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Role of IL-1α and the Nlrp3 / caspase-1 / IL-1β axis in cigarette smoke-induced pulmonary inflammation and COPD

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Running title: Role of the Nlrp3 / caspase-1 / IL-1β axis in COPD

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Abstract

Cigarette smoke (CS), the primary risk factor of COPD, leads to pulmonary inflammation through IL-1Receptor-I (IL-1RI) signalling, as determined using COPD mouse models. It is unclear whether IL-1α or IL-1β – activated by the Nlrp3 / caspase-1 axis – is the predominant ligand for IL-1RI in CS-induced responses.

We exposed wild type mice (treated with anti-IL-1α or anti-IL-1β antibodies) and IL-1RI KO, Nlrp3 KO and caspase-1 KO mice to CS for 3 days or 4 weeks and evaluated pulmonary inflammation. Additionaly, we measured the levels of IL-1α and IL-1β mRNA (in total lung tissue by RT-PCR) and protein (in induced sputum by ELISA) of never smokers, smokers without COPD and patients with COPD.

In CS-exposed mice, pulmonary inflammation was dependent on IL-1RI and could be significantly attenuated by neutralizing IL-1α or IL-1β. Interestingly, CS-induced inflammation occured independent from IL-1β activation by the Nlrp3 / caspase-1 axis. In human subjects, IL-1α and IL-1β were significantly increased, respectively in total lung tissue and induced sputum of patients with COPD, compared to never smokers.

These results suggest that not only IL-1β, but also IL-1α should be considered as an important mediator in CS-induced inflammation and COPD.

Keywords: caspase-1, cigarette smoke, chronic obstructive pulmonary disease, IL-1, inflammasome, Nlrp3

Introduction

Chronic obstructive pulmonary disease (COPD), a primarily cigarette smoke (CS)-related disease, is a leading cause of morbidity and mortality worldwide. Its overall prevalence in adults over 40 years is currently estimated at 10% [1]. Inflammation is present in the lungs of patients with COPD and is critical in the development and progression of the disease, leading to obstructive bronchiolitis and destruction of lung parenchyma (emphysema) [2]. Current COPD therapy mainly focuses on the reduction of symptoms such as cough and breathlessness, whereas effective anti-inflammatory therapies are still lacking. The aim of this study is to unravel the molecular mechanisms involved in CS-induced pulmonary inflammation, which is of vital importance for the development of future anti-inflammatory therapies for patients with COPD.

IL-1α and IL-1β, two pro-inflammatory isoforms of the IL-1 family of ligands, play an important role in modulating both innate and adaptive immune responses [3, 4]. They share a common receptor, IL-1Receptor I (IL-1RI), which activates the transcription factors Nuclear Factor-KappaB (NF-κB) and Activator Protein-1 (AP-1) upon stimulation. Eventually, the ensuing pathway increases the expression of vascular adhesion molecules (e.g. Inter- and Vascular Cellular Adhesion Molecule [ICAM-1 and VCAM-1]) and induces chemokines (e.g. IL-8, a neutrophil chemoattractant) which together promote the inflammatory cell infiltration from the circulation into the affected tissue(s) [3-5]. Importantly, IL-1β is produced as inactive precursor form and its activation is generally performed by active caspase-1 (= ICE, IL-1-β-converting enzyme) [3]. Pro-caspase-1 can in turn be activated by the Nlrp3 (NOD-like receptor family, pyrin domain containing 3) inflammasome, a multiprotein platform activated upon nonmicrobial and stress-associated danger signals, including reactive oxygen species and extracellular ATP [6]. However, cleavage of pro-caspase-1 is not exclusively

performed by the Nlrp3 inflammasome, as it can also be activated by other inflammasomes [7-9]. In contrast with IL-1β, both precursor and cleaved forms of IL-1α are biologically active. IL-1α is released by dying cells acting as a danger signal [10], but it can also be secreted by a caspase-1 dependent mechanism [11].

Animal models that mimic the hallmarks of COPD are a valuable tool to unravel the molecular mechanisms of the inflammatory process and already established an important role for IL-1RI in acute and chronic CS-induced inflammation [12, 13]. Our study demonstrates for the first time that also subacute (4 weeks) CS-induced inflammation is IL-1RI dependent, which reinforces the concept that the IL-1RI pathway is critically implicated in CS-induced responses. However, it is still unclear which pro-inflammatory ligand of IL-1RI, IL-1α or IL-1β, predominantly stimulates its receptor in CS-induced responses. To elucidate this important issue, we injected mice intravenously with antibodies against IL-1α or IL-1β, exposed them to CS for 3 days (acute exposure) and studied pulmonary cell accumulation. Next, we investigated the role of Nlrp3 and caspase-1, upstream regulators of IL-1β, in prolonged CS-induced inflammation, by exposing wild-type (WT), Nlrp3 knockout (KO) and caspase-1 KO mice to CS for 4 weeks (subacute exposure). In addition, we performed a translational study investigating mRNA and protein expression of IL-1α and IL-β (in total lung tissue and sputum supernatants, respectively) of never smokers, smokers without COPD and patients with COPD.

Materials and methods

Animals

Homozygous breeding pairs of IL-1RI knockout (KO) mice (B6.129S7-*Il1r1*^{tm1Imx}) and control WT mice (C57BL/6J) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Nlrp3 KO (and appropriate control WT mice) were kindly provided by Dr J. Tschopp (University of Lausanne, Lausanne, Switzerland). Caspase-1 KO mice were obtained from Dr. P. Vandenabeele from Flanders Institute for Biotechnology (Ghent, Belgium). Except for the caspase-1 KO and control mice who were bred in the animal facility at the Flanders Institute for Biotechnology, all animals were bred in the animal facility at the Faculty of Medicine and Health Sciences, Ghent University (Ghent, Belgium). All mice used in this study were bred on a C57BL6/J background. Animals of 6-10 weeks were maintained in standard conditions under a 12 h light-dark cycle, provided a standard diet (Pavan, Brussels, Belgium) and chlorinated tap water *ad libitum*. All *in vivo* manipulations were approved by the local Ethics Committee for animal experimentation of the Faculty of Medicine and Health Sciences, Ghent University.

Cigarette smoke (CS) exposure

Groups of 8-10 mice were exposed to CS, as described previously [14]. Briefly, the animals received mainstream CS of 5 reference cigarettes (3R4F without filter; University of Kentucky, Lexington, KY, USA) 4 times a day with 30-min smoke-free intervals. An optimal smoke/air ratio of 1/6 was obtained. The mice were exposed for 3 days (acute) or 4 weeks (subacute). The control groups were exposed to room air.

Administration of anti-IL-1α and anti-IL-1β antibodies

C57BL/6 mice were injected intravenously (i.v.) with 40µg of anti-IL-1α, anti-IL-1β or Armenian Hamster IgG isotype control antibodies (Biolegend, San Diego, CA, USA). Mice were injected on day 1 and 3 of an acute (3 days) CS-experiment, 30 minutes before air- or CS-exposure.

Bronchoalveolar lavage (BAL)

Bronchoalveolar lavage was performed as previously described [15-17]. Briefly, lungs were first lavaged using 3 times 300 µl HBSS, free of Ca^{2+} and Mg^{2+} and supplemented with 1% BSA, followed by 3 times 1 ml HBSS supplemented with 0.6 mM EDTA, via a tracheal cannula. The six lavage fractions were pooled, centrifuged, and the cell pellet was finally resuspended in 200 µl buffer (PBS supplemented with 1 % BSA, 5mM EDTA and 0.1 % sodium azide). Subsequently, total cell counts were obtained using a Bürker chamber and differential cell counts (on at least 400 cells) were performed on cytocentrifuged preparations after May-Grünwald (Sigma-Aldrich, St. Louis, MO) and Giemsa staining (VWR, West Chester, PA, USA). Flow cytometric analysis of BAL cells was performed to enumerate macrophages, neutrophils, dendritic cells (DCs) and $CD4^+$ and $CD8^+$ T-lymphocytes.

Lung harvest and preparation of lung single-cell suspensions

Following BAL, the pulmonary and systemic circulation was rinsed with saline, supplemented with 5 mM EDTA. The left lung was used for histology, as previously described [5, 15]. The right lung was harvested for the preparation of lung homogenate (middle lobe) and single-cell suspension (major lobe), as described previously [18]. Briefly, the lung was thoroughly minced, digested, subjected to red blood cell lyses, passed through a

50 µm cell strainer and kept on ice until labelling. Cell counting occurred with a Z2 particle counter (Beckman-Coulter Inc., Fullerton, CA, USA).

Labelling of BAL cells and lung single-cell suspension for flow cytometry

The cells were first incubated with FcR blocking antibody (anti-CD16/CD32, clone 2.4G2) to reduce nonspecific binding. Secondly, the labelling reactions were performed to discriminate macrophages, DCs and T-lymphocytes. All reactions were performed on ice. The macrophages and DCs were discriminated using the methodology described by Vermaelen and Pauwels [19]. Briefly, macrophages are identified as the CD11c-bright (APC-conjugated anti-CD11c; HL3), high autofluorescent cell population. DCs were characterized as CD11c-bright, low autofluorescent and MHC class II-high (PE-conjugated anti-I-A[b]; AF6-120.1) population. Mouse T cell subpopulations in lung single cell-suspensions were identified by the following Abs: FITC-conjugated anti-CD4 (GK1.5), FITC-conjugated anti-CD8 (53-6.7), APC-conjugated anti-CD3 (145-2C11) and PE-conjugated anti-CD69 (H1.2F3), a marker for activation of T-lymphocytes. Finally, all samples were incubated with 7-Amino-actinomycin D for exclusion of dead cells (BD Pharmingen, San Diego, CA, USA). All monoclonal Abs were obtained from BD Pharmingen.

Flow cytometry data acquisition was performed on a dual-laser FACS Calibur™ flow cytometer running CellQuest™ software (BD Biosciences, San Diego, CA, USA). FlowJo Software (Tree Star Inc., Ashland, OR, USA) was used for data analysis.

Preparation of lung tissue homogenate

The middle lobe of the right lung was snap-frozen (in liquid nitrogen) and stored at -80°C until further analysis. The lobes were transferred to tubes containing 1 ml T-PER Tissue Protein Extraction Reagent containing Halt™ Protease Inhibitor Cocktail Kit (Thermo Fisher

Scientific, Waltham, MA, USA) and homogenized on ice using TissueRuptor (Qiagen, Hilden, Germany). The homogenates were centrifuged (10000 x g for 5 min at 4°C) and the middle layer was transferred to microcentrifuge tubes. Total protein concentration was measured using the Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Lung tissue homogenates were diluted with T-PER containing Cocktail Kit to a final protein concentration of 500 μg/ml.

IL-1α and IL-1β ELISA

We determined IL-1α and IL-β in BAL fluid and lung homogenate using commercially available ELISA kits (R&D systems, Minneapolis, MN, USA). ELISA was performed following the manufacturer's instructions.

Human study populations

Lung resection specimens were obtained from 57 patients diagnosed with solitary pulmonary tumors at Ghent University Hospital (Ghent, Belgium). None of the patients were treated with neo-adjuvant chemotherapy. Sputum induction was performed on 53 subjects who were recruited from the outpatient pulmonary clinic of the Ghent University Hospital or by advertising. There was no overlap between the subjects of lung tissue and sputum analysis. All subjects were classified into 3 groups: never smokers, smokers without COPD and patients with COPD. Written informed consent was obtained from all subjects according to protocols approved by the medical ethical committee of the Ghent University Hospital.

Lung tissue isolation, RNA extraction and real-time-PCR analysis

Lung tissue at maximum distance from the pulmonary lesions and without signs of retro-obstructive pneumonia or tumour invasion was collected by a pathologist. Tissue blocks from the resection samples were submersed in RNA-later and stored at -80°C until RNA extraction. Total lung RNA was extracted with Rneasy Mini kit (Qiagen) and cDNA was obtained by the Transcriptor First Strand cDNA synthesis kit (Roche, Basel, Switzerland) following manufacturer's instructions. Expression of target genes IL-1α and IL-1β and reference genes GADPH (Glyceraldehyde-3-phosphate dehydrogenase), HPRT-1 (Hypoxanthine phosphoribosyltransferase-1) and PPIA (Peptidylprolyl isomerase 1) were analyzed using TaqMan Gene Expression Assays (Applied Biosystems, Forster City, CA, USA). Real-time-PCR reactions were set up in duplicate using diluted cDNA using identical amplification conditions for each of the target and reference genes. A standard curve derived from serial dilutions of a mixture of all samples was included in each run. The amplification conditions consisted of: 10 minutes at 95°C, 50 cycles of 10 seconds at 95°C and 15 seconds at 60°C. Reaction samples had a final volume of 20 µl consisting of LightCycler480 Probes Master (Roche), the specific primer/fluorogenic probe mix (Applied Biosystems) and 5 µl cDNA. Amplifications were performed using a LightCycler480 detection system (Roche). Data were processed using the standard curve method. Expression of target genes was corrected by a normalization factor that was calculated based on the expression of the three reference genes (GADPH, HPRT-1, PPIA).

Sputum induction, processing and analysis of IL-1α and IL-1β by ELISA

Sputum induction and processing was performed as described previously [20]. In brief, subjects inhaled sterile, pyrogen-free, hypertonic, nebulized saline at increasing concentrations of NaCl (3%,4% and 5%) over a 5 minute period after inhalation of salbutamol

(2x 200μg). Subsequently, subjects were encouraged to cough and expectorate an adequate sample. Sputum plugs were selected, transferred in a polystyrene tube and mixed with dithiotreitol (DTT; 10% Sputalysin with an amount of four times the weight of the sputum plugs, Boehringer-Calbiochem Corp, San Diego, CA, USA) for 30 seconds by vortex and for 15 minutes by tube rocker. Next, an amount of PBS equal to the volume of DTT was added. The sample was incubated for 5 minutes, filtered and centrifuged for separation between cell fraction and cell-free supernatant fraction. The supernatants was aspirated, aliquoted, and stored at -80°C until further analysis. The cell pellet was processed as previously described [20] and subjected to differential cell counts.

We determined IL-1α and IL-β in sputum supernatants using commercially available ELISA kits (R&D systems). Sputum supernatants were diluted by 4-fold and analyzed according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed with Sigma Stat software (SPSS 15.0, Chicago, IL, USA) using non-parametric tests (Kruskall-Wallis; Mann-Whitney U) for variables without normal distribution, parametric tests (ANOVA; t-test) for variables with normal distribution and Fisher's exact test. Reported values are expressed as mean ± SEM. P-values < 0.05 were considered to be significant.

Results

IL-1RI is critically implicated in subacute CS-induced pulmonary inflammation

To elucidate if the pulmonary inflammation upon 4 weeks (subacute) CS-exposure is IL-1RI dependent, we evaluated inflammatory cells and cytokines in BAL fluid and lung tissue of WT and IL-1RI KO mice. In WT mice, exposure to CS significantly increased the total number of BAL cells and absolute numbers of neutrophils, dendritic cells (DCs) and $CD4^+$ and $CD8^+$ T-lymphocytes in BAL fluid (Figure 1A-F). In contrast, IL-1RI KO mice were significantly protected against CS-induced accumulation of inflammatory cells in BAL fluid (Figure 1A-F). Also in lung digests, CS-induced accumulation of macrophages, neutrophils, DCs and activated ($CD69^+$) $CD4^+$ and activated $CD8^+$ T-lymphocytes was significantly impaired IL-1RI KO mice, compared to WT mice (Figure 2A-E).

Levels of the pro-inflammatory IL-1 family members IL-1α and IL-1β were significantly upregulated in lungs of WT mice upon CS-exposure, as measured by ELISA in lung homogenates (Figure 3A-B). In IL-1RI KO mice, the CS-induced increase of IL-1α and IL-1β was significantly attenuated, compared to WT mice (Figure 3A-B)

CS-induced pulmonary inflammation is attenuated upon IL-1α or IL-1β neutralization

To elucidate whether IL-1α or IL-1β is the predominant ligand for IL-1RI in CS-induced responses, we exposed C57BL/6 mice to CS for 3 days (acute exposure) and injected them i.v. on day 1 and 3 with Hamster IgG (isotype control) or neutralizing antibodies against IL-1α or IL-1β. Acute exposure to CS resulted in a significant increase of BAL neutrophils in isotype treated mice (Figure 4). In contrast, mice treated with anti-IL-1α or anti-IL-1β antibodies were significantly protected against the CS-induced increase of BAL neutrophils (Figure 4). BAL macrophages and $CD4^+$ and $CD8^+$ T-lymphocytes were not (yet) significantly increased upon acute CS-exposure in both isotype or antibody treated groups (data not shown).

CS-induced pulmonary inflammation is independent of the Nlrp3 inflammasome

To investigate if the Nlrp3 / caspase-1 / IL-1β axis is implicated in CS-induced responses, we first evaluated pulmonary inflammation upon subacute CS in Nlrp3 KO versus WT mice. The accumulation of macrophages, neutrophils, dendritic cells (DCs) and $CD4^+$ and $CD8^+$ T-lymphocytes in BAL fluid upon 4 weeks CS-exposure was similar between WT and Nlrp3 KO mice (Figure 5A-F), indicating that the Nlrp3 inflammasome is not critical in subacute CS-induced pulmonary inflammation. Since the Nlrp3 inflammasome is, in contrast to other inflammasomes [8], described to play an important role in acute responses, we also studied the involvement of Nlrp3 upon acute (3 days) CS exposure. We observed that the Nlpr3 deficiency did not affect acute pulmonary inflammation, since numbers of macrophages, neutrophils, DCs and $CD4^+$ and $CD8^+$ T-lymphocytes were not significantly different between WT and Nlrp3 KO mice (data not shown).

CS-induced pulmonary inflammation is not affected by caspase-1 deficiency

Since pro-caspase-1 can be activated by other mechanisms than the Nlrp3 inflammasome [7], we investigated the role of caspase-1 deficiency in CS-induced pulmonary inflammation. Caspase-1 KO mice were not protected against subacute CS-induced pulmonary accumulation of macrophages, neutrophils, dendritic cells (DCs) and $CD4^+$ and $CD8^+$ T-lymphocytes in BAL fluid (Figure 6A-F), indicating that caspase-1 is not critically implicated in CS-induced inflammatory responses.

Expression levels of IL-1α and IL-1β in CS-exposed Nlrp3 and caspase-1 KO mice

We determined IL-1α and IL-1β protein levels upon CS-exposure (in lung homogenate and BAL fluid), because they both exert their biological responses by binding to IL-1RI. IL-1α levels were increased in both BAL fluid and lung homogenate of WT mice (Figure 7A-B).

Importantly, the CS-induced increase of IL-1α was not different between WT, Nlrp3 KO and caspase-1 KO mice (Figure 7A-B). IL-1β levels in BAL fluid were below the detection limit of the ELISA assay in both air- and CS-exposed groups. In lung homogenate, levels of IL-1β were affected by both Nlrp3 and caspase-1 deficiency, since CS-exposed Nlrp3 KO and caspase-1 KO mice had significantly lower levels of IL-1β, compared to CS-exposed WT mice (Figure 7C-D). Since both inflammatory cell recruitment and IL-1α levels are not impaired in CS-exposed Nlrp3 and caspase-1 KO mice, while the levels of IL-1β in these mice are significantly attenuated, IL-1α may be an important trigger for IL-1RI in CS-induced responses.

IL-1α mRNA and IL-1β protein levels are increased in patients with COPD

To evaluate mRNA expression of IL-1α and IL-1β by qRT-PCR, we extracted mRNA from total lung tissue of a study population containing never smokers, smokers without COPD and patients with COPD. The demographic, clinical and lung functional characteristics of the study subjects are presented in Table 1. mRNA levels of IL-1α were significantly and by at least twofold increased in both smokers without COPD and patients with COPD, compared to never smokers (Figure 8A). IL-1β mRNA expression was also increased in smokers with and without COPD, but this increase did not reach statistical significance (Figure 8B).

In addition, protein levels of IL-1α and IL-1β in induced sputum of never smokers, smokers without COPD and patients with COPD were determined by ELISA. The characteristics of the study subjects who underwent sputum induction are listed in Table 2. In accordance with mRNA levels, protein levels of IL-1α increased in induced sputum of smokers and patients with COPD, compared to never smokers, but this did not reach statistical significance (Figure 8C). However, protein levels of IL-1β were significantly increased in patients with COPD, compared to never smokers and smokers without airflow limitation (Figure 8D).

Discussion

Using a mouse model of cigarette smoke (CS) exposure, we reinforced previous findings that CS-induced pulmonary inflammatory cell and cytokine accumulation is IL-1RI dependent. Importantly, our *in vivo* study in mice is the first to report that the impaired inflammatory response in CS-exposed IL-1RI KO mice can not only be mimicked by neutralization of IL-1β, but also with anti-IL-1α antibodies. Interestingly, CS-induced pulmonary inflammation in mice is not critically mediated by the Nlrp3 / caspase-1 / IL-1β axis, again suggesting a role for IL-1α and/or an alternative activation of IL-1β. Finally, using a translational approach, we demonstrated increased levels of IL-1α and IL-1β, respectively in lungs and sputum of patients with COPD, compared to never smokers.

We have shown that IL-1RI KO mice are protected against pulmonary inflammation induced by subacute (4 weeks) CS-exposure, which is in accordance with previous reports about IL-1RI KO mice exposed to acute or chronic CS [12, 13]. However, our study is the first to report protection of IL-1RI KO mice against CS-induced accumulation of DCs and $CD4^+$ and $CD8^+$ T-lymphocytes. Our study hereby reinforces the important role for IL-1RI in CS-induced inflammatory responses. However, it was still unclear which pro-inflammatory ligand, IL-1α or IL-1β, predominantly stimulates its common receptor upon CS-exposure.

We demonstrated that mice treated with anti-IL-1α or anti-IL-1β antibodies were significantly protected against acute CS-induced increases in BAL neutrophils, suggesting a role for both IL-1α and IL-1β in CS-induced inflammation. While the observed protective effect of anti-IL-1β confirms the results of Castro *et al.* [21], this is by our knowledge the first report of an attenuated CS-induced inflammation by neutralization of IL-1α. The involvement of IL-1α in CS-induced inflammatory responses is underappreciated although IL-1α has been described to

play an important role in inflammation; e.g. mice deficient in IL-1α are resistant to experimental colitis [22]. Also in sterile inflammation, IL-1α, but not IL-1β, drives the neutrophilic inflammatory response to cell injury [23]. Multiple *in vivo* studies in mice describe the levels of IL-1β upon CS, but these levels seem to depend strongly on the mouse strain, the specimen tested (BAL fluid or lung homogenate) and the exposure time [12, 13, 21, 24]. In general, IL-1β has been described to be upregulated and implicated in early responses upon CS exposure [12, 21], but not upon chronic CS exposure [13].

We next studied the role of IL-1β activation by the Nlrp3 / caspase-1 pathway in CS-induced inflammation. We found that accumulation of inflammatory cells in the airways and lungs upon subacute CS exposure is independent of Nlrp3 and caspase-1. In contrast, Churg and coworkers previously demonstrated, in an acute CS model, that administration of a selective caspase-1 inhibitor resulted in diminished CS-induced pulmonary inflammation [13]. However, their experiments with caspase-1 inhibition are all in an acute setting, which is different from our prolonged CS exposure. The processes driving pulmonary inflammation upon CS exposure probably change over time with different pathways implicated in the initiation and persistence of inflammatory responses. Similarly, the role for caspase-1 in experimental arthritis also depends on the phase of the disease (acute or chronic), as described by Joosten and coworkers [25]. Since COPD is a chronic inflammatory disease, animal models that mimic and unravel the molecular mechanisms of responses to prolonged CS exposure are of great value and interest. Interestingly, we have found that the pulmonary cell accumulation in CS-exposed Nlrp3 KO and caspase-1 KO mice is not impaired although they have lower pulmonary levels of IL-1β upon CS exposure. This again suggests an important role for IL-1α, the other pro-inflammatory ligand of IL-1RI, which is not impaired in Nlrp3 KO and caspase-1 KO mice upon prolonged exposure to CS.

To support our findings in the mouse COPD model, we performed a translational study and investigated IL-1α and IL-1β in never smokers, smokers without COPD and patients with COPD. We found that IL-1α mRNA expression was significantly increased in total lung tissue of smokers without airflow limitation and patients with COPD, compared to never smokers. Although we observed the same trends for the IL-1α protein levels in induced sputum, these differences did not reach statistical significance. This may be due to the strong heterogeneity in COPD, but may also be explained by the origin of the samples and the different sources of IL-1α and IL-1β [26]. As IL-1α is released as a danger signal by necrotic cells, induced sputum may not be the ideal compartment to measure IL-1α levels, especially since the viability of the cells in our sputum inductions is approximately 90%. In contrast, IL-1β is mainly released by activated macrophages. Therefore, we found significantly increased protein levels of IL-1β in induced sputum of patients with COPD, compared to never smokers and smokers without airflow limitation. Although IL-1β expression is well-studied in patients with COPD [27, 28], the major advantage of this study is that both IL-1RI ligands, IL-1α and IL-1β, were evaluated in the same subjects. Taken together with the *in vivo* findings in the mouse model, these data further suggest an important role for both IL-1α and IL-1β in IL-1RI driven inflammation in COPD.

Our findings could have important implications for the study of anti-inflammatory therapies for smoking-related diseases like COPD. Our data indicate that potential therapies targeting Nlrp3 or caspase-1 might have limited benefit, compared with the efficacy of IL-1α/β or IL-1RI antagonism. Recombinant human IL-1receptor antagonist (IL-1ra, Anakinra, Kineret®) is already marketed for the treatment of rheumatoid arthritis, but it has - to our knowledge - not yet been examined clinically in patients with COPD.

In conclusion, we confirm that pulmonary inflammation upon CS exposure in mice is IL-1RI dependent and demonstrate for the first time a crucial role for both IL-1α and IL-1β. Moreover, we have shown that pulmonary inflammation upon CS exposure is independent from IL-1β activation by the Nlrp3 / caspase-1 axis. Finally, we demonstrated increased levels of both IL-1α and IL-1β in patients with COPD. These results suggest that not only IL-1β, but also IL-1α should be considered as an important mediator in CS-induced inflammation and COPD.

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Footnotes

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Figure legends

Figure 1: Effect of CS exposure and IL-1RI deficiency on the total number of BAL cells and cell subsets in bronchoalveolar lavage (BAL) fluid (A-F). BAL cell counts in WT and IL-1RI KO mice upon 4 weeks exposure to air or CS: (A) total BAL cells, (B) neutrophils, (C) macrophages, (D) dendritic cells, (E) $CD4^+$ T-lymphocytes and (F) $CD8^+$ T-lymphocytes. All cell types were enumerated by flow cytometry, except for the neutrophils which were determined by cytospin counts. Data are expressed as mean ± SEM (N = 10 animals/group; * $p < 0.05$ and *** $p < 0.001$).

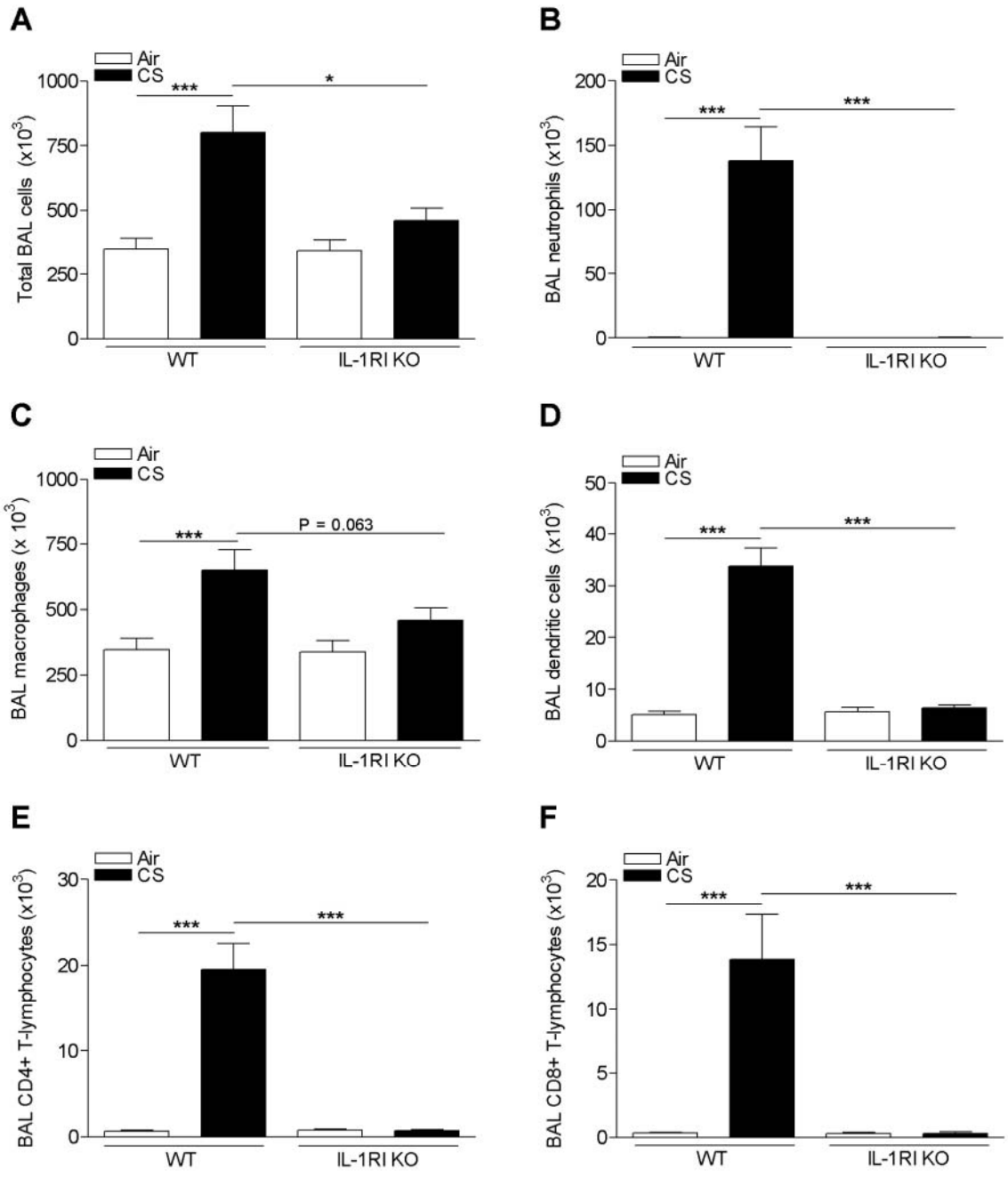

Figure 1

Figure 2: Pulmonary cell accumulation in lung tissue of IL-1RI KO mice and WT mice (A-E). Inflammatory cell counts in WT and IL-1RI KO mice upon 4 weeks air or CS exposure: (A) neutrophils, (B) macrophages, (C) dendritic cells, (D) $CD4^{+}CD69^{+}$ T-lymphocytes, (E)

$CD8^+CD69^+$ T-lymphocytes. All cell types were enumerated by flow cytometry and the data are expressed as mean ± SEM (N = 10 animals/group; * $p < 0.05$ and *** $p < 0.001$).

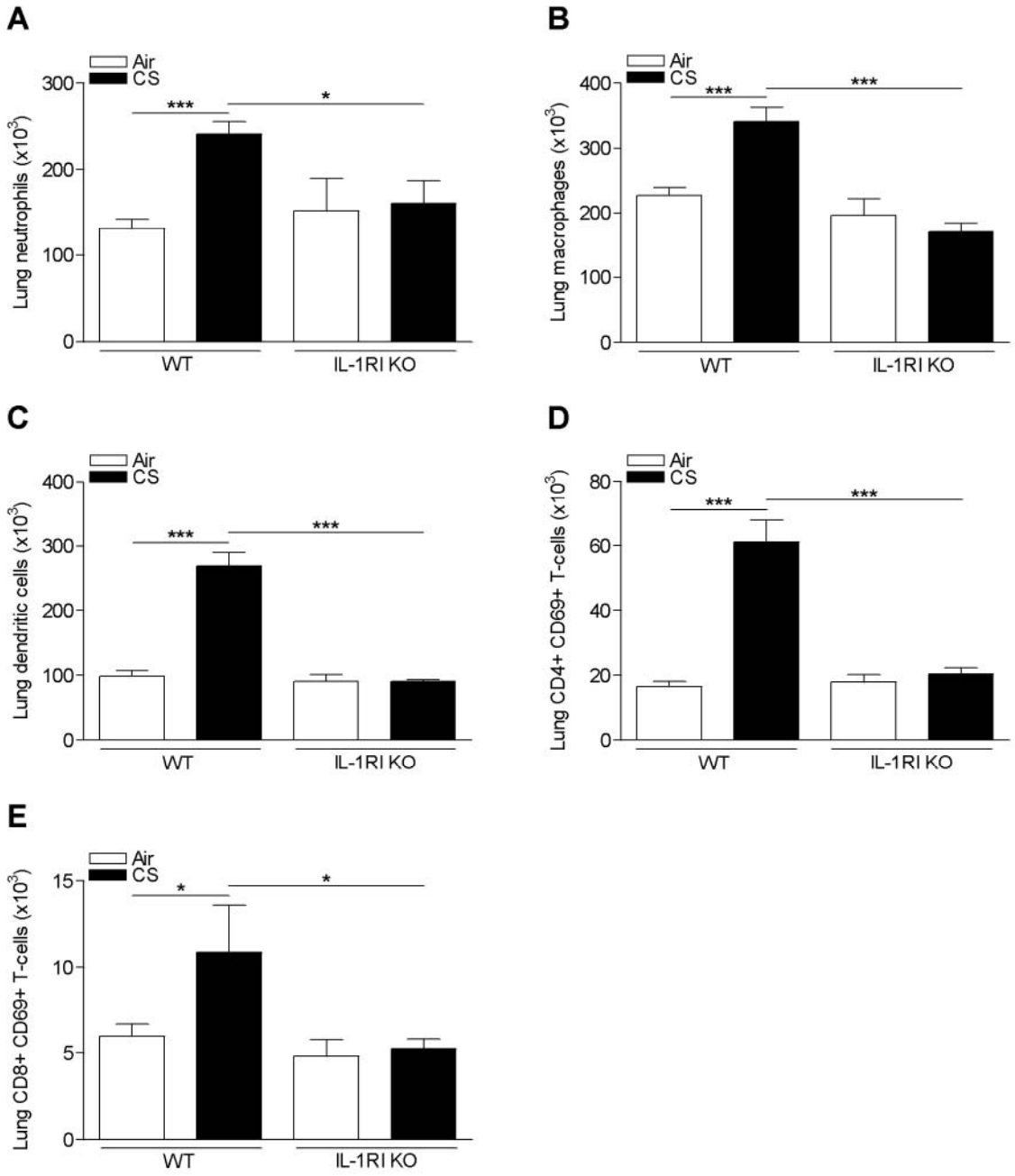

Figure 2

Figure 3: Effect of CS exposure and IL-1RI deficiency on pulmonary levels of IL-1α and IL-1β. (A) IL-1α and (B) IL-1β protein levels in lung homogenate of WT and IL-1RI KO mice upon 4 weeks exposure to air or CS, as measured by ELISA. Data are expressed as mean ± SEM (N = 10 animals/group; * $p < 0.05$, *** $p < 0.01$).

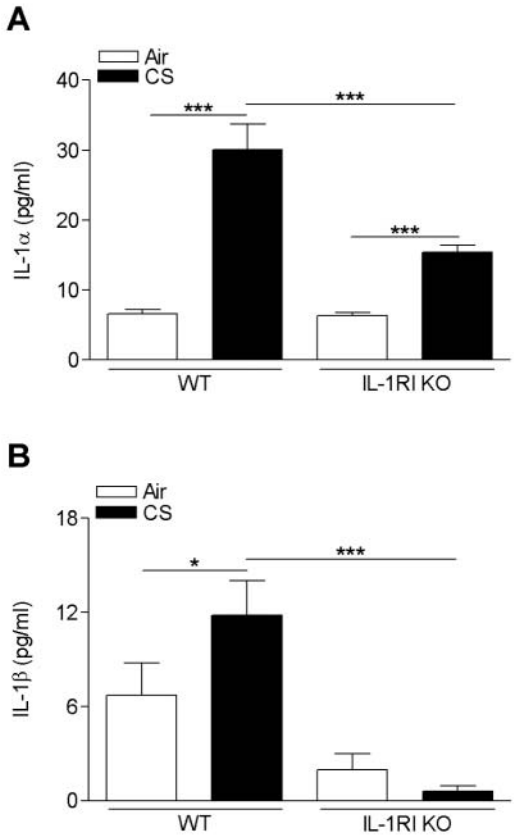

Figure 3

Figure 4: Effect of CS exposure and IL-1α or IL-1β neutralization on the total number of neutrophils in bronchoalveolar lavage (BAL) fluid. BAL neutrophils in WT mice upon 3 days exposure to air or CS, injected intravenously on day 1 and 3 with anti-IL-1α, anti-IL-1β or Hamster IgG isotype control antibodies. Neutrophils were determined by cytospin counts. Data are expressed as mean ± SEM (N = 8 animals/group; * $p < 0.05$).

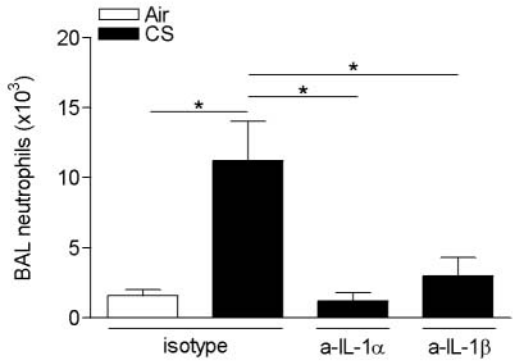

Figure 4

Figure 5: Effect of CS exposure and Nlrp3 deficiency on the total number of BAL cells and cell subsets in bronchoalveolar lavage (BAL) fluid (A-F). BAL cell counts in WT and Nlrp3 KO mice upon 4 weeks exposure to air or CS: (A) total BAL cells, (B) neutrophils, (C) macrophages, (D) dendritic cells, (E) $CD4^+$ T-lymphocytes and (F) $CD8^+$ T-lymphocytes. All cell types were enumerated by flow cytometry, except for the neutrophils which were determined by cytospin counts. Data are expressed as mean ± SEM (N = 10 animals/group; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

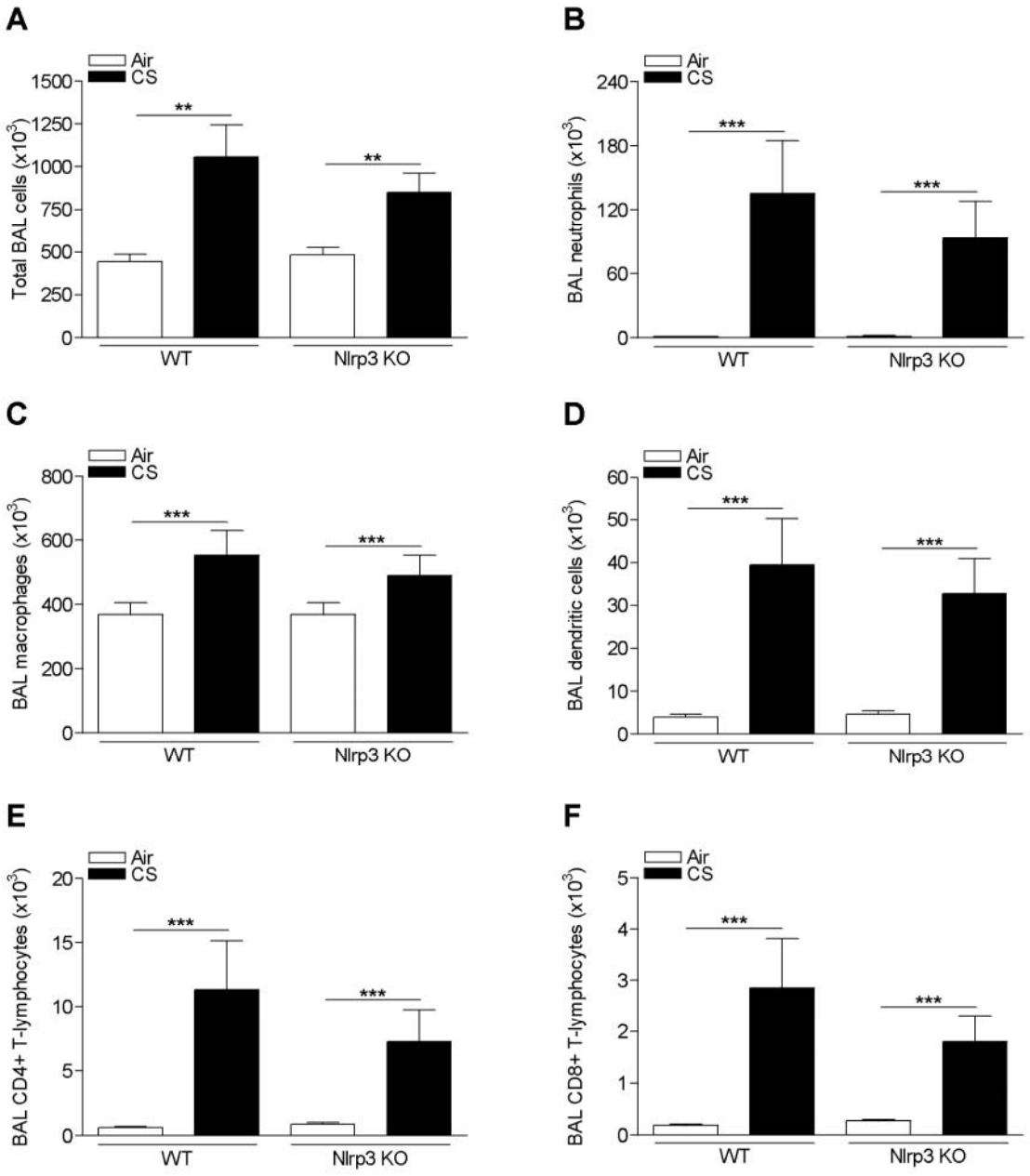

Figure 5

Figure 6: Effect of CS exposure and caspase-1 deficiency on the total number of BAL cells and cell subsets in bronchoalveolar lavage (BAL) fluid (A-F). BAL cell counts in WT and caspase-1 KO mice upon 4 weeks exposure to air or CS: (A) total BAL cells, (B) neutrophils, (C) macrophages, (D) dendritic cells, (E) $CD4^+$ T-lymphocytes and (F) $CD8^+$ T-lymphocytes. All cell types were enumerated by flow cytometry, except for the neutrophils which were

determined by cytospin counts. Data are expressed as mean ± SEM (N = 8 animals/group; * p < 0.05, ** p < 0.01 and *** p < 0.001).

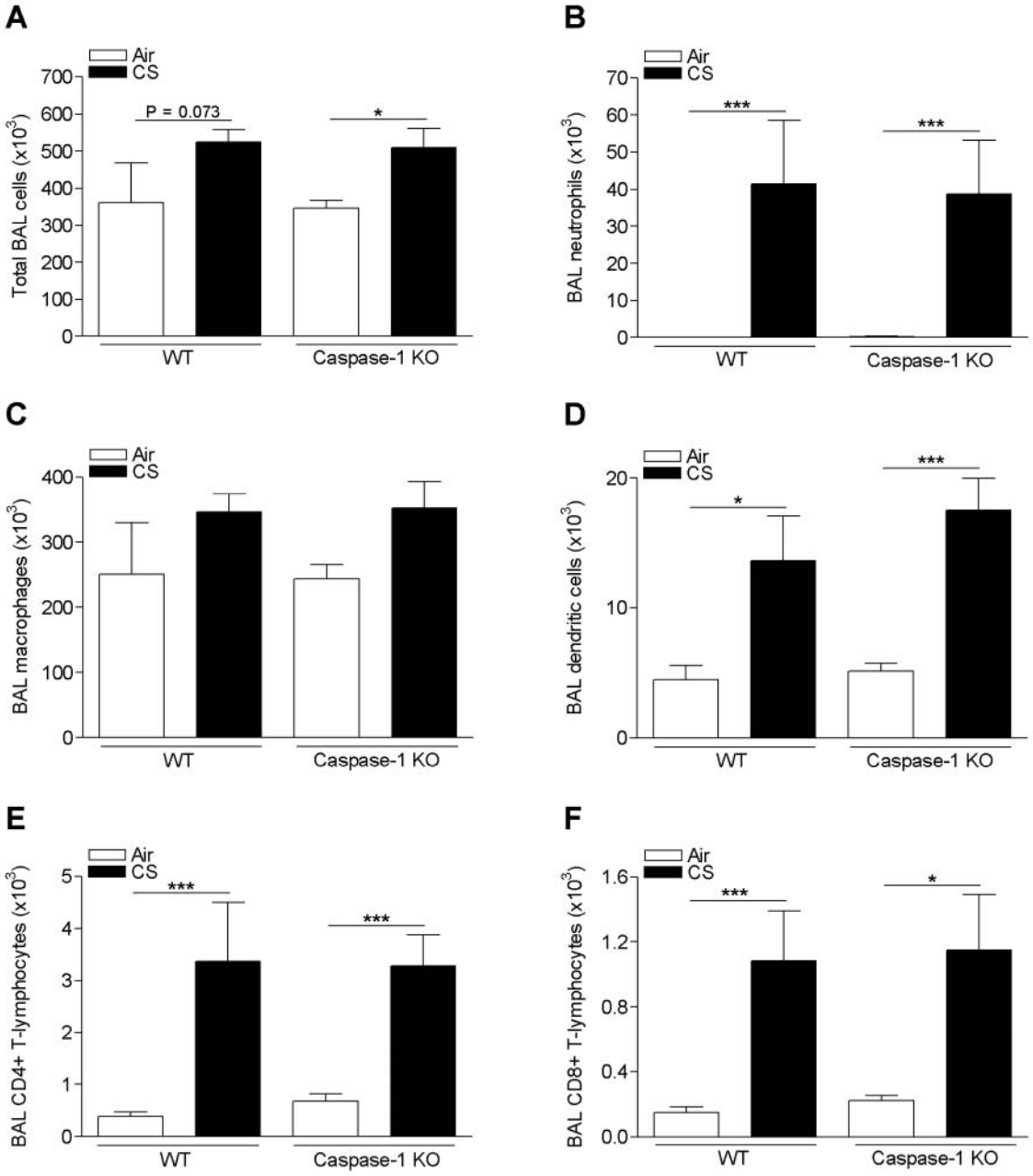

Figure 6

Figure 7: Effect of CS exposure, Nlrp3 deficiency and caspase-1 deficiency on pulmonary levels of IL-1α and IL-1β. IL-1α levels in lung homogenate of (A) Nlrp3 KO and (B) caspase-1 KO mice upon 4 weeks exposure to air or CS, as measured by ELISA. IL-1β levels in lung homogenate of (C) Nlrp3 KO and (D) caspase-1 KO mice upon 4 weeks exposure to air or CS, as measured by ELISA. Data are expressed as mean ± SEM (N = 8-10 animals/group; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

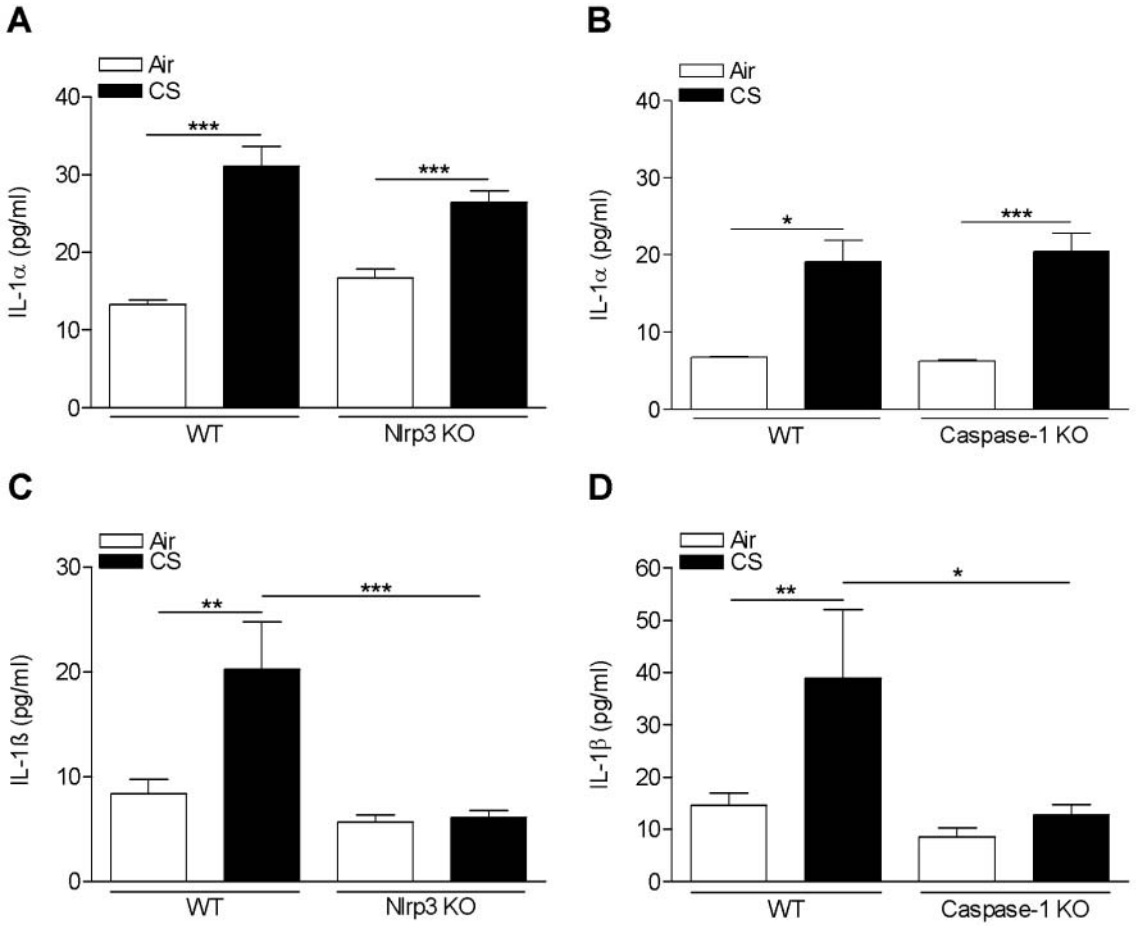

Figure 7

Figure 8: Expression of IL-1RI ligands, IL-1α and IL-1β, in human subjects: never smokers, smokers (without COPD) and patients with COPD. mRNA levels of (A) IL-1α and (B) IL-1β in lung tissue of never smokers (n=10), smokers (n=18) and patients with COPD (n=29), as measured by qRT-PCR. mRNA levels were corrected using a calculated normalization factor based on mRNA expression of three reference genes (GAPDH, PPIA, HPRT-1). Protein levels of (C) IL-1α and (D) IL-1β in induced sputum of never smokers (n=15) smokers (n=20) and patients with COPD (n=18). Data are expressed as mean ± SEM (* $p < 0.05$, ** $p < 0.01$).

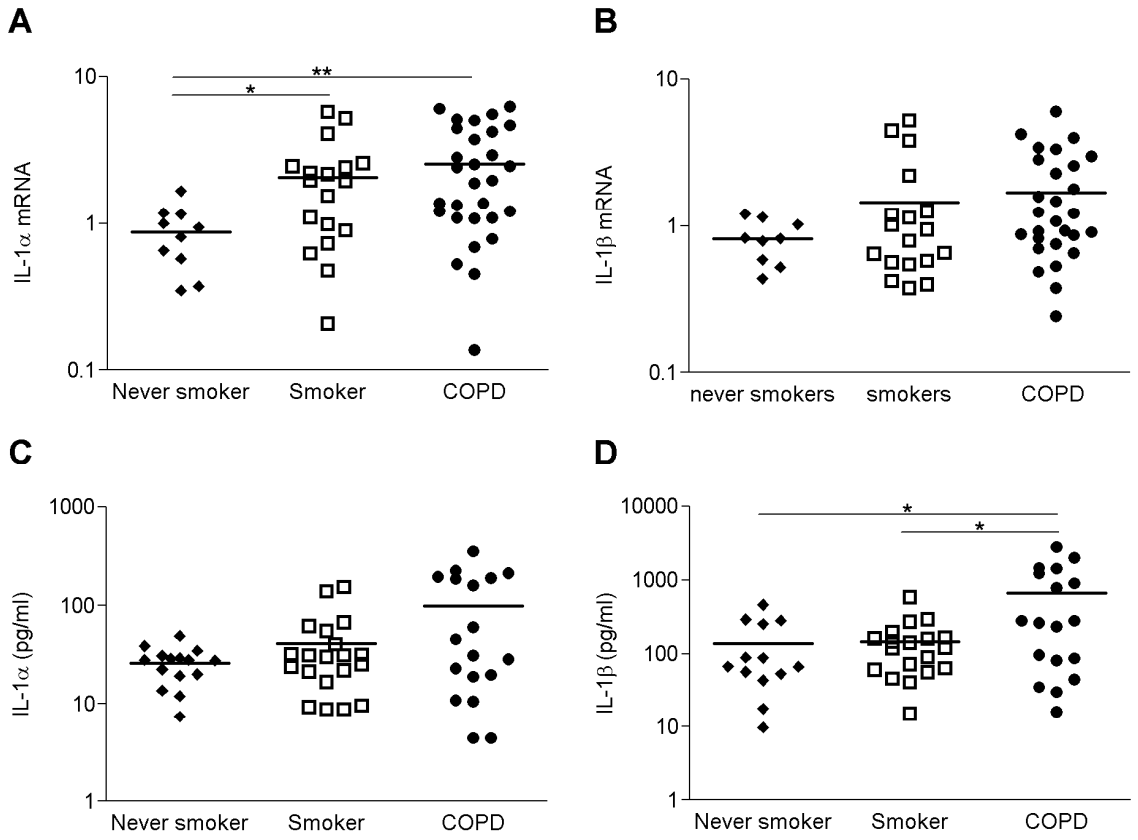

Figure 8

Table 1:

Characteristics of study subjects for lung mRNA analysis (by qRT-PCR)

	never smokers	smokers	COPD
Number	10	18	29
Gender ratio (male/female)	3/7 #	12/6 #	26/3 #
Age (years)	61 (50-70)	61 (52-69)	67 (59-72)
Current-smoker / Ex-smoker	-	7/11	17/12
Smoking history (pack years)	0 (0-0)	35 (19-46)*	40 (30-55)*
FEV1 post-bronchodilator (L)	2,6 (2,0-3,0)	3,2 (2,7-3,5)	2 (1,8-2,6)§
FEV1 post-bronchodilator % predicted	103 (84-104)	108 (93-113)	66 (55-77)*§
FEV1 / FVC post-bronchodilator (%)	77 (73-84)	77 (71-81)	56 (51-61)*§
ICS (yes/no)	0/10 #	0/18 #	11/18 #

Footnote

FEV1 (forced expiratory volume in 1 second); FVC (forced vital capacity); ICS (inhaled corticosteroids)

Data are presented as median (IQR)

Mann-Whitney U test: * $P < 0,05$ versus never smokers; § $P < 0,05$ versus smokers

Fisher's exact test: # $P < 0,001$

Table 2:

Characteristics of study subjects for sputum analysis (by ELISA)

	never smokers	smokers	COPD
Number	15	20	18
Gender ratio (male/female)	6/9	13/7	15/3
Age (years)	52 (40-55)	52 (46-62)	60 (56-71)*§
Current-smoker / Ex-smoker	-	13/7	11/7
Smoking history (pack years)	0,0 (0,0-0,0)	30 (10-48)*	50 (39-84)*§
FEV1 post-bronchodilator (L)	3,2 (2,8-3,7)	3,3 (2,8-3,9)	2,0 (1,5-2,5)*§
FEV1 post-bronchodilator % predicted	104 (97-116)	106 (91-112)	66 (60-73)*§
FEV1 / FVC post-bronchodilator (%)	80 (77-87)	79 (76-83)	58 (49-66)*§
ICS (yes/no)	0/15 #	0/20 #	8/10 #
Sputum differential cell count:			
% Macrophages	57 (39-68)	40 (25-68)	29 (15-39)*§
% Neutrophils	42 (28-59)	58 (30-73)	65 (54-81)*
% Eosinophils	0,2 (0,0-0,3)	0,7 (0,2-1,0)*	0,8 (0,3-2,8)*
% Lymphocytes	1,0 (0,7-2,6)	0,3 (0,0-1,0)*	0,2 (0,1-1,2)*
% Bronchial epithelial cells	0,4 (0,0-1,0)	0,0 (0,0-0,3)	0,0 (0,0-0,5)
% Squamous epithelial cells	1,0 (0,4-3,1)	0,9 (0,5-1,9)	0,5 (0,0-2,4)
Viability (%)	88 (79-92)	90 (85-95)	89 (81-92)

Footnote

FEV1 (forced expiratory volume in 1 second); FVC (forced vital capacity); ICS (inhaled corticosteroids)

Data are presented as median (IQR)

Mann-Whitney U test: * $P < 0,05$ versus never smokers; § $P < 0,05$ versus smokers

Fisher's exact test: # $P < 0,001$