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ON LINE SUPPLEMENT

THE INFLAMMASOME PATHWAY IN STABLE AND EXACERBATED PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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METHODS

IL-1ß, IL-18 and Caspase-1 protein levels in lung and sputum

Total lung protein was extracted from flash frozen lung tissue using the TPER tissue reagent (Pierce, Rockford, USA) with complete Mini Protease Inhibitor Cocktail Tablets (Roche, Germany). Lysates were centrifuged at 4,000 x*g* for 5 min at 4ºC to remove undigested tissue and then supernatants centrifuged at 10,000 x*g* for 5 min at 4ºC. Supernatants containing soluble proteins were frozen at -80ºC until use. Spontaneous sputum was obtained and processed at 4ºC as previously described [35, 36]. The supernatant was stored at -80°C until analysis. All samples and standards were measured in duplicated, and mean values were used for statistical analysis. CASP-1, IL-18 and IL-1β detection assays determined both, the pro-form and mature form. To avoid a downward bias of results, a nominal level of half of the lower limit of quantification was used in individuals with values below the lower limit of quantification [37].

Lung mRNA expression

RNA concentration, purity and integrity were assessed with spectrophotometry using Nanodrop (Thermo Fisher Sientific, USA) and the Eukaryote Total RNA nano Bioanalyzer kit (Agilent Technologies, USA*).* CASP-1, NLRP3, IL18, TGFB1, CCL20 and IL1B transcripts levels were then amplified using real time ready assays (RTR, Roche, Mannheim, Germany) in a LightCycler® 480 (Roche, Mannheim, Germany). CD4, CD69, IL6, IL8, IL2, ELANE and IL1*A were amplified using SybrGreen (*Roche, Mannheim, Germany*)* and primers described in Supplementary Table S1 using a LightCycler® 480 *(*Roche, Mannheim, Germany*).* A mix of all cDNAs was used as calibrator and was run in all reactions. Results are expressed as the ratio of sample expression to GAPDH expression (RQ)[34].

Assessment of active Inflammasome

Briefly, 20 μg of lung homogenate from flash frozen samples, or 20μl of sptum supernatant were subjected to SDS-PAGE (Mini-protean TGX gels, Biorad), transferred to a 0.2μm PVDF membrane (Biorad, US), blocked with 5% non-fat dry milk in PBS/Tween-20 0.3% (PBST) and incubated overnight at 4°C with the specific primary antibody at 1:1000 in 5% BSA-PBST. Blots were washed, incubated with a 1:500 dilution of the secondary HRP-goat anti-rabbit antibody (Molecular probes, Invitrogen, USA) and Luminata Crescento (Millipore, USA). Chemo-luminescent signals were acquired in a LAS4000 Imager (Fujifilm, Japan).

Caspase-1 and ASC lung tissue distribution

Briefly, formalin-fixed paraffin-embebbed tissue sections were deparaffinised, rehydrated and subjected to an antigen retrieval step, blocked with 0.1% BSA (Sigma Aldrich, US) and 1% normal goat or donkey serum (company), washed and incubated overnight at 4ºC with 1:200 of anti-caspase-1 or 1:100 of anti-ASC antibody. Specific staining was detected with secondary biotinylated goat anti-rabbit antibody (BA-1000, Abd-Serotec, Kidlington, UK) followed by Vectorstain ABC Elite reagent and DAB peroxidase substrate (both from Vector laboratories, Burlingame, USA) and counterstained with haematoxylin for caspase-1 staining. For ASC staining secondary Alexa-647-donkey anti-rabbit antibody (Life technologies, USA) was used and slides were mounted with prolong Gold with DAPI (Life technologies, USA) and analyzed in a TCS-SP5 confolcal microscope (Leica).

Table S1. Primers used with for SybrGreen quantification

Gene	*Primer*	Sequence
GAPDH	*Fw*	TCTTCTTTTGCGTCGCCAG
	Rv	AGCCCCAGCCTTCTCCA
IL6	*Fw*	GGCACTGGCAGAAAACAACC
	Rv	GCAAGTCTCCTCATTGAATCC
ELANE	*Fw*	CGTCTGCACTCTCGTGAGG
	Rv	GAGGCAATTCCGTGGATTAG
IL2	*Fw*	AACTCACCAGGATGCTCACATTTA
	Rv	TCCCTGGGTCTTAAGTGAAAGTTT
CD69	*Fw*	GGAGAGTGGACAAGAAAATGAT
	Rv	AGGACAGGAACTTGGAAGGA
IL8	*Fw*	TCCTTGTTCCACTGTGCCTTG
	Rv	TGCTTCCACATGTCCTCACAA
IL1A	Fw	GAGATGCCTGAGATACCCAA
	Rv	TAGTTCTTAGTGCCGTGAGTTT
CD4	Fw	GCGAAAACAGGAAAGTTGCAT
	Rv	GTCAAATTTTTCTGGAGCTGAGT

ON-LINE SUPPLEMENT FIGURE LEGENDS

Figure S1. Smoking status in COPD patients does not determine inflammasome mediated citokines. (A) NLRP3 expression in current and former smoker COPD patients. (B) IL-1RA protein levels in lung tissue of current and former smoker COPD patients. (C) IL-18 protein levels in lung tissue of current and former smoker COPD patients. (D) IL-18 protein levels in lung tissue of current and former smoker COPD patients.

Figure S2. Expression levels of inflammasome related mediators. (A) Expression levels in relation to GAPDH (RQ) of the following immune mediators: *IL1, ELANE, CCL20, IL2, IL6, TGFB1, IL8, CD69* and *CD4*, in the three studied groups. Results are presented as mean ± SEM of n=15 non-smokers, n=15 smokers and n=38 COPD patients. (B) Spearman Correlation of *NLRP3* mRNA with the studied immune mediators, Rho and *p values* are indicated in the text. Red indicates a positive correlation; green indicates the lack of correlation.