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Steroids induce a disequilibrium of sIL-1Ra and IL-1β synthesis by human neutrophils

Steroids affect the IL-1Ra/IL-1β ratio in PMNs

Jeroen D. Langereis, Erik-Jan Oudijk, Rene C. Schweizer, Jan-Willem J. Lammers, Leo Koenderman, Laurien H. Ulfman

Department of Respiratory Medicine, University Medical Center Utrecht, the Netherlands

Heidelberglaan 100, 3584 CX Utrecht, the Netherlands

Correspondence: Laurien Ulfman, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands. Tel.; 0031 88 755 3795, Fax; 0031 88 755 5415, E-mail; l.ulfman@umcutrecht.nl

Abstract

Chronic obstructive pulmonary disease (COPD) is characterized by neutrophilic inflammation in the airways and these cells contribute to the production of inflammatory mediators. Dampening the production of pro-inflammatory mediators might be an important strategy to treat COPD and glucocorticosteroids are known to do so via inhibition of NF-κB. However, this pathway is important for the control of pro- and anti-inflammatory genes. Therefore, we studied the effects of dexamethasone on production and secretion of pro-inflammatory IL-1β and anti-inflammatory sIL-1Ra by human neutrophils activated with TNFα.

In vitro, TNFα-stimulated neutrophils produced significant amounts of IL-1β and sIL-1Ra, which 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 changed ratio resulted in a more pro-inflammatory condition as visualized by increased ICAM-1 expression on human endothelial cells. *In vivo*, moderate–to-severe COPD patients using inhaled glucocorticosteroids have decreased plasma sIL-Ra levels compared to 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

COPD

Glucocorticosteroids

Interleukin-1β

Interleukin-1 receptor antagonist

Neutrophil

Introduction

The incidence of chronic obstructive pulmonary disease (COPD) is increasing and is estimated to be the third cause of death in the world in 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 poorly effective [2]. The chronic inflammatory response found in the lungs is characterized predominantly by an accumulation of neutrophils but also macrophages, B-cells and CD8+ T-cells [3]. Furthermore, enhanced neutrophil numbers are found in bronchial alveolar lavage (BAL) fluid, induced sputum [4] and bronchial biopsy specimens [5]. These neutrophils synthesize cytokines, chemokines and other inflammatory mediators that are known to contribute to the inflammation in the lungs and beyond [6-8]. Limited data on the effects of inhaled GCS on these extrapulmonary effects of COPD are present in literature [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) on the DNA and subsequently regulates GR target genes [12]. GRα is also described to interact with other transcription factor such as activator protein-1 (AP-1) [13] and nuclear factor-κB (NF-κB)[14, 15] and, thereby, modulates gene transcription. In contrast, GRβ does not activate GR-responsive genes [16] but is described to inhibit the repressive capacity of GRα in a dose-dependent manner via mechanisms that are yet unclear. It may involve competition for GRE, co-factors 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 pro-inflammatory 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]. An increased number of reports describe that GCS elicit pro-inflammatory effects on granulocytes such as increased IL-1RI 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 IgA binding for eosinophils [25] and increased secretion of lysosomal enzymes by neutrophils [26]. Because not only pro-inflammatory but also anti-inflammatory mediators are controlled by the transcription factor NF-κB, GCS are expected to affect the expression of anti-inflammatory response as well, which is often not assessed.

In this paper, we report an immune-modulatory role for the glucocorticosteroid dexamethasone on the secretion of IL-1β and sIL-1Ra by neutrophils. Activation of neutrophils with TNFα induced significant IL-1β and sIL-1Ra protein synthesis and secretion by neutrophils *in vitro*. As expected, dexamethasone dampened the production and secretion of pro-inflammatory IL-1β by neutrophils, however, secretion of sIL-1Ra was inhibited more efficiently than IL-1β. This difference resulted in a decreased IL-1β: sIL-1Ra ratio, which allowed an increase of 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 a decreased plasma IL-1Ra level compared to non-treated COPD patients and healthy controls which might either indicate that disease severity or 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) [27] (see for demographics and inhaled GCS use table I and II respectively). All patients had stable COPD without an exacerbation in the last four weeks before entering the study. Patients with other inflammatory conditions, heart failure and treatment with oral glucocorticosteroids were excluded. Healthy age-matched subjects and asymptomatic smokers without COPD symptoms were included. 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) and diluted in ethanol. Recombinant human TNFα was purchased from Roche (Indianapolis, IN). Recombinant IL-1β, recombinant sIL-1Ra, anti-IL-1β and anti-IL-1Ra were from R&D systems (Abingdon, United Kingdom). Anti-actin (I-19) was from Santa Cruz (Santa Cruz, CA). HRP-coupled second antibodies were from DAKO (Denmark). Anti-ICAM-1 PE conjugated (clone MEM-111) was from Caltag (Burlingame, CA). HEPES-buffered RPMI 1640 was purchased from Invitrogen (Carlsbad, CA). 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) exactly as described. In short, blood was diluted 2.5:1 with PBS containing trisodium citrate (0.4% w/v, pH 7.4) and human pasteurized plasma-protein solution (4 g/L). Granulocytes were separated by centrifugation using Ficoll-Paque. Erythrocytes were lysed in isotonic ice-cold NH_4Cl solution followed by centrifugation at 4°C. After isolation, granulocytes were washed in PBS containing trisodium citrate (0.4% w/v, pH 7.4) and human pasteurized plasma-protein solution (4 g/L) and taken up in HEPES-buffered RPMI 1640 supplemented with 0.5% (w/v) HSA. Purity of neutrophils was >95% with eosinophils as major contaminant but less than 1% monocytes.

Western blot analysis

Neutrophils ($5x10^6$ 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 TNFα (100 U/mL), dexamethasone (10^{-6} M, 10^{-8} M, 10^{-10} M, 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-HCL (pH 6.8), 2% 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). The membranes were blocked in hybridization 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 first specific antibody in hybridization buffer with 0.5% (w/v) milk powder overnight at 4°C. The membranes were washed three times 5 min in hybridization buffer, incubated for 2 h with the second antibody followed by three 5 min washings in hybridization

buffer and a last wash step in PBS. Detection of all Western blots was performed by ECL plus (GE Healthcare) and detected using a Typhoon 9410 (GE Healthcare). Spot density analysis was performed with Imagequant TL (GE Healthcare).

sIL-1Ra and IL-1β ELISAs

For plasma samples, sIL-1Ra (catalog # ELH-IL1Ra-001, RayBio, Norcross, GA) and a high sensitivity IL-1β ELISA were performed following the manufacturers protocol (catalog # HSLB00C, R&D systems, Abingdon, United Kingdom). For medium samples, sIL-1Ra (catalog # ELH-IL1Ra-001C, RayBio, Norcross, GA) and IL-1β (catalog # DLB50, R&D systems, Abingdon, United Kingdom) ELISAs were performed following the manufacturers protocol.

HUVEC culture and stimulation

HUVECs were isolated from human umbilical cord veins as previously described [28]. The cells were cultured in endothelial cell growth medium-2 (Lonza, Walkersville, MD). Cell monolayers were grown to confluence in 5–7 days. HUVECs of the second or third passage were activated with IL-1β in combination with IL-1Ra for 3 h, stained with anti-ICAM PE-conjugated for 30 min, washed twice and analyzed in a FACSCalibur flow cytometer (Becton-Dickinson).

Statistical analysis

The results are expressed as standard error of the mean. Normal data without significant heterogeneous variances were analyzed using one-way ANOVA followed by Tukey test as method of post hoc analysis or Student *t* test (statistical software package SPSS version 15.0 or Graphpad Prism 4). $p < 0.05$ was considered statistically significant.

Results

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

Various pro-inflammatory mediators are linked to the severity of COPD, but little is known regarding 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 synthesize *de novo* IL-1β and IL-1Ra upon stimulation with pro-inflammatory mediator TNFα. First, we investigated dose and time dependency of TNFα-induced pro-IL-1β and sIL-1Ra protein synthesis by human neutrophils. TNFα induced intracellular pro-IL-1β (31 kDa) and sIL-1Ra (23 kDa) in a dose dependent manner (Figure 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). TNFα-stimulated neutrophils synthesized pro-IL-1β and sIL-1Ra already after 1 h, which increased to a maximum at 3 h – 4 h and declined at 5 h. Neutrophils activated with vehicle control did not synthesize pro-IL-1β or sIL-1Ra (Figure 1C-F). Based on these results, 100 U/mL TNFα for 3 h was used in further experiments.

Dexamethasone inhibits TNFα-induced IL-1β and IL-1Ra protein production in neutrophils.

Anti-inflammatory therapy based on GCS is aimed to reduce the synthesis of pro-inflammatory mediators by inflammatory cells. In order to evaluate the effect of the GCS dexamethasone on TNFα-induced synthesis of IL-1β and IL-1Ra we stimulated neutrophils with TNFα (100 U/mL)

alone and TNFα in combination with different concentrations of dexamethasone (10^{-6} M, 10^{-8} M, 10^{-10} M and 10^{-12} M). Neutrophil stimulation with TNFα induced synthesis of pro-IL-1β and sIL-1Ra at the protein level (Figure 2A and B). Dexamethasone inhibited the TNFα-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 and sIL-1Ra (Figure 2A and B). Remarkably, dexamethasone (10^{-6}M) decreased TNFα-induced sIL-1Ra production with 82% however it decreased the TNFα-induced IL-1β production by only 52%. Inhibition of TNFα-induced pro-IL-1β and sIL-1Ra by dexamethasone was mediated through the GR because addition of an excess RU38486, a competitive GR antagonist [29], antagonized the inhibition of dexamethasone significantly whereas it had no effect on the TNFα-induced pro-IL-1β and sIL-1Ra in the absence of dexamethasone (Figure 2C and D respectively). Thus, dexamethasone-induced GR signaling had a more potent inhibitory effect on TNFα-induced sIL-1Ra than IL-1β production implying a pro-inflammatory balance under these conditions as a result.

Dexamethasone inhibits TNFα-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 TNFα stimulation in the presence or absence of dexamethasone. In order to quantify secreted sIL-1Ra and IL-1β, we performed Enzyme-Linked Immuno Sorbent Assays (ELISAs). Stimulation of neutrophils with TNFα (100 U/mL, 3 h) resulted in the production and secretion of 9 pg/mL IL-1β and 4 ng/mL sIL-1Ra (Figure 3A and B, respectively). Pre-treatment of neutrophils with dexamethasone inhibited the TNFα-induced secretion of both sIL-1Ra and IL-1β. However, secretion of sIL-1Ra

from neutrophils stimulated with TNFα was inhibited at lower concentrations of dexamethasone (10^{-8}M). When molar concentrations of secreted IL-1β and sIL-1Ra were calculated, TNFα-stimulated neutrophils synthesized 377 times more sIL-1Ra over IL-1β indicating that a strong anti-inflammatory action is initiated upon a pro-inflammatory stimulus. Interestingly, the combination of TNFα and dexamethasone decreased the IL-1β: sIL-1Ra ratio to 1:238, 1:206 and 1:267 for 10^{-10} M, 10^{-8} M and 10^{-6} M dexamethasone, respectively (Figure 3C). This indicates that the inhibitory capacity of IL-1Ra strongly decreases when glucocorticosteroids are present.

Different ratios of recombinant human sIL-1Ra and IL-1β modulate TNFα-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 recombinant human IL-1β increased ICAM-1 expression in a dose-dependent manner (Figure 4A). Recombinant human IL-1Ra dose-dependently antagonized the IL-1β (100 pg/mL) -induced ICAM-1 expression on HUVECs with an IC_{50} of 1.47 ng/mL (Figure 4B). A 100-fold excess of IL-Ra was needed to inhibit the IL-1β-induced ICAM-1 by 80%. To evaluate the change in IL-1β: sIL-1Ra molar ratio, we stimulated HUVECs with 10 times the measured IL-1β and sIL-1Ra with the ELISA (Figure 3A and B) since this enabled ICAM-1 induction on HUVECs (see Figure 4A). Corresponding to TNFα-stimulated neutrophils, 88.5 pg/mL rhIL-1β and 39.1 ng/mL rhIL-1Ra was not able to induce significant ICAM-1 expression on HUVECs (Figure 4C). However, corresponding to neutrophils stimulated with TNFα in combination with 10^{-8}M dexamethasone, 47.9 pg/mL rhIL-1β and 12.4 ng/mL rhIL-1Ra induced significant ICAM-1 expression. These results show that even with lower IL-1β

concentrations (47.9 pg/mL versus 88.5 pg/mL), a decreased 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 IL-1Ra in plasma of COPD patients on inhaled glucocorticosteroids

We measured IL-1β and IL-1Ra in plasma of healthy controls, asymptomatic smokers and COPD patients with and without daily inhaled GCS use to assess the effects of smoking and GCS usage. The amount of inhaled steroids used is depicted in Table II. All study subjects were age-matched and no difference in FEV_1 was present between healthy controls and asymptomatic smokers (Table I). COPD patients without GCS therapy ranged between GOLD I and III, whereas COPD patients on GCS ranged from GOLD 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 (Figure 5A and table 1). IL-1Ra, however, showed a trend to be 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 (Figure 5B). IL-1Ra expression of COPD patients with and without GCS usage did not correlate with FEV_1 (Figure 5C-D).

Table I. Characteristics of study subjects.

	Controls (n=29)	Asymptomatic Smokers (n=17)	COPD no GCS (n=16)	COPD GCS (n=16)
Age (Years)	60.2 (7.9)	60.4 (6.8)	62.1 (5.0)	66.0 (8.6)
FEV_1 (% predicted)	104.9 (14.5)	98.2 (13.0)	67.5 (10.9)	46.4 (17.0)
GOLD I			1	0
II			13	6
III			2	8
IV			0	2
Plasma IL-1β (pg/mL)	0.31 (0.38)	0.21 (0.28)	0.28 (0.34)	0.29 (0.34)
Plasma IL-1Ra (pg/mL)	51.2 (50.0)	37.3 (40.0)	37.5 (23.2)	22.2 (17.1)

Data is represented as mean ± standard deviation COPD: chronic obstructive pulmonary disease, FEV_1:.Forced expiratory volume in 1 second, GOLD: Global Initiative for Chronic Obstructive Lung Disease, GCS: Glucocorticosteroids

Table II. Amount of inhaled glucocorticosteroids in COPD patients.

Inhaled GCS (μg/day)	*Number of patients in group*
Fluticasone propionate (500)	4
Fluticasone propionate (1000)	9
Alvesco (160)	1
Budesonide (800)	2
Clinical comparable dose of inhaled GCS;*	*Number of patients in group*
Low	1
Medium	4
High	11

* According to [30] clinical comparable doses of inhaled GCS are classified in low, medium and high dose depending on type and dose of GCS and the application form (dry powder inhaler or metered dose inhaler).

Discussion

Treatment of inflammatory diseases with GCS are generally aimed to reduce pro-inflammatory cytokine production by inflammatory cells, although their efficacy to inhibit neutrophil inflammation is poor [2]. Recent work has demonstrated that GCS are able to induce pro-inflammatory responses in these cells [20-26]. We tested the hypothesis that GCS affect TNFα-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 TNFα-induced neutrophils in the absence and presence of different amounts of dexamethasone. Several mediators, such as serum amyloid A, GM-CSF, LPS and TNFα can induce pro- and anti-inflammatory mediators by neutrophils [31-38]. We selected TNFα 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 that both IL-1Ra [32-35] and IL-1β [36, 37] synthesis is increased in neutrophils following TNFα stimulation whereas controversy exists on 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 not to have found 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 paper of Schröder et al it is difficult to know whether low IL-1β levels were actually observed [33] whereas Altstaedt et al did find a low but detectable increase in IL-1β upon zymosan stimulation. Indeed, we also found low but detectable levels (9 pg/mL) of IL-1β upon TNFα stimulation which was in a similar range as observed by Marucha et al. [37] These concentrations were within the detection range of our ELISA (1 – 250 pg/mL) but are much lower than 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 are often several folds higher compared to monocytes, such as found in BAL of COPD patients and synovial fluid in rheumatoid arthritis patients [44, 45]. Therefore, we propose that neutrophils are a significant source of pro-inflammatory IL-1β and anti-inflammatory sIL-1Ra.

Neutrophils are capable to synthesize *de novo* sIL-1Ra and IL-1β, which is thought to be mediated through activation of NF-κB [46-48]. Glucocorticosteroids have shown to inhibit the activity of this transcription factor [14, 15] which primed 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 [49] protein translation [50] and/or protein stability [22]. A shifted balance between pro-inflammatory IL-1β and anti-inflammatory sIL-1Ra has been found in several chronic inflammatory diseases such as rheumatoid arthritis, ulcerative colitis, Crohn's disease and COPD *in vivo* [51-54]. Recently, Aksentijevich et al. and Reddy et al showed mutation in the IL-1Ra gene that resulted in hyperresponsiveness to IL-1β [55, 56].

In agreement with Sapey et al, we observed a decreased sIL-1Ra in COPD patients that use daily inhaled GCS [53] when compared to healthy controls, asymptomatic smokers, and COPD patients without glucocorticosteroid therapy. This might indicate that also in vivo inhaled GCS downregulate anti-inflammatory cytokine production. However, neither inhaled nor oral GCS have been shown to be of any influence on IL-1β and IL1Ra levels in serum whereas other

cytokines such as IP-10, MCP-1 and sTNF-R2 were affected [9]. Alternatively, the low levels of IL-1Ra were due to severity of disease since the COPD patients that were on inhaled glucocorticosteroid therapy had the lowest FEV_1. However, the observation that no correlation was found between FEV_1 and plasma IL-1Ra levels in both COPD groups did not support this hypothesis (Figure 5C-D). Thus, our data cannot discriminate between the hypotheses that the low IL-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 IL-1Ra levels in vivo remains to be established. Further research should be performed to address these hypotheses.

The pro-inflammatory 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:IL-1Ra ratio in supernatants of neutrophils could not be determined because A) many other putative inflammatory factors are present in supernatants that might influence ICAM-1 expression on HUVEC cells and B) supernatants contained TNFα and dexamethasone that will influence ICAM-1 expression on HUVEC cells. Therefore, we tested different ratios of IL-1β/IL-1Ra by using recombinant proteins. Recombinant human IL-1β was able to induce ICAM-1 expression on HUVECs in a dose-dependent manner (Figure 5A). The rhIL-1β (100 pg/mL) -induced ICAM-1 expression was inhibited with rhIL-1Ra, with an IC_{50} of 1.47 ng/mL (1:15 ratio), which was in accordance to previous published data [57, 58]. As HUVEC cells responded potently to 100 pg/mL rhIL-1β we multiplied the IL-1β and IL-1Ra concentrations measured in the ELISA 10-times. Concentrations of over 100 pg/mL IL-1β are present in the synovial fluid of arthritis patients [59] and BAL of COPD patients [41] and depending on the amount of neutrophils present at the site of inflammation these concentrations might be reached. The

combination of 88.5 pg/mL rhIL-1β and 39.1 ng/mL rhIL-1Ra that corresponded to TNFα-stimulated neutrophils did not induce significant ICAM-1 expression on HUVECs. However, 47.9 pg/mL rhIL-1β in combination with 12.4 ng/mL rhIL-1Ra, corresponding to TNFα with 10^{-8}M dexamethasone, induced ICAM-1 expression of HUVECs significantly (Figure 4C). These results show that even with reduced rhIL-1β, the ratio to rhIL-1Ra determines the biological effect. The importance of IL-1Ra is demonstrated by the natural occurring IL-1Ra deficient individuals that suffer from severe autoimmune diseases [55, 56]. Recombinant sIL-1Ra (Anakinra) was very effective in this disease and is also used in the treatment of chronic inflammatory diseases [60] such as rheumatoid arthritis [61] and juvenile idiopathic arthritis [62]. Naturally occurring receptor antagonist are potent mediators in dampening pro-inflammatory signals during inflammation and 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 auto-inflammatory disorders, emphasizing the importance of a tight balance between IL-1β and IL-1Ra [63, 64].

Overall, we would like to emphasize 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 [65], despite the fact that anti-inflammatory mediators such as sIL-1Ra are inhibited by this approach as well. Carefully addressing the effects of glucocorticosteroids on anti-inflammatory cytokine production in vivo will be important because a low endogenous anti-inflammatory host response might aggravate the inflammatory disease.

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Figure legends

Figure 1. TNFα induces pro-IL-1β and IL-1Ra protein synthesis in a time and dose dependent manner. Neutrophils ($5x10^6$/mL) were stimulated with indicated concentrations of TNFα for 3 h at 37°C (A-B) or for indicated time with 100 U/mL TNFα at 37°C (C-F). Neutrophils were lysed in sample buffer and protein samples were analyzed by Western blotting with anti-IL-1β (A, C, E), anti-IL-1Ra (B, D, F) or anti-actin as loading control. Mean values are represented ± SEM (n=6). One-way ANOVA followed by Tukey test as method of post hoc analysis was used to perform statistical analysis (NS = not significant, * = P<0.05, ** = P<0.01, *** = P<0.001).

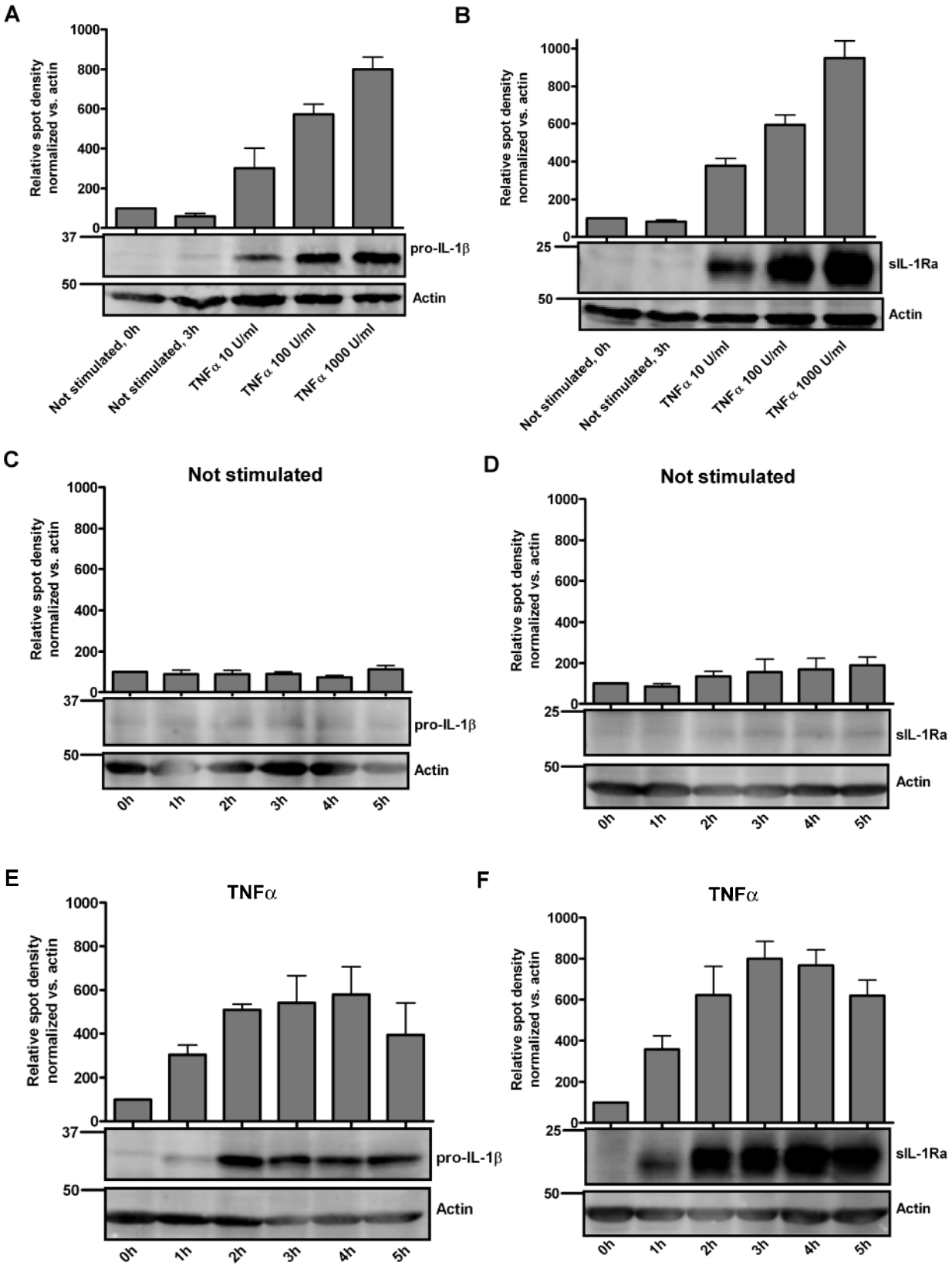
A
Relative spot density normalized vs. actin
1000
800
600
400
200
0
37
50
pro-IL-1β
Actin
Not stimulated, 0h
Not stimulated, 3h
TNFα 10 U/ml
TNFα 100 U/ml
TNFα 1000 U/ml
B
Relative spot density normalized vs. actin
25
sIL-1Ra
C
Not stimulated
0h
1h
2h
3h
4h
5h
D
Not stimulated
E
TNFα
F
TNFα

Figure 1

Figure 2. Dexamethasone inhibits TNFα-induced IL-1β and IL-1Ra protein synthesis in neutrophils. Neutrophils ($5x10^6$/mL) were pre-incubated with indicated amounts dexamethasone or RU38486 for 15 min and stimulated with vehicle control or TNFα (100 U/mL) for 3 h at 37°C. Neutrophils were lysed in sample buffer and protein samples were analyzed by Western blotting with, anti-IL-1β (A, C) anti-IL-1Ra (B, D) or anti-actin as loading controls. The experiment shown is representative for at least three experiments. Mean values are represented ± SEM (n=6). One-way ANOVA followed by Tukey test as method of post hoc analysis was used to perform statistical analysis (NS = not significant, * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$).

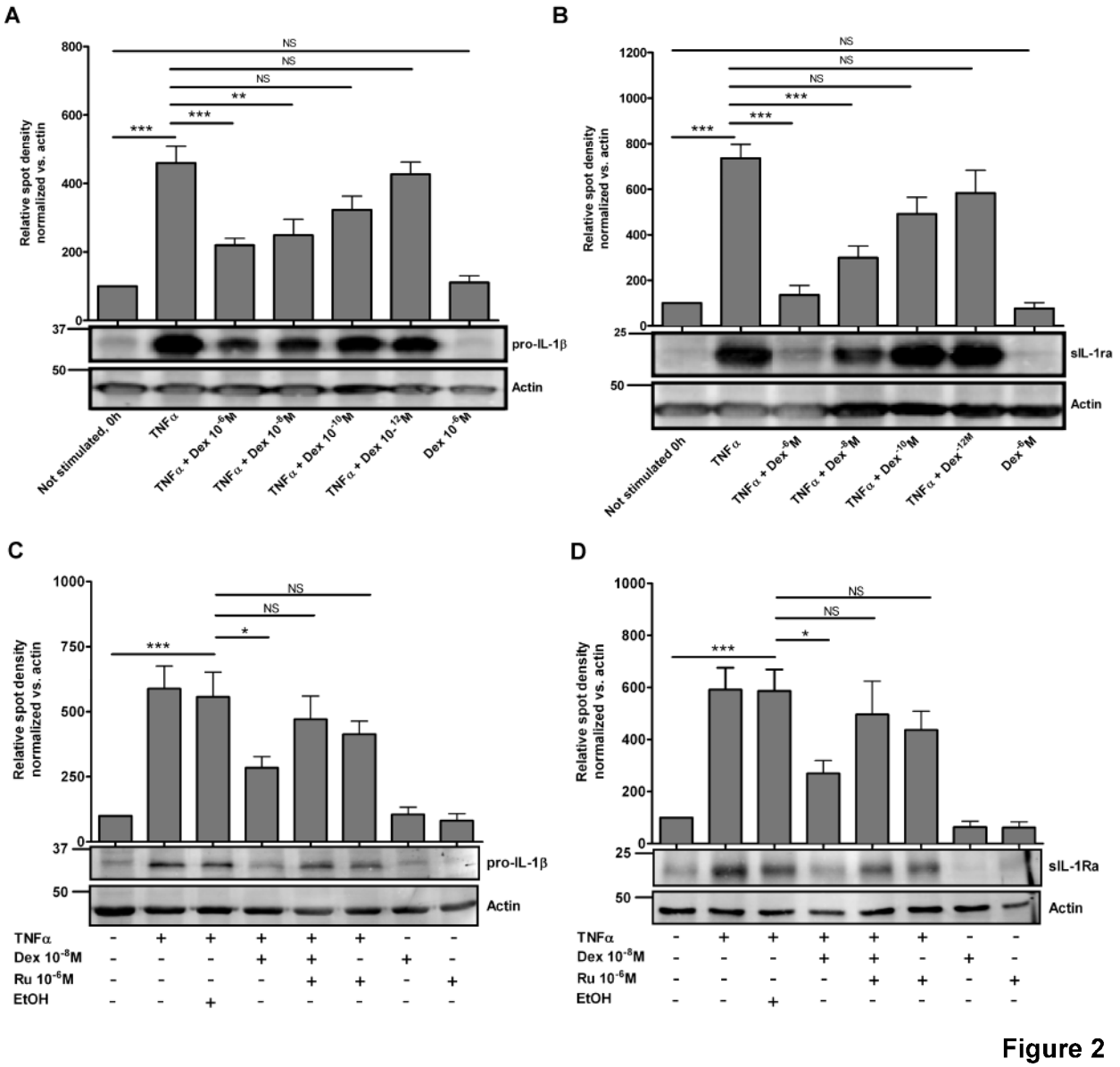

Figure 2

Figure 3. Dexamethasone inhibits TNFα-induced IL-1β and sIL-1Ra protein secretion by neutrophils. Neutrophils ($5x10^6$/mL) were pre-incubated with indicated amounts dexamethasone for 15 min and stimulated with vehicle control or TNFα (100 U/mL) for 3 h at 37°C. IL-1β (A) and sIL-1Ra (B) were measured via ELISA following manufacturer's protocol. Mean values are presented ± SEM (n=6). Ratio between secreted IL-1β and sIL-1Ra measured by ELISA was calculated (C). One-way ANOVA followed by Tukey test as method of post hoc analysis was

used to perform statistical analysis (NS = not significant, * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$).

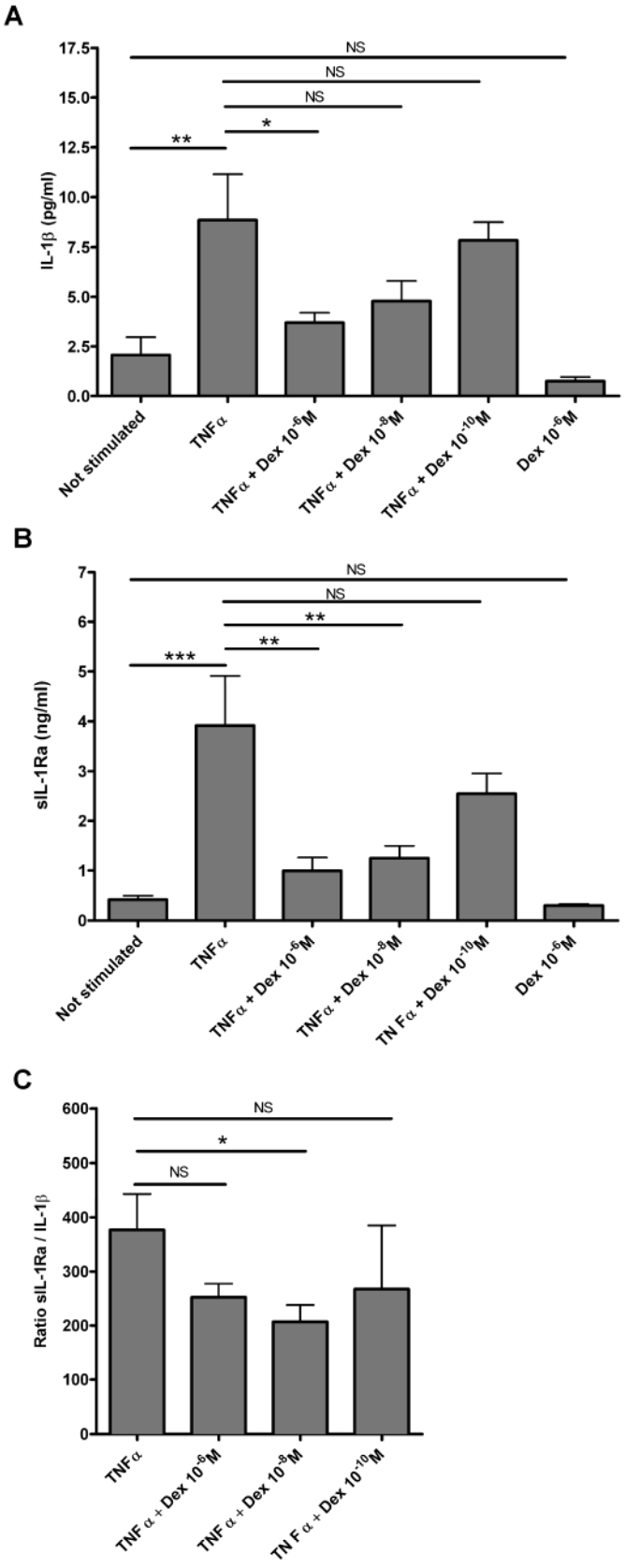
A
IL-1β (pg/ml)
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
NS
NS
NS
*
**
Not stimulated
TNFα
TNFα + Dex 10-6M
TNFα + Dex 10-8M
TNFα + Dex 10-10M
Dex 10-6M
B
sIL-1Ra (ng/ml)
7
6
5
4
3
2
1
0
NS
NS
**
**
***
Not stimulated
TNFα
TNFα + Dex 10-6M
TNFα + Dex 10-8M
TN Fα + Dex 10-10M
Dex 10-6M
C
Ratio sIL-1Ra / IL-1β
600
500
400
300
200
100
0
NS
*
NS
TNFα
TNF α + Dex 10-6M
TNF α + Dex 10-8M
TN F α + Dex 10-10M

Figure 3

Figure 4. Changed IL-1β: sIL-1Ra ratio increases ICAM-1 expression on HUVECs. (A), HUVECs were stimulated with indicated concentrations rhIL-1β for 3 h and ICAM-1 expression was measured with 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 rhIL-1β in combination with the indicated concentrations rhIL-1Ra for 3 h and ICAM-1 expression was measured with flow cytometry. Relative inhibition of rhIL-1β induced ICAM-1 expression by rhIL-1Ra on HUVECs was depicted (n=3). (C), HUVECs were stimulated with rhIL-1β in combination with a rhIL-1Ra corresponding to the amounts measured in the ELISA for 3 h and ICAM-1 expression was measured with flow cytometry (n=5). One-way ANOVA followed by Tukey test as method of post hoc analysis was used to perform statistical analysis (NS = not significant, * = P<0.05, ** = P<0.01, *** = P<0.001).

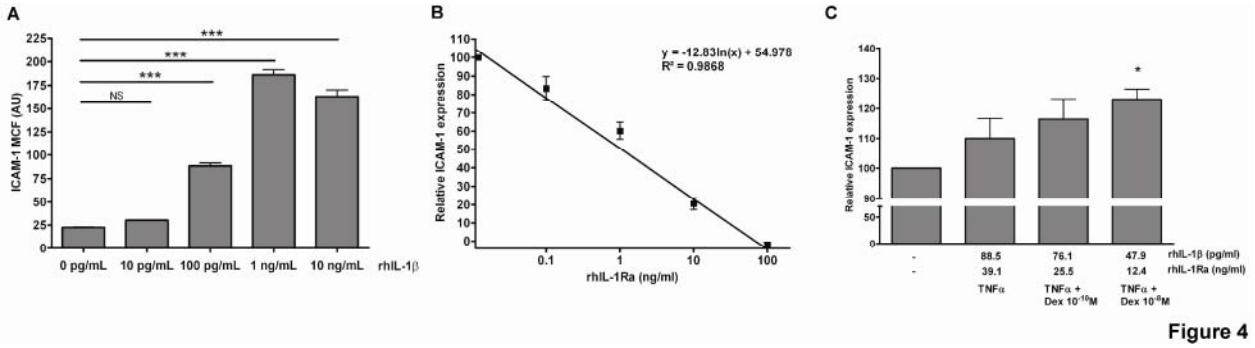

Figure 4

Figure 5. IL-1β and IL-1Ra ELISA on plasma samples of healthy controls, asymptomatic smokers and COPD patients. Plasma samples were collected and IL-1β (A) and IL-1Ra (B) were measured by ELISA. A non-paired Student's t-test was used to perform statistical analysis (NS =

not significant, * = P<0.05). Correlation of plasma IL-1Ra of COPD patients without inhaled steroid (C) and with inhaled steroid treatment (D) with FEV_1 (% predicted).

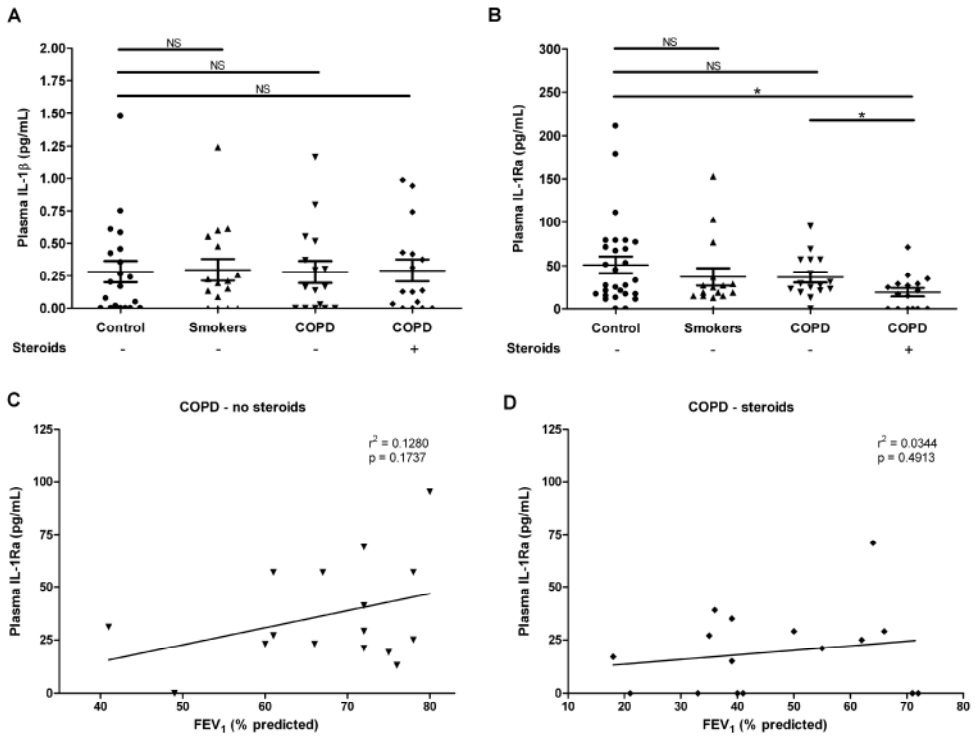

Figure 5