

Imbalanced secretion of IL-1 β and IL-1RA in *Chlamydia pneumoniae*-infected mononuclear cells from COPD patients

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Imbalanced secretion of IL-1 β and IL-1RA in Chlamydia pneumoniae-infected mononuclear cells from COPD patients. J. Rupp, H. Kothe, A. Mueller, M. Maass, K. Dalhoff. ©ERS Journals Ltd 2003.

ABSTRACT: Balanced secretion of pro- and anti-inflammatory cytokines is essential in limiting pulmonary inflammation in respiratory infections. It was hypothesised that, in acute infection with *Chlamydia pneumoniae*, mononuclear cells from chronic obstructive pulmonary disease (COPD) patients lack the opportunity to compensate for the inflammatory immune response by secreting adequate amounts of anti-inflammatory cytokines.

Alveolar macrophages (AMs) and peripheral blood mononuclear cells (PBMCs) from eight COPD patients and eight healthy controls were infected with *C. pneumoniae* in order to determine interleukin (IL)-1 β , IL-1 receptor antagonist (IL-1RA) and IL-8 expression and messenger ribonucleic acid levels.

Secretion of IL-1 β was significantly enhanced in AMs (six-fold) and PBMCs (four-fold) from COPD patients after infection with *C. pneumoniae*. Compared to the control group, release of its anti-inflammatory counterpart IL-1RA was diminished in COPD patients, resulting in a significantly higher IL-1 β /IL-1RA ratio in *C. pneumoniae*-infected AMs and PBMCs from COPD patients.

Mononuclear cells from chronic obstructive pulmonary disease patients have less capacity for balancing the pro-inflammatory immune response caused by *Chlamydia pneumoniae* infection than those from healthy controls. These findings suggest that, during acute exacerbation with intracellular pathogens, chronic obstructive pulmonary disease patients are predisposed to inflammatory changes in the lungs.

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Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death in industrialised countries. The precise role of bacterial infection in the pathogenesis of COPD is the subject of ongoing controversy. Approximately one-half of acute exacerbations are caused by bacteria. Besides *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*, the obligate intracellular bacterium *Chlamydia pneumoniae* is associated with 5–10% of acute exacerbations in COPD patients [1, 2]. *C. pneumoniae* has become established as a cause of acute episodes of upper and lower respiratory tract infection, whereas its association with persistent inflammation in COPD is less certain [3]. The evidence of serological studies correlating *C. pneumoniae*-specific immunoglobulin (Ig)G and IgM levels to acute exacerbations in COPD patients is limited due to the high incidence of IgG-seropositivity in adults [4]. Nevertheless, *C. pneumoniae* IgA is found more frequently and in higher concentrations in COPD patients with acute exacerbation than in disease-free controls [5].

Following recognition of bacterial pathogens, generation of inflammation in the respiratory tract involves the coordinated expression of pro- and anti-inflammatory cytokines. Pro-inflammatory cytokines such as interleukin (IL)-1 β and tumour necrosis factor (TNF)- α are secreted predominantly by alveolar macrophages (AMs), which represent the first line of defence in the host response against pulmonary pathogens [6], and are known to be potent triggers for an effective immune response [7]. Apart from the beneficial effects of IL-1 β and TNF- α in

promoting inflammatory cell accumulation and stimulating the antimicrobial capacity of neutrophils [8], an imbalance between pro- and anti-inflammatory cytokines in the pulmonary compartment might contribute to airway wall remodelling and fibrosis of the small airways. The significance of the relationship between IL-1 β , TNF- α and their anti-inflammatory counterparts IL-1 receptor antagonist (IL-1RA) and soluble TNF receptor I has been well established in lung diseases such as sarcoidosis [9] and pulmonary tuberculosis [10]. To date, data concerning the effect of bacterial pathogens on the balance between pro- and anti-inflammatory cytokines in COPD patients are lacking. In order to differentiate between local and systemic abnormalities in the host response against *C. pneumoniae* infection, release of IL-1 β and IL-1RA from AMs and peripheral blood mononuclear cells (PBMCs) from COPD patients and healthy volunteers was evaluated.

Methods

Subjects

For *in vitro* experiments, PBMCs and AMs were isolated from COPD patients who underwent fiberoptic bronchoscopy for diagnostic evaluation of peripheral solitary pulmonary nodules. All COPD patients and controls (healthy male volunteers) were without clinical and laboratory signs of acute infection at the time of bronchoscopic examination.

Diagnosis of COPD was made according to American Thoracic Society guidelines [11]. Spirometry (BodyScope; Ganshorn Medizin, Niederlauer, Germany) was performed in the sitting position, and forced expiratory volume in one second (FEV₁) and forced vital capacity measured. At the same time point, venous blood from patients and volunteers was drawn from a cubital vein into four 10-mL tubes containing ethylene diamine tetra-acetic acid (Sarstedt, Nümbrecht, Germany).

The protocol was approved by the Ethical Committee of the Medical University of Lübeck (01-084). All volunteers gave written informed consent.

BAL

Bronchoalveolar lavage (BAL) was performed as described previously [12]. In brief, a fiberoptic bronchoscope was wedged into the right middle lobe. A total of 200 mL phosphate-buffered isotonic saline (pH 7.4, 25°C) was instilled sequentially and gently aspirated. The amount of returned fluid was >60% of the total infused volume in all patients and volunteers. Aliquots were retained for further microbiological testing. The chilled BAL fluid (BALF) was strained through a single layer of coarse gauze and centrifuged for 15 min at 400×g at 4°C to recover cells. After washing steps with saline, the cell pellet was resuspended in serum-free Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, Taufkirchen, Germany) and adjusted to an AM density of 1×10⁶ cells·mL⁻¹.

C. pneumoniae nested PCR using BAL fluid

BALF was analysed for genomic *C. pneumoniae* deoxyribonucleic acid (DNA) using a nested polymerase chain reaction (PCR) protocol [13]. Briefly, DNA was purified from BALF *via* proteinase K digestion and cetyltrimethylammonium bromide treatment. Nested PCR was then performed using the species-specific HL-1/HR-1 primer pair and the nested primer pair IN-1/2 that yields a product of 128 base pairs. Nonradioactive DNA hybridisation was performed using oligonucleotide HM-1 3'-labelled with digoxigenin-deoxyuridine triphosphate (Boehringer Mannheim, Mannheim, Germany) as the probe.

Isolation of human PBMCs

Isolation of PBMCs was performed by density centrifugation of blood diluted 1:2 in pyrogen-free saline over Histopaque (Sigma). PBMCs were washed twice in saline, resuspended in RPMI 1640 medium and adjusted to 1×10⁶ cells·mL⁻¹ as described for AMs.

Culture of C. pneumoniae

The pulmonary *C. pneumoniae* strain CWL-029 (American Type Culture Collection (ATCC) VR-1310) was grown on HEp-2 monolayers as described previously [14]. Briefly, immortalised laryngeal epithelial cells (HEp-2, ATCC CLL 23) were grown in tissue culture plates in Eagle minimal essential medium (Sigma) containing 10% foetal calf serum (Biochrom KG, Berlin, Germany), L-glutamine (Invitrogen, Leek, the Netherlands; 2 mM), nonessential amino acids (Invitrogen), gentamicin (Sigma; 10 mg·L⁻¹), vancomycin (Sigma; 50 mg·L⁻¹) and amphotericin B (Sigma; 2 mg·L⁻¹).

Confluent monolayers were infected with *C. pneumoniae* and growth medium was replaced by an antibiotic-free medium.

Infection of AMs and PBMCs with C. pneumoniae

AMs and PBMCs were seeded into six-well plates (Invitrogen) for determination of messenger ribonucleic acid (mRNA) expression (at 4, 12, 24 and 48 h); cover slips were inserted into the plates to allow mononuclear cell adherence and facilitate staining procedures. After 2 h, the nonadherent population was removed and fresh medium containing *C. pneumoniae* (50% tissue culture infective dose=1×10^{-2.7} dilution/1×10⁶ inclusion forming units·mL⁻¹) added. Supernatants were used for cytokine measurements and stored at -70°C.

Real-time PCR

Total ribonucleic acid (RNA) from AMs and PBMCs from COPD patients was isolated using a NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany). Extracted RNA was reverse transcribed to give complementary DNA using random primers and reverse transcriptase according to the manufacturer's protocol (First-Strand PCR kit; Roche, Mannheim, Germany). PCR amplification and quantification was performed using the LightCycler Detection System (Roche Molecular Biochemicals, Mannheim, Germany). Primers were designed using computer software containing minimal internal structure and having compatible melting temperatures. The sequences of the primers are as follows: forward glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5'-TCTGCCCTCTGCTGATGCCCC-3'; reverse GAPDH: 5'-CCATCACGCCACAGTTTCCCGGAG-3'; forward IL-1β: 5'-CTGATGGCCCTAAACAGATGAAG-3'; reverse IL-1β: 5'-GGTCCGAGATTCGTAGCAGCTGGAT-3'; forward IL-1RA: 5'-GGAATCCATGGAGGGAAGAT-3'; and reverse IL-1RA: 5'-CCTTCGTCAGGCATATTGGT-3'. The PCR product was monitored in real-time by measuring the increase in fluorescence caused by the binding of SYBR Green I Dye (Roche Molecular Biochemicals) to double-stranded DNA.

Determination of cytokine concentrations

Concentrations of IL-1β, IL-1RA and IL-8 were determined in cell supernatants by means of enzyme-linked immunosorbent assays with detection limits of 3.9, 31.2 and 16 pg·mL⁻¹, respectively (Biosource International, Camarillo, CA, USA; Bender MedSystems, Vienna, Austria; and R&D Systems, Minneapolis, MN, USA).

Table 1.—Characteristics of study subjects

	COPD patients	Healthy controls
Subjects n	8	8
Age yrs	57.3±12.1	28.0±6.1
Current smokers n	8	8
FEV ₁ L	2.5±1.1	4.5±0.7
% pred	73.5±11.5	91.8±9.7
FEV ₁ /FVC %	65.9±18.3	82.4±6.0
Bronchoalveolar lavage fluid		
Cell density 10 ⁷ cells·mL ⁻¹	1.2±0.7	0.9±0.5
Alveolar macrophages %	92±7	95±4
PMNs %	4±2	3±2
Cell viability %	95±4	97±3

Data are presented as mean±SD. COPD: chronic obstructive pulmonary disease; FEV₁: forced expiratory volume in one second; FVC: forced vital capacity; PMN: polymorphonuclear neutrophils; % pred: percentage of the predicted value.

Fluorescence microscopy

A two-colour fluorescence assay (LIVE/DEAD® kit; MolecularProbes, Eugene, OR, USA) was used to determine the viability of *C. pneumoniae* and PBMCs over 48 h. Green fluorescence indicated viable cells as the highly membrane permeable dye (Syto 9) labels cells with intact plasma membranes. In contrast, the red fluorescent nucleic acid stain (propidium iodide) labels only cells with disrupted membranes. *C. pneumoniae*-infected PBMCs and AMs were seeded on to sterile glass coverslips and incubated with both

fluorescent dyes (at 1:300 dilution) for 45 min in darkness. Dye solution was removed, cells were fixed in 1% paraformaldehyde for 10 min and stored at -4°C. Infection rates were determined using a fluorescein isothiocyanate-conjugated antichlamydial antibody (Dako, Hamburg, Germany).

Statistical analysis

For statistical analysis, nonparametric tests were used throughout the study. As maximum IL-1 β secretion from

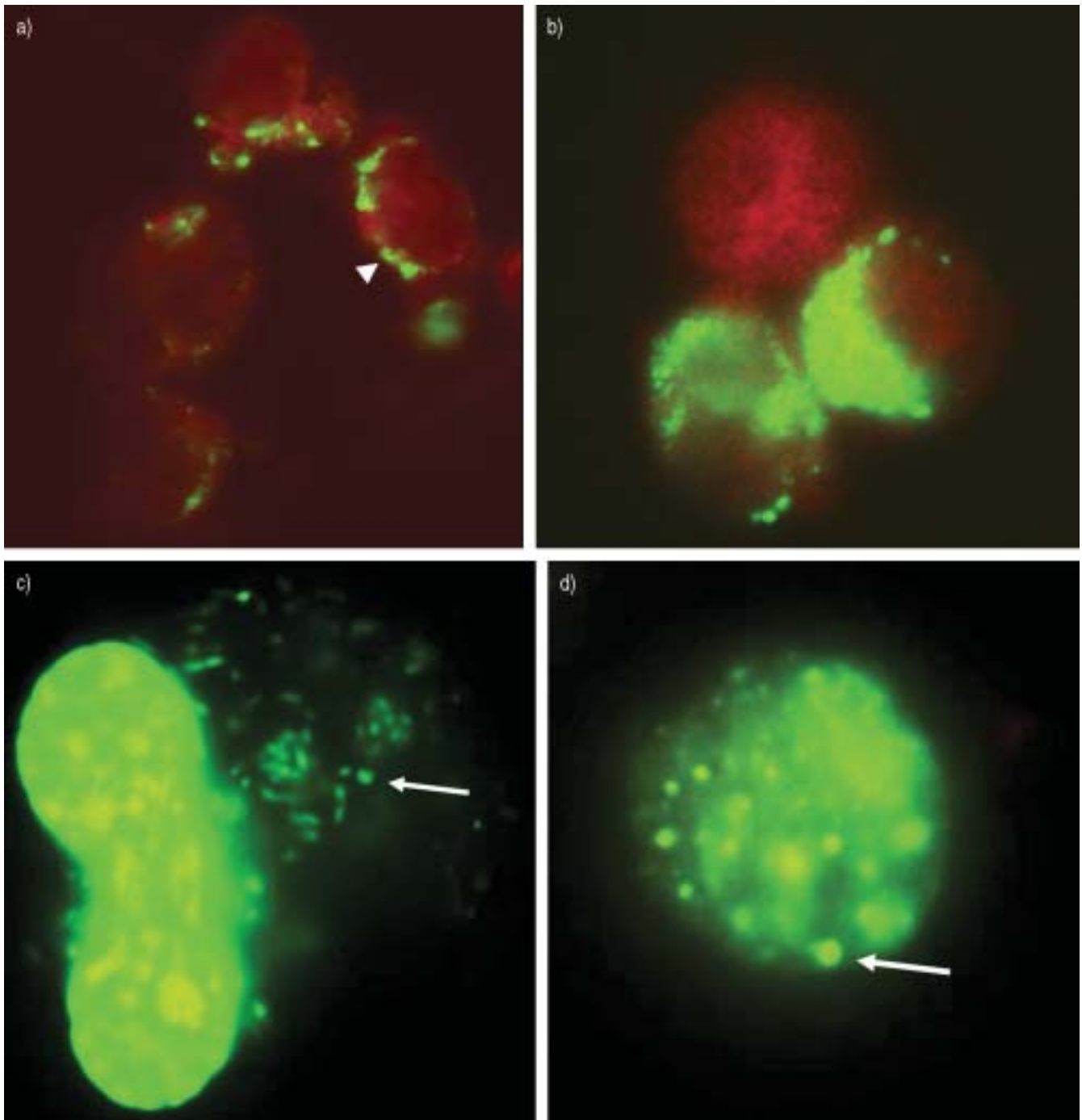


Fig. 1. – Fluorescence microscopy with fluorescein isothiocyanate-conjugated antichlamydial antibody showing the development of intracellular chlamydial infection in peripheral blood mononuclear cells after a) 24 h and b) 48 h. Increasing viability of *Chlamydia pneumoniae* was demonstrated in alveolar macrophages after c) 24 h and d) 48 h by live/dead staining. Arrows/arrowheads indicate *C. pneumoniae*.

C. pneumoniae-infected PBMCs was detected after 24 h, this time was appointed for the statistical analysis of samples from the COPD patients and healthy controls (Mann-Whitney U-test). A $p < 0.05$ was considered significant.

Results

Study subjects

The clinical characteristics of the subjects are shown in table 1. All COPD patients and volunteers were male current smokers with a cumulative cigarette consumption of ≥ 6 pack-yrs. None of the COPD patients were receiving systemic steroids or antibiotic treatment at the time of the study. No potentially pathogenic microorganisms were isolated from BALF, and PCR was negative for *C. pneumoniae* in all COPD patients and healthy controls included in the study.

Infection rates and viability of *C. pneumoniae*-infected AMs and PBMCs

Inclusion-forming bodies of *C. pneumoniae* were observed 24 and 48 h after infection of AMs and PBMCs (fig. 1a and b), indicating an intracellular infection. In mononuclear cells from COPD patients and healthy volunteers, infection rates ranged from $65 \pm 10\%$ in AMs to $73 \pm 8\%$ in PBMCs without differences between the two groups. Viable *C. pneumoniae* could be detected in viable AMs (fig. 1c and d) and PBMCs over 48 h with live/dead staining.

Expression of IL-1 β and IL-1 receptor antagonist in *C. pneumoniae*-infected AMs and PBMCs from COPD patients

In order to evaluate the time course of pro- and anti-inflammatory cytokine production in AMs and PBMCs from COPD patients, IL-1 β and IL-1RA expression and mRNA levels were determined 4, 12, 24 and 48 h after chlamydial infection in a subgroup of six patients. Levels of IL-1 β mRNA were significantly enhanced four-fold 4 h after infection of AMs and PBMCs with *C. pneumoniae*. Secretion of IL-1 β from AMs reached maximum levels 24 h after chlamydial infection with continual decrease up to 48 h (fig. 2a). In contrast, infection with *C. pneumoniae* did not result in enhanced levels of IL-1RA mRNA. There was no significant difference in release of IL-1RA from *C. pneumoniae*-infected and noninfected AMs (fig. 2b) and PBMCs at 24 h.

IL-1 β /IL-1 receptor antagonist ratio in *C. pneumoniae*-infected AMs and PBMCs from COPD patients and healthy volunteers

Infection of AMs and PBMCs from COPD patients with *C. pneumoniae* resulted in increased secretion of IL-1 β . Compared to healthy controls, the maximum increase was 2.5-fold in AMs and 2.3-fold in PBMCs, starting from similar baseline levels. Strikingly, enhanced release of its anti-inflammatory counterpart IL-1RA was absent in preactivated AMs and PBMCs from COPD patients after chlamydial infection (fig. 3). In contrast, mononuclear cells from healthy volunteers possessed the capacity to functionally balance the pro-inflammatory immune response by secreting increased amounts of IL-1RA (106.8 ± 40.6 versus 69.0 ± 38.7 ng·mL⁻¹ from AMs and 16.9 ± 6.0 versus 5.8 ± 4.2 ng·mL⁻¹ from PBMCs; $p < 0.05$) after chlamydial infection.

Taken together, the IL-1 β /IL-1RA ratio ($\times 1,000$) in AMs (and PBMCs) from COPD patients increased significantly from 6.3 ± 2.9 to 27.1 ± 4.1 (170.6 ± 65.2 to 511.4 ± 196.5 in PBMCs) in response to *C. pneumoniae* infection. In the control group, infection of AMs with *C. pneumoniae* did not significantly affect the IL-1 β /IL-1RA ratio (4.6 ± 2.5 versus 7.3 ± 1.2), whereas there was a tendency for it to decrease in *C. pneumoniae*-infected PBMCs (186.6 ± 92.7 versus 85.4 ± 20.6).

Release of IL-8 from *C. pneumoniae*-infected AMs from COPD patients and healthy volunteers

In both groups, infection of AMs with *C. pneumoniae* resulted in a 4.5-fold increase in IL-8 secretion. In contrast to other cytokines, basal IL-8 levels in unstimulated AMs from COPD patients were significantly enhanced compared to the control group (283.0 ± 112.0 versus 6.5 ± 2.3 ng·mL⁻¹; $p < 0.05$). As the inducibility of IL-8 secretion from AMs was similar in both groups, infection of AMs from COPD patients with *C. pneumoniae* resulted in significantly enhanced IL-8 levels (1324.4 ± 498.3 versus 43.9 ± 14.9 ng·mL⁻¹; $p < 0.05$).

Discussion

Five to ten per cent of acute exacerbations in COPD patients are associated with *C. pneumoniae* infection [1, 2, 15].

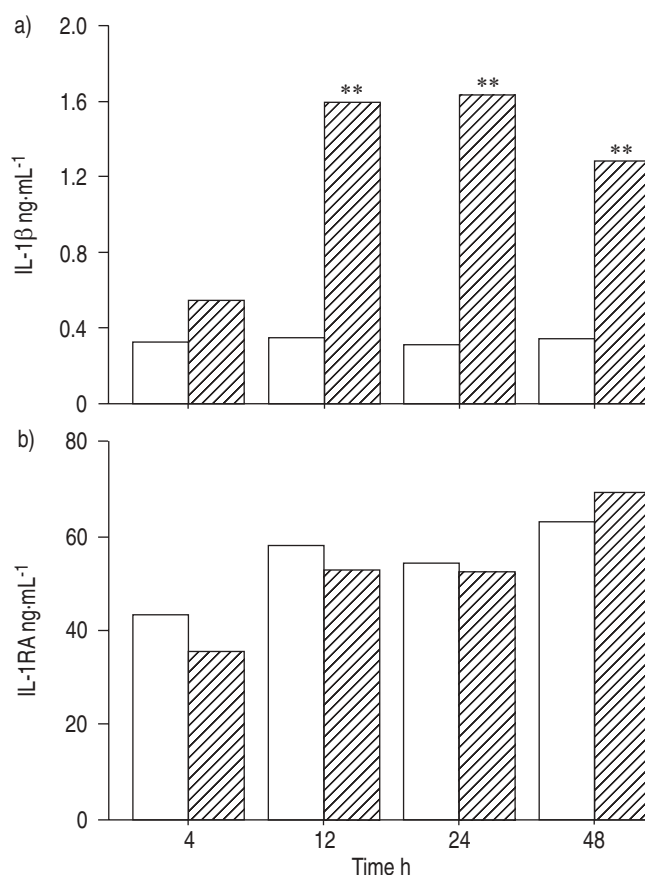


Fig. 2. – Time course of a) interleukin (IL)-1 β and b) IL-1 receptor antagonist (IL-1RA) secretion from *Chlamydia pneumoniae*-infected (▨) and noninfected (□) alveolar macrophages (AMs) from chronic obstructive pulmonary disease patients (n=6). Within 12 h, secretion of IL-1 β was significantly enhanced in infected AMs, whereas release of IL-1RA from AMs remained unaffected by infection over 48 h. **: $p < 0.01$ versus noninfected AMs.

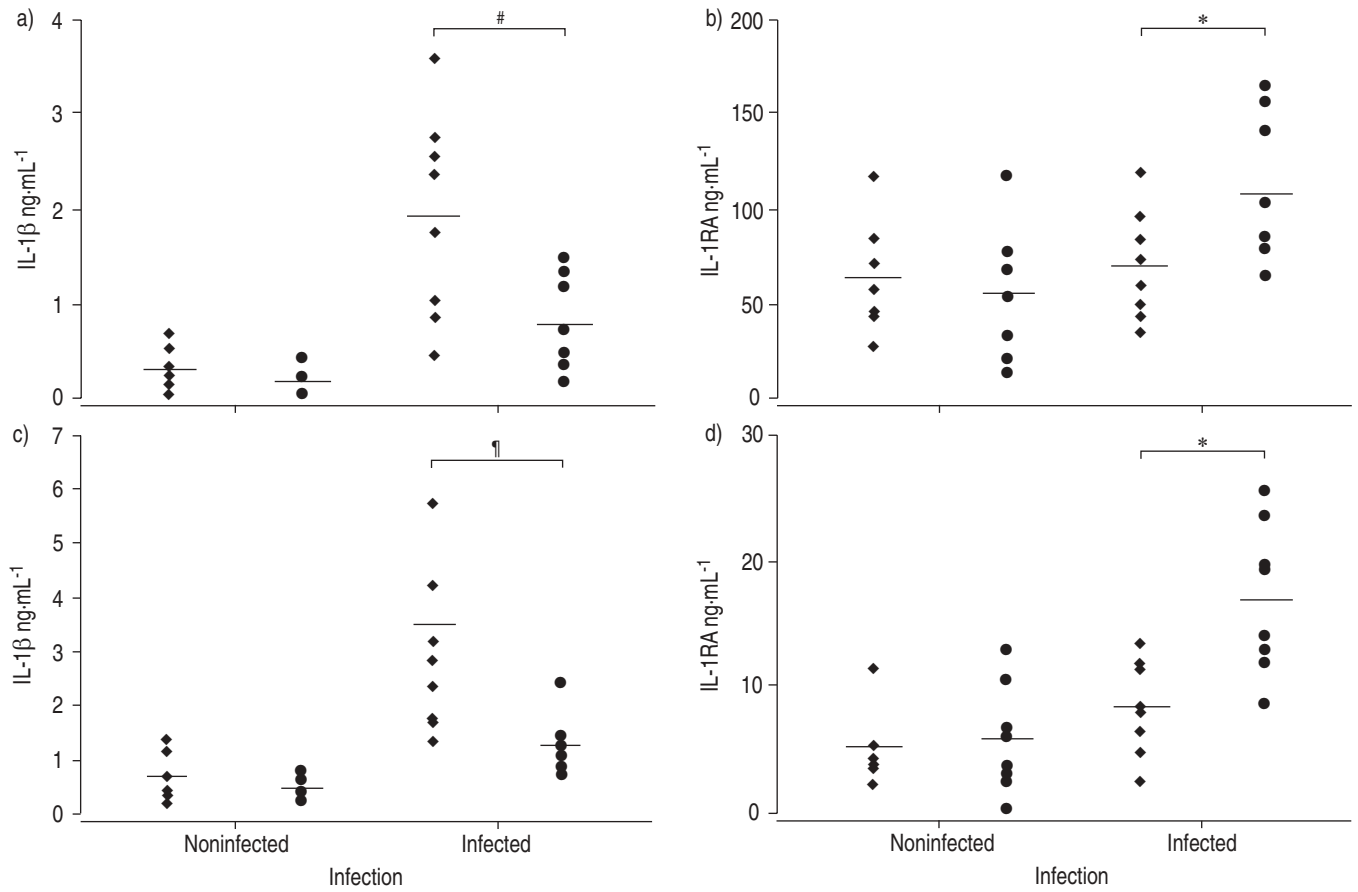


Fig. 3.—Secretion of a, c) interleukin (IL)-1 β and b, d) IL-1 receptor antagonist (IL-1RA) from a, b) alveolar macrophages (AMs) and c, d) peripheral blood mononuclear cells (PBMCs) from chronic obstructive pulmonary disease (COPD) patients (\blacklozenge) and healthy controls (\bullet) 24 h after infection with *Chlamydia pneumoniae*. Horizontal bars represent means (n=8). Infection of AMs and PBMCs from COPD patients resulted in an increased IL-1 β /IL-1RA ratio compared to healthy volunteers due to enhanced expression of IL-1 β but unchanged secretion of IL-1RA. *: p<0.05; #: p=0.016; †: p=0.009.

In acute exacerbation of COPD, the pulmonary compartment represents a site of localised inflammation, in which AMs play a pivotal role in the host defence against bacterial pathogens [16]. Initially, *C. pneumoniae* infects AMs, which results in enhanced secretion of the pro-inflammatory cytokines IL-1 β and TNF- α [17]. Besides the beneficial effects of pro-inflammatory cytokine secretion in infectious diseases, some of their biological activities may contribute to structural tissue damage if an adequate anti-inflammatory immune response is lacking [18].

In the present study, the relationship between release of IL-1 β and its anti-inflammatory counterpart IL-1RA was investigated in *C. pneumoniae*-infected AMs and PBMCs in order to assess the role of a balanced immune response to intracellular pathogens in respiratory infections in COPD patients. Infection of AMs and PBMCs from COPD patients with *C. pneumoniae* resulted in a predominant increase in the pro-inflammatory cytokine IL-1 β in a dose- and time-dependent manner. Interestingly, this increase was significantly more distinct in COPD than in the control group, starting from similar baseline levels.

The pro-inflammatory cytokine secretion pattern of *C. pneumoniae*-infected AMs and PBMCs from healthy persons has been described previously [17, 19], whereas data concerning the release of anti-inflammatory cytokines are lacking.

It is widely accepted that IL-1RA is a successful inhibitor of IL-1 β bioactivity *in vivo* [20, 21]. The importance of a balanced immune response has been described in detail as

regards the regulation of IL-1 β and IL-1RA, which competitively block the binding of IL-1 α and IL-1 β without inducing a cellular signal of their own [22]. Patients with active pulmonary tuberculosis and large necrotic cavities are characterised by elevated IL-1 β /IL-1RA ratios in BALF, giving rise to the assumption that an imbalance in IL-1 β /IL-1RA contributes to severe tissue damage [10]. The present results indicate that AMs and PBMCs from healthy volunteers have a greater capacity to balance IL-1 β activity by increased production of IL-1RA. Even though cytokine levels may differ in elderly patients, it seems unlikely that the greater age in this patient group is decisive for the observed mismatch in IL-1 β and IL-1RA secretion. Population studies revealed increased production of IL-1RA from blood mononuclear cells in the elderly, whereas IL-1 β levels remained unchanged on ageing [23].

PBMCs and AMs have been reported to play an important role in the regulation of the systemic and local inflammatory response by expression of high IL-1RA levels [24]. Joos *et al.* [25] showed that polymorphisms in the IL-1 β and IL-1RA genes are linked to the rate of decline in lung function in smokers, indicating a genetic predisposition for enhanced susceptibility to lung tissue damage. The absence of adequate IL-1RA secretion in COPD patients may explain the sustained inflammatory responses in acute exacerbations with intracellular pathogens, by interference with IL-1 β -dependent adhesion molecule expression and IL-8 generation, resulting in enhanced neutrophil recruitment and activation [26].

Significantly enhanced IL-8 levels were found in *C. pneumoniae*-infected and unstimulated AMs from COPD patients in the present study. Increased sputum levels of IL-8 associated with enhanced neutrophil activation have been previously reported during acute exacerbation in COPD patients [27, 28].

The precise role of exacerbations in patients suffering from chronic obstructive lung disease in tissue destruction and FEV1 decline is still the subject of controversy. LEWIS *et al.* [29] found, in a population study, that increased blood monocyte counts were related to reduced FEV1, suggesting a central role of monocytes and tissue macrophages in the pathogenesis of COPD. To the present authors' knowledge, the current data are the first demonstrating an imbalance in the release of pro- and anti-inflammatory cytokines from inflammatory cells from COPD patients in response to infection with an intracellular pathogen. The present findings indicate a possible pathophysiological mechanism for perpetuating the inflammatory process in the lungs, since a well balanced anti-inflammatory cytokine response is indispensable for limiting inflammation in the pulmonary compartment. Further studies are needed to evaluate the balance of pro- and anti-inflammatory cytokines in BALF from COPD patients with acute exacerbations with respect to their status regarding acute or persistent *C. pneumoniae* infection, and to clarify whether the higher exacerbation rate in patients with persistent *C. pneumoniae* infection [30] is associated with the *in vitro* observed cytokine mismatch. In patients with *C. pneumoniae* DNA-positive pneumonia without underlying pulmonary disease, balanced secretion of IL-1 β and IL-1RA was observed in BALF (unpublished data).

Finally, the present study demonstrates that the imbalance in the release of pro- and anti-inflammatory cytokines in response to *Chlamydia pneumoniae* infection is not limited to the lungs since it can also be detected in infected blood monocytes from chronic obstructive pulmonary disease patients. This finding may suggest that genetic differences in the regulation of the inflammatory response to intracellular pathogens in chronic obstructive pulmonary disease are equally as important as environmental factors, which primarily affect the airways.

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