. IDO knockdown significantly tipped the balance of the expression of these two cytokines towards IL-17A whereas control siRNAs had no effect (IL-17A:  $28.8 \pm 2.4\%$  for siRNA IDO vs  $14.1 \pm 1.6\%$  for scrambled siRNA,  $p=0.01$ ; IL-10:  $1.1 \pm 0.38\%$  vs  $3.9 \pm 0.7\%$ ,  $p=0.031$ ; figure 4C,D). In addition, CSE significantly reduced IDO activity in monocyte-derived macrophages (CSE-treated macrophages  $0.004 \pm 0.0006$  vs CSE-untreated macrophages



**Figure 3** Relationship between sputum interleukin (IL)-17A levels and (A) IL-6, (B) CXCL8, (C) sputum neutrophil percentage in patients with chronic obstructive pulmonary disease of all Global Initiative for Obstructive Lung Disease (GOLD) stages, illustrating a positive correlation of IL-17A in patients with IL-6 and with CXCL8 but no correlation with sputum neutrophil percentage. (D) A positive correlation is shown between CXCL8 levels and sputum neutrophil percentage in patients with all GOLD stages.



**Figure 4** Effects of siRNA knockdown of indoleamine 2,3-dioxygenase (IDO) in macrophages treated with siIDO compared with scramble (Scr) siRNA. (A) IDO protein expression, (B) IDO activity, (C) interleukin (IL)-17A expression, (D) IL-10 expression, (E) IDO activity in monocyte-derived macrophages exposed to cigarette smoke extract (CSE). Mean±SEM values of three independent experiments are shown. Kyn/Trp ratio, kynurenine/tryptophan ratio.

0.014±0.001,  $p=0.001$ ; figure 4E). These in vitro results complemented our in vivo finding that cigarette smoke contributed to the loss of IDO activity which resulted in IL-17A/IL-10 imbalance.

## DISCUSSION

In this study we demonstrate for the first time that IDO activity and expression is reduced in the sputum of patients with COPD and that this is related to increasing clinical severity. In addition, patients with COPD of increasing clinical severity had progressive upregulation of IL-17A and reduction in IL-10 concentrations. Increased sputum IL-17A concentrations were in turn positively correlated with IL-6 and IL-8 concentrations and with sputum neutrophils.

IDO mediates T cell suppressive and antioxidant effects. It inhibits T cell-mediated responses in several inflammatory disorders, all of which are associated with either neutrophilic or eosinophilic inflammation. It uses superoxide anions as a substrate and a cofactor in its catalytic process.<sup>16</sup> On consumption of superoxide anions, IDO initiates the formation of tryptophan catabolites which act as potent radical scavengers.<sup>17</sup> Because of its ability to convert a pro-oxidant into antioxidants, IDO modulates oxidative stress.<sup>17</sup> IDO suppresses the formation of intracellular reactive oxygen species in acute lung allograft injury and enhances the resistance of lung cells to oxidative stress.<sup>20</sup> Although no data exist on the expression of IDO in COPD, the presence of oxidative stress in patients with COPD,<sup>21</sup> at least in part due to increasingly impaired antioxidant enzymes,<sup>22</sup> may

imply a reduction in IDO activity. Our study showed that IDO activity was indeed reduced in normal smokers who did not have airflow obstruction compared with healthy non-smokers, and progressively reduced with increasing severity of COPD. This reduction in IDO activity was, at least in part, due to decreased IDO expression in sputum macrophages.

Kynurenine, the major metabolite of IDO activity, may tip the balance of IL-10-producing Treg cells to IL-17-producing Th17 cells.<sup>14</sup> Our data confirm that this occurs in COPD patients and that the reduction of IDO activity is linked to the change in balance of IL-10/IL-17 towards IL-17A in induced sputum. A greater imbalance of IL-10/IL-17A levels was found if the clinical severity of COPD was greater, consistent with a previous study showing that the degree of IL-17 upregulation in the bronchial mucosa of patients with stable COPD was correlated with disease severity.<sup>9</sup> In addition, patients with more severe COPD had a significantly greater reduction in kynurenine concentrations. These data suggest that the IL-10/IL-17A balance may be regulated by IDO through the effects of kynurenine.

IL-17A was upregulated in the bronchial mucosa of patients with COPD<sup>9 10</sup> and may contribute to the regulation of chronic inflammation in COPD.<sup>9</sup> IL-17 induced the release of CXCL1, CXCL8 and granulocyte-macrophage colony-stimulating factor from airway epithelial cells and smooth muscle cells, thereby orchestrating neutrophilic inflammation.<sup>8 23 24</sup> Mice exposed to cigarette smoke show upregulation of IL-17 production and increased airway neutrophilia,<sup>25</sup> and the inhibition of IL-17 by a specific neutralising antibody attenuates neutrophilic airway inflammation.<sup>26</sup> However, the present study is consistent with previous studies<sup>9 10</sup> that found no correlation between sputum IL-17A levels with neutrophilic airway inflammation, whereas sputum IL-17A concentrations were negatively associated with airflow obstruction (FEV<sub>1</sub>/FVC%) in patients with COPD. Sputum IL-17A levels were also associated with IL-6 and CXCL8 concentrations which correlated with the percentage of sputum neutrophils. These data suggest that IL-17A-induced neutrophilic airway inflammation in COPD is mediated, at least in part, through IL-6 and CXCL8. However, we cannot exclude the possibility that CXCL8 might be regulated independently from IL-17A as several stimulants are involved in CXCL8 production through the activation of Toll-like receptor (TLR) 9 on neutrophils or via the activation of airway epithelial cells by human rhinovirus and cigarette smoke.<sup>27–29</sup>

The reduction in sputum kynurenine and IL-10 appears to be smoking-dependent. In patients with COPD, although the regulation of IL-10 production is complex, this, at least in part, is associated with smoking status and is dependent on cell type. Cigarette smoking may promote IL-10 release from the airway that is primarily defective in patients with COPD.<sup>30</sup> Cigarette smoke exposure could induce IL-10 production through the upregulation and activation of TLR4 and TLR9 on lung CD8 T cells from patients with COPD.<sup>31</sup> The number of mucosal mature dendritic cells that produce IL-10 was decreased in healthy smokers and current smokers with COPD,<sup>32 33</sup> and this might reduce IL-10 concentrations in the airways. In addition, kynurenine induces the development of tolerogenic dendritic cells<sup>14</sup> and the differentiation of CD4 T cells towards Treg cells<sup>15</sup> through the interaction with aryl hydrocarbon receptors that results in the induction of IL-10 and tryptophan catabolism. This suggests that the IL-10 reduction in smokers, which was not seen in patients with COPD, may be due to the fact that the majority of them in this study were ex-smokers.

In contrast, IL-17A and IDO activity were COPD-dependent. The IDO molecule contains heme, which is essential for its

enzymatic activity. The activation of IDO requires the single electron reduction of ferric to ferrous iron that promotes the binding of IDO to L-tryptophan. Intracellular reducing cofactors including superoxide anion radical are required for IDO heme iron reduction. Cigarette smoke-induced oxidative stress increases superoxide anion radical levels<sup>34</sup> and particulate matter in cigarette smoke causes accumulation of iron,<sup>35</sup> both of which might affect IDO activity. This was consistent with our in vitro study which showed that CSE significantly suppressed IDO activity in macrophages. Although the number of pack-years was positively correlated with IDO activity, there was no difference in IDO activity between patients with COPD who were current smokers and patients with COPD who were ex-smokers. This may explain the higher IDO activity in healthy subjects than in patients with COPD, most of whom were ex-smokers. The mechanisms of IDO suppression in patients with COPD could involve other alternative pathways that warrant further investigation.

Statin treatment upregulates IDO activity in patients with asthma<sup>36</sup> and tips the balance between Treg and Th17-derived cytokines towards IL-10, with downregulation of IL-17.<sup>37</sup> In addition, the use of statins has been associated with a reduced number of exacerbations and the rate of FEV<sub>1</sub> decline in patients with COPD.<sup>38 39</sup> Statins may therefore be beneficial in suppressing airway inflammation in patients with COPD, and this requires future investigation in controlled trials.

In summary, our data provide evidence that IDO activity is progressively reduced in patients with COPD of increasing clinical severity which results in the induction of IL-17A and concomitant loss of IL-10 secretion. This suggests that inflammation in patients with COPD may be amplified, and that this may be a factor determining disease severity and long-term progression of the disease. It also raises the possibility that increasing IDO activity may be a novel approach to treating inflammation in COPD.

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**Decreased indoleamine 2, 3-dioxygenase activity and IL-10/IL-17A ratio in  
COPD patients: *Online Supplement***

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## **SUPPLEMENTAL METHODS**

### **Sputum induction and processing**

Sputum induction was performed as previously described.[E1] The supernatants were kept frozen at  $-70^{\circ}\text{C}$  until further analysis. For immunocytochemistry, cytopins were fixed with 4% paraformaldehyde (BDH Ltd., Poole, UK) and stored at  $-20^{\circ}\text{C}$ . Total cell counts were recorded with on a hemocytometer, using Kimura staining. Cell viability was determined by Trypan blue exclusion before cytopins were undertaken. The slides were stained with May-Grunwald-Giemsa stain and differential cell counts were made by a blinded observer. Four hundred inflammatory cells were counted on two slides for each sample in a blinded manner. Differential cell counts are expressed as the percentages of total inflammatory cells. Samples with cell viability of greater than 70% and less than 30% squamous cell contamination were considered adequate for analysis.

### **Alkaline phosphatase immunostaining**

Indirect staining of sputum cells was performed using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method using a commercial kit (Vectastain Laboratories, Burlingame, CA), as previously described.[E2] Cells were permeabilised for 10 minutes with 0.5% Nonidet P-40 (NP-40, Sigma Chemicals, St Louis, MO) and blocked in 20% normal swine nonimmune serum (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. Polyclonal anti-rabbit antibodies were used to detect IDO (1:100; Genescript International Inc, Temecula, CA). After incubating with the secondary biotinylated goat anti-rabbit antibody, the immunoreaction was detected using the APAAP system to produce red staining. Slides were counterstained with hematoxylin for cellular identification, and examined under light microscopy. Anti-IDO positive cells, which were identified by red immunoreactive signals on sputum cytopins, were counted by an experienced observer blind to the clinical characteristics of the subjects. Immunoreactive macrophages were counted among 300 cells and expressed as a percentage of total cells.

### **ELISA**

For detection of human IL-6, CXCL8, IL-10 and IL-17A sandwich ELISAs were performed according to the manufacturer's instructions (R & D Systems, Minneapolis, MN). The sensitivity was 0.7 pg/mL for IL-6, 3.5 pg/mL for CXCL8, 3.9 pg/mL for IL-10 and 15 pg/mL for IL-17A. Cytokine output was normalised to the concentration of protein.

### Determination of IDO enzymatic activity

Enzymatic activity of IDO was determined by measuring kynurenine (Kyn) and tryptophan (Trp) concentrations in sputum supernatants, as previously described.[E2]

### Preparations of cigarette smoke extract

Cigarette smoke extract (CSE) was prepared using two full-strength Marlboro cigarettes with filters removed (Phillip Morris, VA), which were combusted through a modified 60-ml syringe apparatus into 20 ml RPMI 1640 as described.[E3] Macrophages were treated with CSE for 24 h. Supernatants were collected and stored at -80°C for determination of IDO activity.

### Knockdown of IDO expression

RNAi was used to suppress IDO in macrophages. Two ds 21-mer RNA oligonucleotides directed against IDO were synthesized by Sigma-Proligo (St. Louis, MO). The sequences are AAGAAGTGGGCTTTGCTCTG and CAGAGCAAAGCCCACTTCTT. Cells were transfected with siRNA using DOTAP Liposomal Transfection Reagent (Roche Applied Science, USA), as described by the manufacturer. IDO protein expression was monitored by flow cytometry after 48 h.

### Fluorescence-activated cell sorting (FACS) analysis

Flow cytometry was performed as described.[E4] Macrophages were incubated with the corresponding primary monoclonal antibodies for 60 min at 4°C, washed twice, and resuspended in PBS, 1% BSA. Analysis on 20,000 cells was performed using a FACSsort (Becton Dickinson, CA). The employed primary monoclonal antibodies were Alexa Fluor 488-conjugated anti-IDO (R&D Systems, MN), phycoerythrin-conjugated anti-IL-10 (eBioscience, CA) and Allophycocyanin-conjugated anti-IL-17A mAbs (R&D Systems, MN). The respective negative controls were matched isotype controls. Data were analyzed using WinMDI version 2.9.

### RNA isolation and real-time RT-PCR analysis

Total RNA was extracted from monocyte-derived macrophages by using an RNeasy Mini kit (Qiagen, Valencia, Calif), according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA by using Improm-II Reverse Transcription system (Promega,

Madison, Wis). Real-time RT-PCR was performed with FastStart Universal SYBR Green Master (Roche, Mannheim, Germany) with an ABI PRISM 7900 thermal cycler (Applied Biosystems, Warrington, United Kingdom), according to the manufacturer's instructions. IDO primers were purchased from First BASE Laboratory (Singapore): sense: AGT CCG TGA GTT TGT CCT TTC AA, antisense: TTT CAC ACA GGC GTC ATA AGC T, and glyceraldehyde-3-phosphate dehydrogenase (GADPH) sense: GAA ATC CCATCA CCATCT TCC, antisense: AAA TGA GCC CCA GCC TTC TC. The 7300 System SDS software version 1.4.0.25 was used to analyse the relative quantity of the target cDNA, according to the  $\Delta\Delta C_t$  method.

#### Whole protein extraction and Western blot analysis

Whole cell protein extraction and western blot analysis were performed as previously described.[E5] Whole cell lysates were prepared in NP-40 lysis buffer (0.5% Nonidet P-40, 20 mM Tris-HCl [pH 7.5], 150 mM NaCl) in the presence of complete protease cocktail inhibitor. Lysates were centrifuged at 4°C for 10 min at 12,000 rpm in an Eppendorf microcentrifuge to remove cellular debris. Western blot analysis was performed using polyclonal anti-rabbit antibody against IDO (Genescript International, Inc, Temecula, Calif).



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