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Steroids induce a disequilibrium of secreted interleukin-1 receptor antagonist and interleukin-1β synthesis by human neutrophils

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ABSTRACT: Chronic obstructive pulmonary disease (COPD) is characterised by neutrophilic inflammation in the airways and these neutrophils contribute to the production of inflammatory mediators. Dampening the production of proinflammatory mediators might be an important strategy to treat COPD and glucocorticosteroids are known to do so *via* inhibition of nuclear factor-κB. However, this pathway is important for the control of pro- and anti-inflammatory genes.

We studied the effects of dexamethasone on production and secretion of pro-inflammatory interleukin (IL)-1β and anti-inflammatory secreted IL-1 receptor antagonist (sIL-1Ra) by human neutrophils activated with tumor necrosis factor (TNF)-α.

***In vitro*, TNF-α-stimulated neutrophils produced significant amounts of IL-1β and sIL-1Ra; this production was inhibited by dexamethasone. However, synthesis and secretion of sIL-1Ra was inhibited at lower concentrations dexamethasone compared to IL-1β, which changed the IL-1β:sIL-1Ra ratio significantly. This altered ratio resulted in a more pro-inflammatory condition, as visualised by increased intercellular adhesion molecule-1 expression on human endothelial cells. *In vivo*, moderate-to-severe COPD patients using inhaled glucocorticosteroids have decreased plasma sIL-Ra levels compared with mild-to-moderate patients not on glucocorticosteroid treatment.**

In conclusion, dexamethasone induces a pro-inflammatory shift in the IL-1β:sIL-1Ra cytokine balance in neutrophils *in vitro*, which might contribute to a lack of endogenous anti-inflammatory signals to dampen inflammation *in vivo*.

KEYWORDS: Chronic obstructive pulmonary disease, glucocorticosteroids, interleukin-1β, interleukin-1 receptor antagonist, neutrophil

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The incidence of chronic obstructive pulmonary disease (COPD) is increasing and has been predicted to become the third most common cause of death in the world by 2020 [1]. COPD is an inflammatory disease of the lungs and treatment of stable COPD patients with conventional anti-inflammatory treatment, such as inhaled glucocorticosteroids (GCS), is ineffective [2]. The chronic inflammatory response found in the lungs is characterised predominantly by an accumulation of neutrophils, but macrophages, B-cells and CD8+ T-cells are also involved [3]. Furthermore, increased neutrophil numbers are found in bronchial alveolar lavage (BAL) fluid, induced sputum [4] and bronchial biopsy specimens [5]. These neutrophils synthesise cytokines, chemokines and other inflammatory mediators that are known to contribute to the inflammation in the lungs and other organs [6–8]. Limited data on the effects of inhaled GCS on these extrapulmonary effects of COPD are available [9, 10].

Glucocorticosteroids elicit their function through binding to the glucocorticoid receptor (GR). Two main variants of GR, GRα and GRβ, are expressed in various inflammatory cells and tissues, including neutrophils [11]. GRα is a ligand-dependent transcription factor, which binds glucocorticoid response elements (GRE) in DNA and, subsequently, regulates GR target genes [12]. GRα has also been shown to interact with other transcription factors, such as activator protein (AP)-1 [13] and nuclear factor (NF)-κB [14, 15], and, thereby, modulate gene transcription. In contrast, GRβ does not activate GR-responsive genes [16],

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but has been shown to inhibit the repressive capacity of GRα in a dose-dependent manner *via* mechanisms that are, to date, unclear. It may involve competition for GREs or cofactors, or the formation of GRα/GRβ transcriptionally inactive heterodimers [17].

Inhibition of transcription factors AP-1 and NF-κB by GCS has been identified to be a major mechanism to inhibit proinflammatory cytokine production by immune cells [18]. However, despite the strong capacity of GCS to inhibit inflammation, inhibition of neutrophil-driven inflammation seems to be less effective [19]. Other studies have shown that GCSs elicit proinflammatory effects on granulocytes, such as increased interleukin (IL)-1 receptor (IL-1R) type I expression on human neutrophils [20], prolonged neutrophil survival *in vitro* [21, 22], leukocytosis *in vivo* [23], p38 activation in neutrophils and eosinophils [24, 25], increased immunoglobulin A binding by eosinophils [25], and increased secretion of lysosomal enzymes by neutrophils [26]. Because not only proinflammatory, but also anti-inflammatory, mediators are controlled by the transcription factor NF-κB, GCSs would be expected to affect the expression of anti-inflammatory response as well, which is not often assessed.

In this paper, we report an immune-modulatory role for the GCS dexamethasone on the secretion of IL-1β and secreted IL-1R antagonist (sIL-1Ra) by neutrophils. Activation of neutrophils with TNF-α induced significant IL-1β and sIL-1Ra protein synthesis and secretion by neutrophils *in vitro*. As expected, dexamethasone dampened the production and secretion of proinflammatory IL-1β by neutrophils; however, secretion of sIL-1Ra was inhibited more efficiently than that of IL-1β. This difference resulted in a decreased IL-1β:sIL-1Ra ratio, which allowed an increase of intercellular adhesion molecule (ICAM)-1 expression on human umbilical vein endothelial cells (HUVECs) *in vitro*. Interestingly, the most severe COPD patients that were treated with inhaled GCS showed decreased plasma IL-1Ra concentrations compared to untreated COPD patients and healthy controls, which might indicate either that disease severity, inhaled GCS or both downregulate anti-inflammatory cytokine production *in vivo*.

METHODS

Patients and healthy control subjects

We included 32 patients with a diagnosis of COPD according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria [27]. Demographics and inhaled GCS use are shown in tables 1 and 2, respectively. All patients had stable COPD without an exacerbation in the 4 weeks prior to the study. Patients with other inflammatory conditions, heart failure or treatment with oral GCS were excluded. Healthy, age-matched subjects and asymptomatic smokers without COPD symptoms were included in the study. The medical ethics committee of the University Medical Center Utrecht (Utrecht, the Netherlands) approved the study, and all patients provided written, informed consent.

Reagents

Ficoll-Paque was obtained from GE Healthcare (Uppsala, Sweden). Human serum albumin (HSA) was from Sanquin (Amsterdam, the Netherlands). Dexamethasone and RU38486 (mifepristone) were obtained from Sigma–Aldrich (St Louis, MO, USA) and diluted in ethanol. Recombinant human (rh)TNF-α was purchased from Roche (Indianapolis, IN, USA). rhIL-1β, rhsIL-1Ra, anti-IL-1β and anti-IL-1Ra were all from R&D systems (Abingdon, UK). Anti-actin (I-19) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-coupled secondary antibodies were from Dako (Glostrup, Denmark). Phycoerythrin (PE)-conjugated anti-ICAM-1 (clone MEM-111) was from Caltag (Burlingame, CA, USA). Hydroxyethylpiperazine ethane sulfonic acid (HEPES)-buffered RPMI 1640 was purchased from Invitrogen (Carlsbad, CA, USA). All other materials were reagent-grade.

Granulocyte isolation

Granulocytes were isolated from 100 mL whole blood from healthy donors anticoagulated with trisodium citrate (0.4% w/v, pH 7.4). Blood was diluted 2.5:1 with PBS containing trisodium citrate (0.4% w/v, pH 7.4) and human pasteurised plasma protein solution (4 $g \cdot L^{-1}$). Granulocytes were separated by centrifugation using Ficoll-Paque. Erythrocytes were lysed in isotonic, ice-cold ammonium chloride solution (8.3 $g \cdot L^{-1}$ NH_4CL, 1 $g \cdot L^{-1}$ $KHCO_3$ and 37 $mg \cdot L^{-1}$ EDTA), followed by centrifugation at 4°C. After isolation, granulocytes were washed in PBS containing trisodium citrate and human pasteurized plasma protein solution, and resuspended in HEPES-buffered RPMI 1640 supplemented with 0.5% (w/v) HSA. Purity of neutrophils was >95%, with eosinophils as major contaminant, but <1% monocytes.

Western blot analysis

Neutrophils (5×10^6 cells per sample) in HEPES-buffered RPMI 1640 supplemented with 0.5% (w/v) HSA were allowed to recover for 30 min at 37°C. Subsequently, cells were stimulated with TNF-α (100 $U \cdot mL^{-1}$), dexamethasone (10^{-6}, 10^{-8}, 10^{-10} and 10^{-12} M) or combinations for 3 h at 37°C, washed once with PBS at 4°C, lysed in sample buffer (60 mM tris-(hydroxymethyl)-aminomethane hydrochloride (Tris-HCL) (pH 6.8), 2% sodium dodecylsulphate (SDS), 10% glycerol, 2% β-mercaptoethanol) and boiled for 5 min. Protein samples were separated on 12% SDS-polyacrylamide gels and transferred to Immobilon-P (Millipore, Amsterdam, the Netherlands). The membranes were blocked in hybridisation buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Tween 20) containing 5% (w/v) milk powder (ELK, Campina, the Netherlands) for 1 h, followed by incubation with primary antibody in hybridisation buffer with 0.5% (w/v) milk powder overnight at 4°C. The membranes were washed 3×5 min in hybridization buffer then incubated for 2 h with the secondary antibody, followed by 3×5 min washes in hybridisation buffer and a final wash in PBS. Detection of HRP activity on all Western blots was performed using ECL Plus (GE Healthcare) and detected using a Typhoon 9410 imager (GE Healthcare). Spot density analysis was performed using Imagequant TL (GE Healthcare).

sIL-1Ra and IL-1β ELISAs

For plasma samples, sIL-1Ra (RayBio, Norcross, GA, USA) and a high-sensitivity IL-1β ELISA kits were used according to the manufacturers protocol (R&D systems). For medium samples, sIL-1Ra (RayBio) and IL-1β (R&D systems) ELISA kits were used according to the manufacturers protocol.

TABLE 1 Characteristics of study subjects

	Controls	Asymptomatic smokers	COPD no GCS	COPD GCS
Subjects n	29	17	16	16
Age yrs	60.2±7.9	60.4±6.8	62.1±5.0	66.0±8.6
FEV1 % pred	104.9±14.5	98.2±13.0	67.5±10.9	46.4±17.0
GOLD stage				
I			1	0
II			13	6
III			2	8
IV			0	2
Plasma IL-1β pg·mL^{-1}	0.31±0.38	0.21±0.28	0.28±0.34	0.29±0.34
Plasma sIL-1Ra pg·mL^{-1}	51.2±50.0	37.3±40.0	37.5±23.2	22.2±17.1

Data are presented as mean±SD, unless otherwise stated. COPD: chronic obstructive pulmonary disease, GCS: glucocorticosteroids; FEV1: forced expiratory volume in 1 s; % pred: % predicted; GOLD: Global Initiative for Chronic Obstructive Lung Disease; IL: interleukin; sIL-1Ra: secreted IL-1 receptor antagonist.

HUVEC culture and stimulation

HUVECs were isolated from human umbilical cord veins as described previously [29]. The cells were cultured in endothelial cell growth medium-2 (Lonza, Walkersville, MD, USA). Cell monolayers were grown to confluence over 5–7 days. Second- or third-passage HUVECs were activated with IL-1β in combination with IL-1Ra for 3 h, stained with PE-conjugated anti-ICAM for 30 min, washed twice and analysed in a FACSCalibur flow cytometer (Becton-Dickinson, Breda, the Netherlands).

Statistical analysis

Data are presented as mean±SEM. Normal data without significant heterogeneous variances were analysed using one-way ANOVA, followed by Tukey test as the method of *post hoc* analysis, or t-test, using SPSS 15.0 (SPSS Inc., Chicago, IL, USA) or Graphpad Prism 4 (GraphPad Software, La Jolla, CA, USA). A p-value of <0.05 was considered statistically significant.

RESULTS

TNF-α induces synthesis of pro-IL-1β and sIL-1Ra in neutrophils in a dose- and time-dependent manner

Various proinflammatory mediators are linked to the severity of COPD, but little is known about the anti-inflammatory cytokines in this process. The ratio between pro- and anti-inflammatory mediators will ultimately determine the effect of the inflammatory response. Because neutrophils play an important role in the pathogenesis of COPD, we investigated whether neutrophils synthesise IL-1β and IL-1Ra *de novo* upon stimulation with the proinflammatory mediator TNF-α. First, we investigated dose and time dependency of TNF-α-induced pro-IL-1β and sIL-1Ra protein synthesis by human neutrophils. TNF-α induced intracellular pro-IL-1β (31 kDa) and sIL-1Ra (23 kDa) in a dose-dependent manner (fig. 1a and b, respectively). No intracellular or cell-associated cleaved IL-1β (17 kDa) was detected by Western blotting after stimulation of neutrophils (data not shown). TNF-α-stimulated neutrophils synthesised pro-IL-1β and sIL-1Ra after 1 h, which increased to a maximum at 3–4 h and declined at 5 h after stimulation. Neutrophils treated with vehicle control did not synthesise pro-IL-1β or sIL-1Ra (fig. 1c–f). Based on these results, 100 U·mL^{-1} TNF-α for 3 h was used in further experiments.

TABLE 2 Amount of inhaled glucocorticosteroids (GCS) in chronic obstructive pulmonary disease patients

	Patients n
500 μg·day^{-1} fluticasone propionate	4
1000 μg·day^{-1} fluticasone propionate	9
160 μg·day^{-1} Alvesco®	1
800 μg·day^{-1} budesonide	2
Clinical comparable dose of inhaled GCS#	
Low	1
Medium	4
High	11

#: depending on type and dose of GCS, and the application form (dry-powder inhaler or metered-dose inhaler), according to the criteria in [28].

Dexamethasone inhibits TNF-α-induced IL-1β and IL-1Ra protein production in neutrophils

Anti-inflammatory therapy based on GCS is intended to reduce the synthesis of proinflammatory mediators by inflammatory cells. In order to evaluate the effect of the GCS dexamethasone on TNF-α-induced synthesis of IL-1β and sIL-1Ra, we stimulated neutrophils with TNF-α (100 U·mL^{-1}) alone or TNF-α in combination with different concentrations of dexamethasone (10^{-6}, 10^{-8}, 10^{-10} and 10^{-12} M). Neutrophil stimulation with TNF-α-induced synthesis of pro-IL-1β and sIL-1Ra at the protein level (fig. 2a and b). Dexamethasone inhibited the TNF-α-induced pro-IL-1β as well as sIL-1Ra protein synthesis in a dose-dependent manner, whereas dexamethasone alone showed no significant inhibitory effect on pro-IL-1β or sIL-1Ra (fig. 2a and b). Remarkably, dexamethasone (10^{-6} M) decreased TNF-α-induced sIL-1Ra production by 82%; however, it decreased the TNF-α-induced IL-1β production by only 52%. Inhibition of TNF-α-induced pro-IL-1β and sIL-1Ra synthesis by dexamethasone was mediated through the GR, because addition of an excess RU38486, a competitive GR antagonist [30], antagonised the inhibition of dexamethasone significantly, whereas it had no effect on TNF-α-induced pro-IL-1β and sIL-1Ra synthesis in the absence

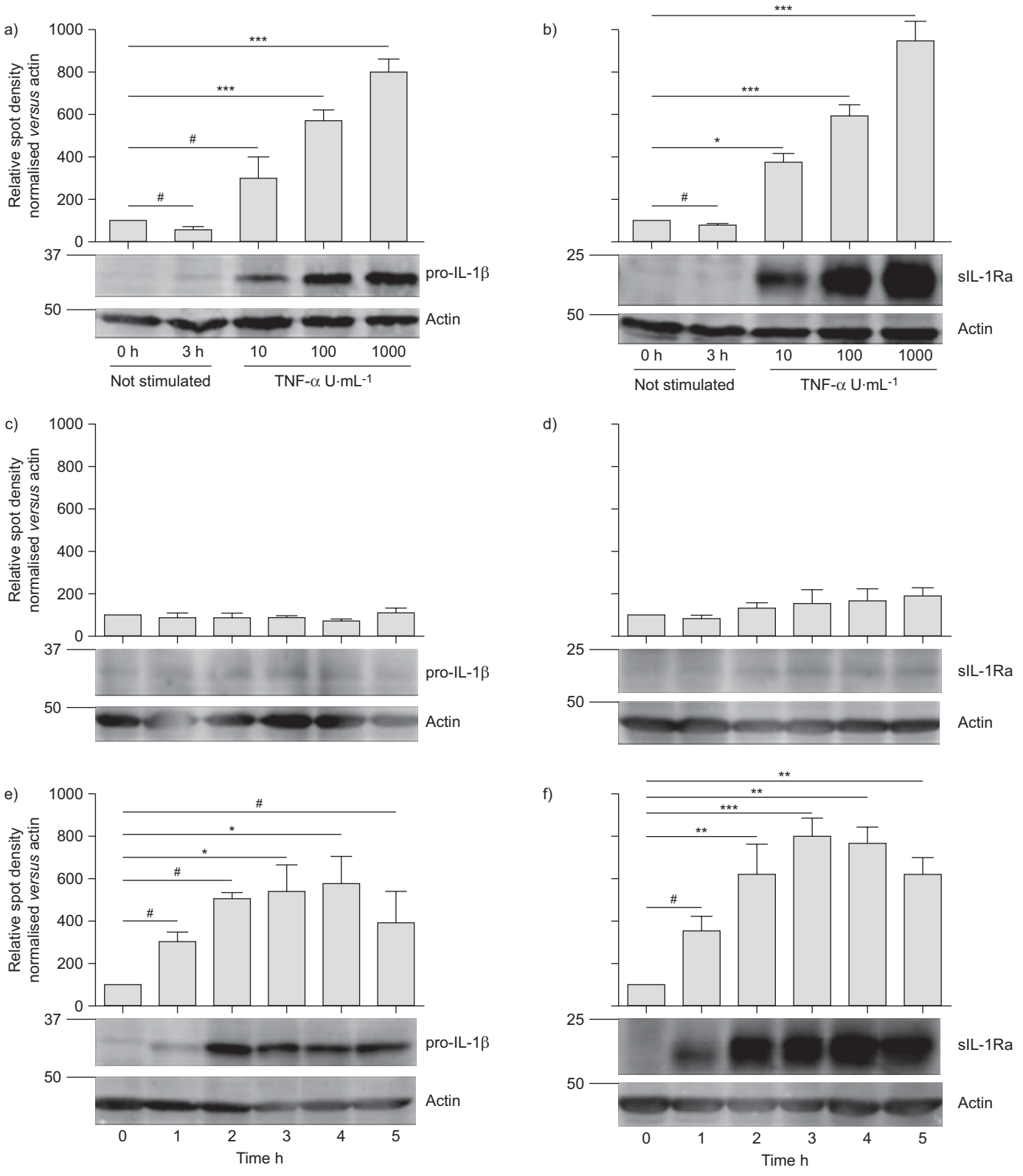

FIGURE 1. Tumour necrosis factor (TNF)-α induces pro-interleukin (IL)-1β and secreted IL-1 receptor antagonist (sIL-1Ra) protein synthesis in a time- and dose-dependent manner. Neutrophils (5×10^6 cells·mL^{-1}) were stimulated a–b) with indicated concentrations of TNF-α for 3 h at 37°C or c–f) the indicated time e, f) with or c, d) without 100 U·mL^{-1} TNF-α at 37°C. Neutrophils were lysed in sample buffer and protein samples were analysed by Western blotting with a, c, e) anti-IL-1β or b, d, f) anti-sIL-1Ra, and anti-actin as loading control. Molecular mass markers are indicated to the left of each blot. Data are presented as mean±SEM (n=6). One-way ANOVA, followed by Tukey test as method of *post hoc* analysis, was used to perform statistical analysis. #: not significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

of dexamethasone (fig. 2c and d, respectively). Thus, dexamethasone-induced GR signalling had a more potent inhibitory effect on TNF-α-induced sIL-1Ra than IL-1β production, implying a proinflammatory balance under these conditions.

Dexamethasone inhibits TNF-α-induced secretion of sIL-1Ra and IL-1β from neutrophils

As both sIL-1Ra and IL-1β are active in the extracellular environment, intracellular production does not necessarily reflect secretion of the cytokines by neutrophils. Therefore, we investigated secretion of both sIL-1Ra and IL-1β from neutrophils into the medium after TNF-α stimulation in the presence or absence of dexamethasone. In order to quantify secreted sIL-1Ra and IL-1β, we performed ELISAs. Stimulation of neutrophils with TNF-α (100 U·mL^{-1} for 3 h) resulted in the production and secretion of 9 pg·mL^{-1} IL-1β and 4 ng·mL^{-1} sIL-1Ra (fig. 3a and b, respectively). Pre-treatment of neutrophils with dexamethasone inhibited the TNF-α-induced secretion of both sIL-1Ra and IL-1β. However, secretion of sIL-1Ra from neutrophils stimulated with TNF-α was inhibited at

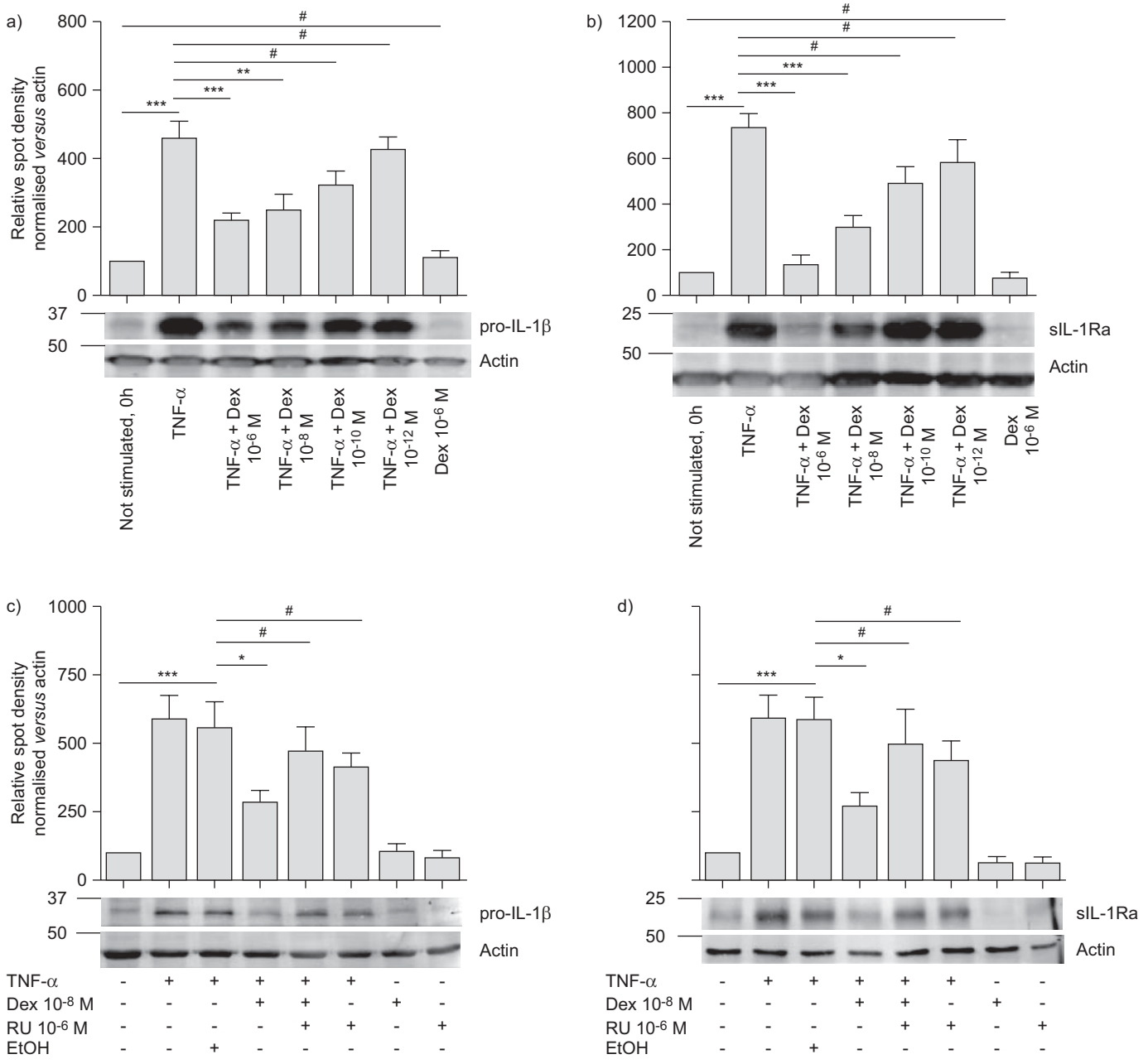

FIGURE 2. Dexamethasone (Dex) inhibits tumour necrosis factor (TNF)-α-induced interleukin (IL)-1β and secreted IL-1 receptor antagonist (sIL-1Ra) protein synthesis in neutrophils. Neutrophils (5×10^6 cells·mL^{-1}) were pre-incubated with indicated amounts of Dex or RU38486 (RU) for 15 min and stimulated with vehicle control or TNF-α (100 U·mL^{-1}) for 3 h at 37°C. Neutrophils were lysed in sample buffer and protein samples were analysed by Western blotting with a, c) anti-IL-1β or b, d) anti-IL-1Ra, and anti-actin as a loading control. The experiment shown is representative of at least three experiments. Data are presented as mean±SEM (n=6). One-way ANOVA, followed by Tukey test as method of *post hoc* analysis, was used to perform statistical analysis. EtOH: ethanol. #: not significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

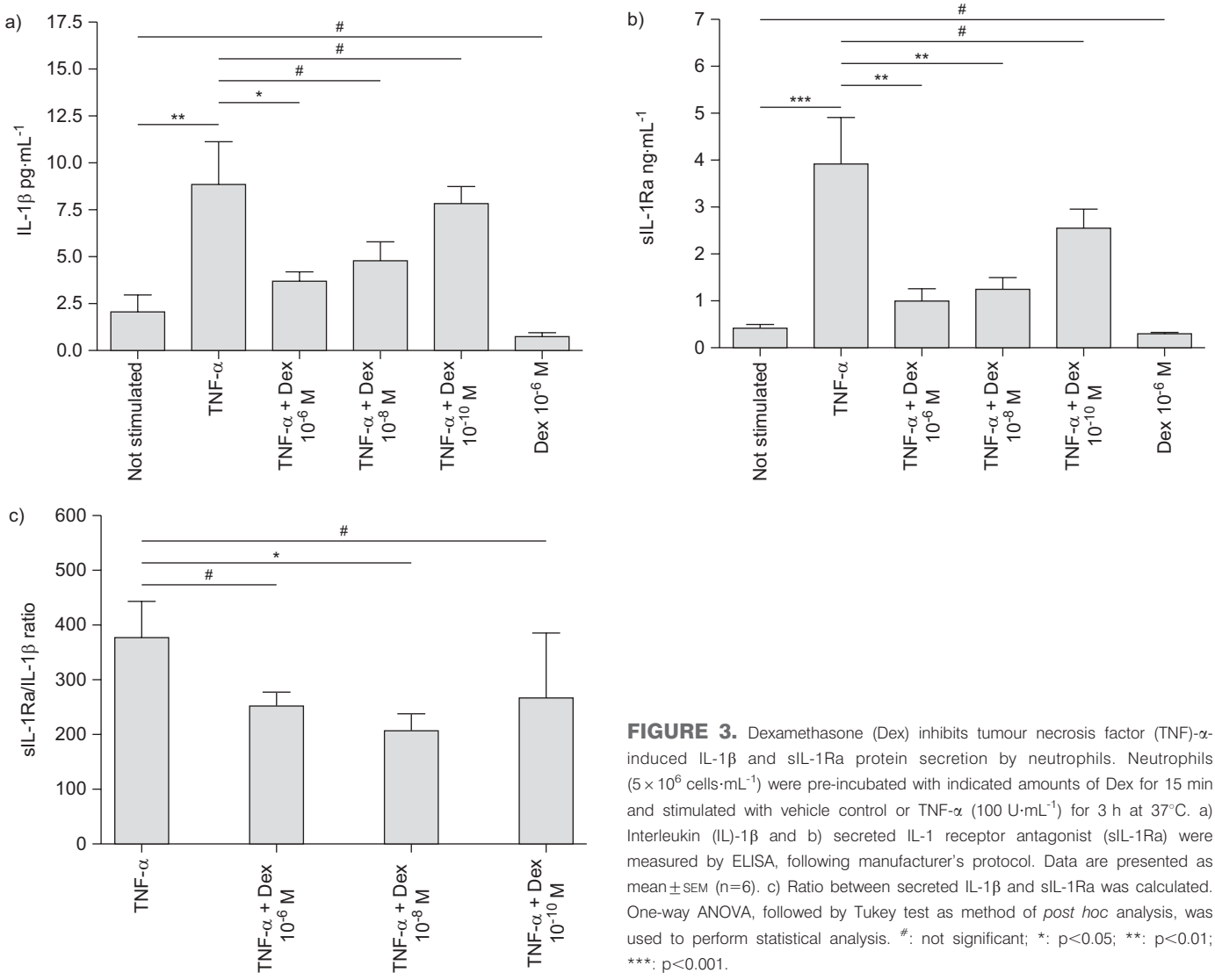

FIGURE 3. Dexamethasone (Dex) inhibits tumour necrosis factor (TNF)-α-induced IL-1β and sIL-1Ra protein secretion by neutrophils. Neutrophils (5×10^6 cells·mL^{-1}) were pre-incubated with indicated amounts of Dex for 15 min and stimulated with vehicle control or TNF-α (100 U·mL^{-1}) for 3 h at 37°C. a) Interleukin (IL)-1β and b) secreted IL-1 receptor antagonist (sIL-1Ra) were measured by ELISA, following manufacturer's protocol. Data are presented as mean±SEM (n=6). c) Ratio between secreted IL-1β and sIL-1Ra was calculated. One-way ANOVA, followed by Tukey test as method of *post hoc* analysis, was used to perform statistical analysis. #: not significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

lower concentrations of dexamethasone (10^{-8} M). When molar concentrations of secreted IL-1β and sIL-1Ra were calculated, TNF-α-stimulated neutrophils synthesized 377 times more sIL-1Ra than IL-1β, indicating that a strong anti-inflammatory response is initiated upon a pro-inflammatory stimulus. Interestingly, the combination of TNF-α and dexamethasone decreased the IL-1β:sIL-1Ra ratio to 1:238, 1:206 and 1:267 for 10^{-10}, 10^{-8} and 10^{-6} M dexamethasone, respectively (fig. 3c). This indicates that the inhibitory capacity of IL-1Ra strongly decreases when GCSs are present.

Different ratios of recombinant human sIL-1Ra and IL-1β modulate TNF-α-induced ICAM-1 expression on endothelial cells

To illustrate the effect of different IL-1β:sIL-1Ra ratios on the pro-inflammatory microenvironment, we used ICAM-1 expression on HUVECs as a biological read-out for IL-1β activity. HUVECs activated by rhIL-1β increased ICAM-1 expression in a dose-dependent manner (fig. 4a). rhsIL-1Ra (100 pg·mL^{-1}) dose-dependently antagonised the IL-1β-induced ICAM-1 expression on HUVECs with a median inhibitory concentration (IC_{50}) of 1.47 ng·mL^{-1} (fig. 4b). A 100-fold excess of rhsIL-1Ra was needed to inhibit the IL-1β-induced ICAM-1 expression by 80%. To evaluate the change in IL-1β:sIL-1Ra molar ratio, we stimulated HUVECs with 10 times the ELISA-measured IL-1β and sIL-1Ra (fig. 3a and b), since this induced ICAM-1 expression on HUVECs (fig. 4a). Corresponding to TNF-α-stimulated neutrophils, 88.5 pg·mL^{-1} rhIL-1β and 39.1 ng·mL^{-1} rhIL-1Ra were not able to induce significant ICAM-1 expression on HUVECs (fig. 4c). However, similarly to neutrophils stimulated with TNF-α in combination with 10^{-8} M dexamethasone, 47.9 pg·mL^{-1} rhIL-1β and 12.4 ng·mL^{-1} rhIL-1Ra induced significant ICAM-1 expression. These results show that, even with lower IL-1β concentrations (47.9 *versus* 88.5 pg·mL^{-1}), a decreasing ratio of IL-1β:sIL-1Ra increased ICAM-1 expression on HUVECs. These results clearly demonstrate that a tight balance between pro- and anti-inflammatory mediators dictate the inflammatory response.

Decreased sIL-1Ra in plasma of COPD patients taking inhaled GCSs

To assess the effects of smoking and GCS usage, we measured IL-1β and sIL-1Ra in the plasma of healthy controls, asympto-

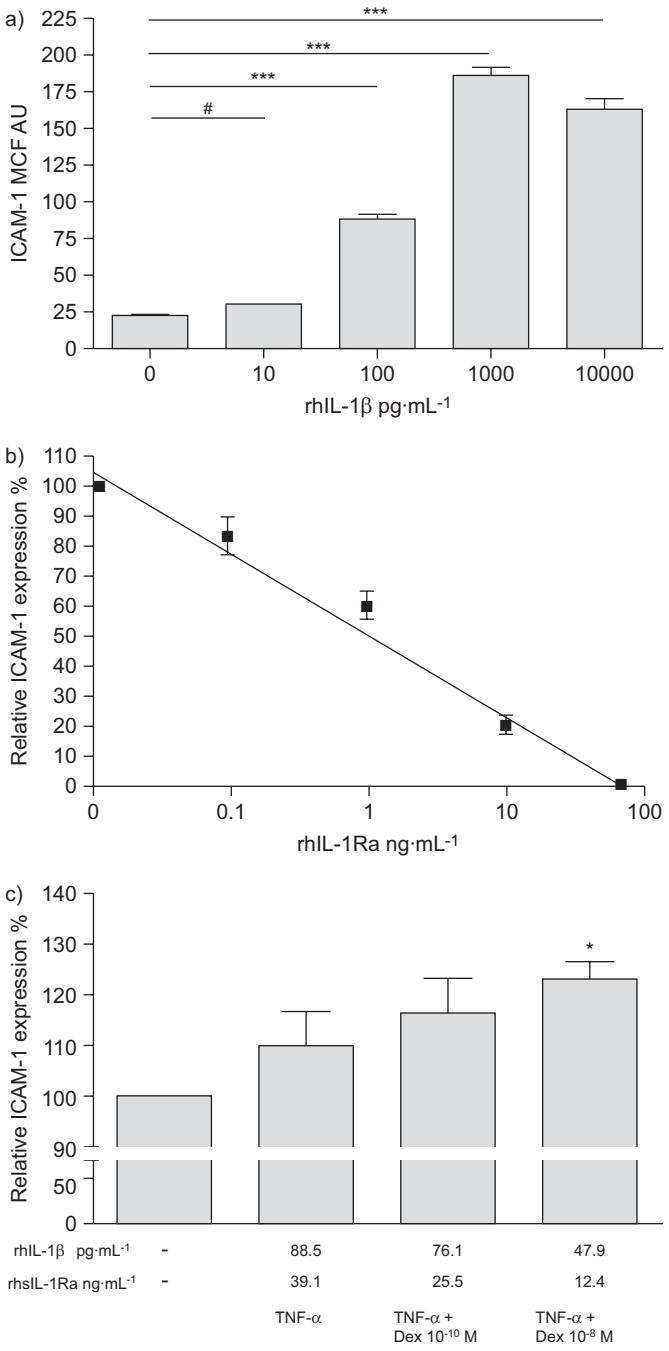

FIGURE 4. Changed interleukin (IL)-1β:secreted IL-1 receptor antagonist (sIL-1Ra) ratio increases intercellular adhesion molecular (ICAM)-1 expression on human umbilical vein endothelial cells (HUVECs). a) HUVECs were stimulated with indicated concentrations of recombinant human (rh)IL-1β for 3 h and ICAM-1 expression was measured by flow cytometry (n=3). Data are depicted as median channel fluorescence (MCF) and given in arbitrary units (AU). b) HUVECs were stimulated with 100 pg·mL^{-1} rhIL-1β in combination with the indicated concentrations rhsIL-1Ra for 3 h and ICAM-1 expression was measured by flow cytometry. Relative inhibition of rhIL-1β induced ICAM-1 expression by rhsIL-1Ra on HUVECs is shown (n=3). y= -12.83ln(x)+54.978, r^2=0.9868. c) HUVECs were stimulated with rhIL-1β in combination with a rhsIL-1Ra corresponding to the amounts measured in the ELISA for 3 h and ICAM-1 expression was measured by flow cytometry (n=5). One-way ANOVA, followed by Tukey test as method of *post hoc* analysis was used to perform statistical analysis. #: not significant; *: p<0.05; **: p<0.01; ***: p<0.001.

matic smokers and COPD patients with and without daily inhaled GCS use. The amounts of inhaled steroids used are shown in table 2. All study subjects were age-matched and no difference in forced expiratory volume in 1 s (FEV_1) was found between healthy controls and asymptomatic smokers (table 1). COPD patients without GCS therapy ranged between GOLD stages I and III, whereas COPD patients on GCS ranged from GOLD stages II to IV, which resulted in a significant difference in FEV_1 between the two groups. Expression of IL-1β was low and not significantly different between the measured groups (fig. 5 and table 1). sIL-1Ra, however, showed a trend for being lower in asymptomatic smokers and COPD patients without GCS therapy compared to healthy controls, whereas it was significantly lower in patients with COPD on a regimen of daily inhaled GCS (fig. 5b). sIL-1Ra expression of COPD patients with and without GCS usage did not correlate with FEV_1 (fig. 5c–d).

DISCUSSION

Treatment of inflammatory diseases with GCS are generally intended to reduce pro-inflammatory cytokine production by inflammatory cells, although their efficacy in inhibiting neutrophil inflammation is poor [2]. Recent work has demonstrated that GCS are able to induce proinflammatory responses in these cells [20–26]. We tested the hypothesis that GCS affect TNF-α-induced synthesis and secretion of pro- and anti-inflammatory cytokines by neutrophils. Therefore, we monitored production of pro-inflammatory IL-1β and anti-inflammatory sIL-1Ra in TNF-α-induced neutrophils in the absence and presence of different amounts of dexamethasone. Several mediators, such as serum amyloid A, granulocyte–macrophage colony-stimulating factor (GM-CSF), lipopolysaccharide (LPS) and TNF-α, can induce pro- and anti-inflammatory mediators by neutrophils [31–38]. We selected TNF-α because this cytokine has been shown to be elevated in serum and BAL of stable COPD patients [39–41] and its action on NF-κB is well documented [42, 43].

Our study corroborates data indicating that both sIL-1Ra [32–35] and IL-1β [36, 37] synthesis is increased in neutrophils following TNF-α stimulation, whereas controversy exists as to the actual secretion of IL-1β by neutrophils. IL-1β secretion from neutrophils upon stimulation with LPS has been observed [35], whereas other studies report no LPS-, zymosan- or GM-CSF- induced IL-1β secretion [33, 35]. The reason for this discrepancy might be due to differences in interpretation of the relevance of the concentrations found. From the study of SCHRÖDER *et al.* [33], it is difficult to know whether low IL-1β levels were actually observed, whereas ALTSTAEDT *et al.* [44] did find a low, but detectable, increase in IL-1β upon zymosan stimulation. Indeed, we also found low but detectable levels (9 pg·mL^{-1}) of IL-1β upon TNF-α stimulation, which was in a similar range as that observed by MARUCHA *et al.* [37]. These concentrations were within the detection range of our ELISA (1–250 pg·mL^{-1}), but are much lower than those produced by monocytes *in vitro* [33]. The low levels produced by neutrophils might still be physiologically relevant in inflammatory conditions *in vivo*, because neutrophil numbers present at sites of inflammation, such as BAL of COPD patients and synovial fluid in rheumatoid arthritis patients [45, 46], are often several times those of monocytes. Therefore, we propose that neutrophils are a significant source of pro-inflammatory IL-1β and anti-inflammatory sIL-1Ra.

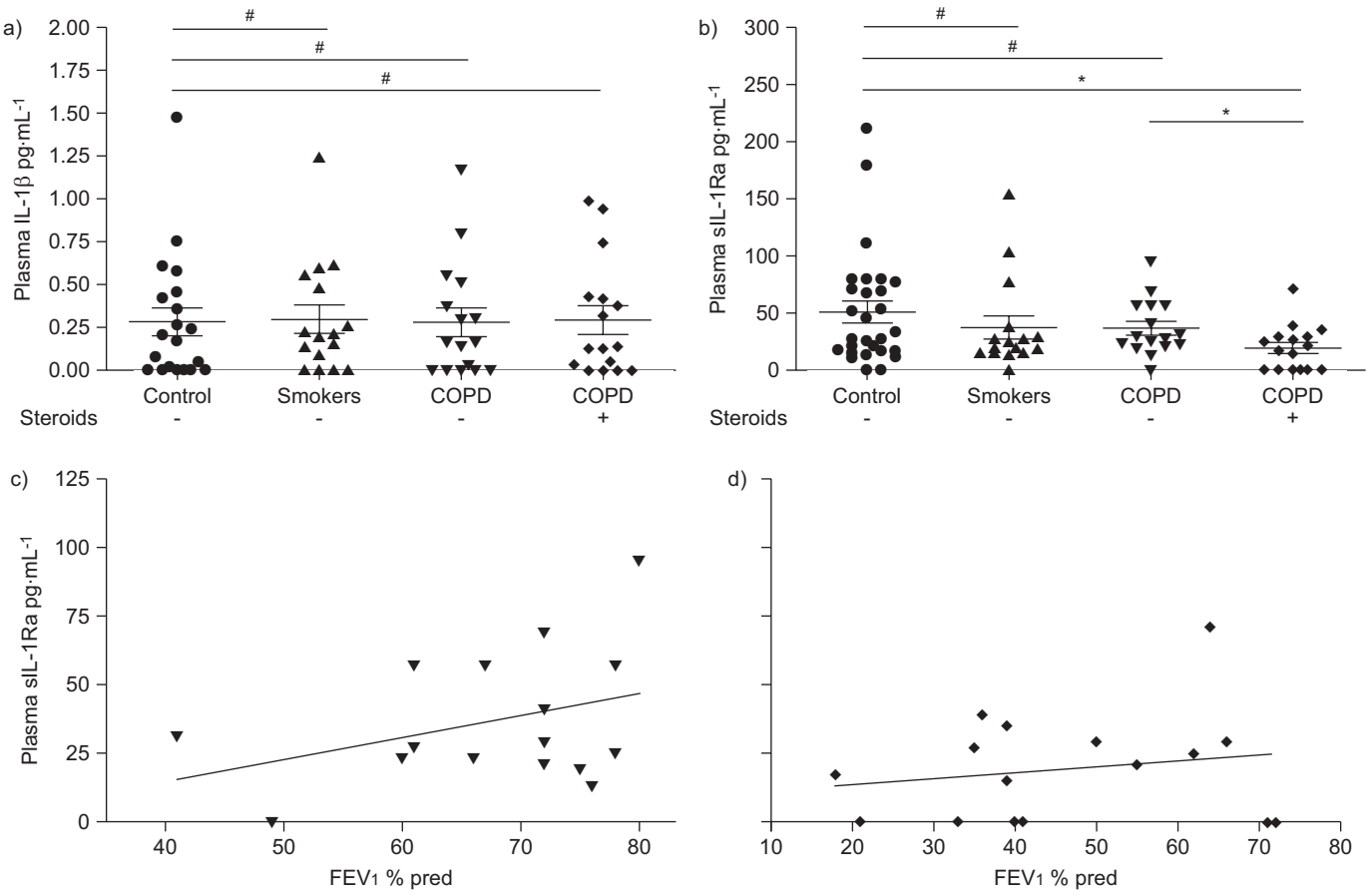

FIGURE 5. Interleukin (IL)-1β and secreted IL-1 receptor antagonist (sIL-1Ra) ELISA on plasma samples of healthy controls, asymptomatic smokers and chronic obstructive pulmonary disease (COPD) patients. Plasma samples were collected, and a) IL-1β and b) sIL-1Ra were measured by ELISA. —: mean. Whiskers represent SEM. An unpaired t-test was used to perform statistical analysis. #: not significant, *: $p<0.05$. Correlation of plasma sIL-1Ra of COPD patients c) without ($r^2=0.1280$, $p=0.1737$) and d) with ($r^2=0.344$, $p=0.4913$) inhaled steroid treatment with forced expiratory volume in 1 s (FEV_1). % pred: % predicted.

Neutrophils are capable of synthesising sIL-1Ra and IL-1β *de novo*, which is thought to be mediated through activation of NF-κB [47–49]. GCSs have been shown to inhibit the activity of this transcription factor [14, 15], which prompted us to investigate the effects of dexamethasone on IL-1β and sIL-1Ra protein synthesis. Interestingly, we found that dexamethasone treatment reduced synthesis and secretion of sIL-1Ra more efficiently than IL-1β, thereby affecting the pro- and anti-inflammatory balance of these secreted IL-1 family members. The mechanism by which dexamethasone shows differential effects on (pro-)IL-1β and sIL-1Ra protein synthesis and secretion is unknown, but could lie in post-transcriptional mechanisms, such as mRNA stability [50], protein translation [51] and/or protein stability [22]. A shifted balance between proinflammatory IL-1β and anti-inflammatory sIL-1Ra has been found *in vivo* in several chronic inflammatory diseases, such as rheumatoid arthritis, ulcerative colitis, Crohn's disease and COPD [52–55]. Recently, REDDY *et al.* [56] and AKSENTIJEVICH *et al.* [57] characterised a mutation in the sIL-1Ra gene that resulted in hyperresponsiveness to IL-1β.

In agreement with SAPEY *et al.* [54], we observed a decreased sIL-1Ra in COPD patients that use daily inhaled GCS when compared with healthy controls, asymptomatic smokers and COPD patients without GCS therapy. This might indicate that *in vivo* inhaled GCS downregulate anti-inflammatory cytokine production. However, neither inhaled nor oral GCS have been shown to influence IL-1β and sIL-1Ra levels in serum, whereas other cytokines, such as interferon-γ-induced protein-10, monocyte chemotactic protein-1 and soluble TNF receptor 2, were affected [9]. Alternatively, the low levels of sIL-1Ra could be due to severity of disease, since the COPD patients that were on inhaled GCS therapy had the lowest FEV_1. However, the observation that no correlation was found between FEV_1 and plasma sIL-1Ra levels in both COPD groups did not support this hypothesis (fig. 5c–d). Thus, our data cannot discriminate between the hypotheses that the low sIL-1Ra levels found in serum of COPD patients could be explained by use of inhaled steroids or by severity of disease. Furthermore, the contribution of neutrophils to these low sIL-1Ra levels *in vivo* remains to be established. Further research is needed in order to address these hypotheses.

The proinflammatory capacity of the IL-1β:sIL-1Ra ratios was shown using ICAM-1 expression by HUVECs as a biological read out. A limitation of our study is that the net inflammatory effect of the IL-1β:sIL-1Ra ratio in supernatants of neutrophils could not be determined because 1) many other putative

inflammatory factors are present in supernatants that might influence ICAM-1 expression on HUVEC cells and 2) supernatants contained TNF-α and dexamethasone that will influence ICAM-1 expression on HUVEC cells. Therefore, we tested different ratios of IL-1β:sIL-1Ra by using recombinant proteins. rhIL-1β was able to induce ICAM-1 expression on HUVECs in a dose-dependent manner (fig. 5a). The rhIL-1β-induced (100 $pg \cdot mL^{-1}$) ICAM-1 expression was inhibited with rhIL-1Ra, with an IC_{50} of 1.47 $ng \cdot mL^{-1}$ (1:15 ratio), which was in accordance to previous published data [58, 59]. As HUVECs responded strongly to 100 $pg \cdot mL^{-1}$ rhIL-1β, we multiplied the IL-1β and sIL-1Ra concentrations measured in the ELISA by a factor of 10. Concentrations of >100 $pg \cdot mL^{-1}$ IL-1β are present in the synovial fluid of arthritis patients [60] and BAL of COPD patients [41], and, depending on the number of neutrophils present at the site of inflammation, these concentrations might be reached. The combination of 88.5 $pg \cdot mL^{-1}$ rhIL-1β and 39.1 $ng \cdot mL^{-1}$ rhsIL-1Ra, which corresponded to the concentrations measured by ELISA for TNF-α-stimulated neutrophils, did not induce significant ICAM-1 expression on HUVECs. However, 47.9 $pg \cdot mL^{-1}$ rhIL-1β in combination with 12.4 $ng \cdot mL^{-1}$ rhsIL-1Ra, corresponding to TNF-α with 10^{-8} M dexamethasone, induced ICAM-1 expression of HUVECs significantly (fig. 4c). These results show that, even with reduced rhIL-1β, the ratio to rhsIL-1Ra determines the biological effect. The importance of sIL-1Ra is demonstrated by the naturally occurring sIL-1Ra-deficient individuals who suffer from severe autoimmune diseases [56, 57]. rhsIL-1Ra (Anakinra) is very effective in treating these diseases and is also used in the treatment of chronic inflammatory diseases [61], such as rheumatoid arthritis [62] and juvenile idiopathic arthritis [63]. Naturally occurring receptor antagonists are potent mediators of the dampening of proinflammatory signals during inflammation. This suggests that an imbalance of IL-1β over sIL-1Ra leads to an exacerbation of inflammation. Furthermore, a three-fold increase in IL-1β secretion by activated human macrophages due to a mutation in the inflammasome protein NALP3 resulted in serious autoinflammatory disorders, emphasising the importance of a tight balance between IL-1β and sIL-1Ra [64, 65].

Overall, we would like to emphasise that it is important to evaluate the inhibitory effect of new drugs on the production of both pro- and anti-inflammatory mediators. Inhibition of NF-κB is one of the most important targets for innovative anti-inflammatory therapy to date [66], despite the fact that anti-inflammatory mediators, such as sIL-1Ra, are also inhibited by this approach. Carefully addressing the effects of GCS on anti-inflammatory cytokine production *in vivo* will be important, because a low endogenous anti-inflammatory host response might aggravate inflammatory disease.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

A statement of interest for L. Koenderman can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

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