Supplementary File

Supplementary detailed methodology

Patients

Patients were classified as current smokers, former smokers (defined as having smoked a minimum of one cigarette a day for a minimum of 1 year, stopping at least 6 months before presentation) or non-smokers. No significant differences arose when we analyzed smoking-matched groups in all demographical parameters.

Thirty-three (33) consecutive subjects were enrolled in this study.

IPF group (n=16): The diagnosis of IPF was based on recent criteria according to ATS/ERS [1]. All IPF patients were newly diagnosed and had not received previous treatment.

RA-UIP group (n=8): Criteria for the diagnosis of CTD included the American College of Rheumatology (ACR) 1987 revised criteria for the classification of rheumatoid arthritis (RA) [2]. RA-UIP patients had HRCT findings indicative of definite interstitial lung disease [3].

Control group (n=9): Patients undergoing bronchoscopy for the investigation of haemoptysis, without any overt pulmonary comorbidities and with normal bronchoscopic findings and cytology results. Subjects who had experienced respiratory infections during the 6 prior weeks were excluded.

No patients had received any immunosuppressive therapy prior to the bronchoscopy.

Informed consent was obtained from all patients. The study was approved by the Ethics Committee of the University Hospital of Heraklion, Crete, Greece.

Pulmonary function tests

Pulmonary function tests, performed within 1 month of CT, included FEV_1, FVC, and DLCO corrected for hemoglobin concentration, expressed as percentages of the predicted normal values.

All patients were evaluated with complete pulmonary function tests (PFTs): Spirometry, lung volumes using the helium-dilution technique and DLCO (corrected for hemoglobin) using the single breath technique were performed using a computerized system (Jaeger 2.12; MasterLab, Würzburg, Germany). Predicted

values were obtained from the standardized lung function testing of the European Coal and Steel Community, Luxembourg (1993).

Age matching

The present study was performed by age matching all groups, in order to eliminate any misguidance caused by extreme differences in age between the tested groups.

Biological samples and processing

BALF was obtained from all patients and controls as previously described [4]. Briefly, a flexible bronchoscope was wedged into a subsegmental bronchus of a predetermined region of interest based on radiographical findings. A BALF technique was performed by instilling a total of 240 mL of normal saline in 60-mL aliquots, each retrieved by low suction. Volume received was the same for all samples. The BALF fractions were pooled and split equally into two samples. One sample was sent to the clinical microbiology and cytology laboratory and the other was kept at room temperature (RT) and used for this research. We then proceeded to the isolation of macrophage population.

Briefly, BALF was passed through a Millipore filter to isolate cells in suspension from debris and mucus. To pellet cells, samples were centrifuged at 1,500 rpm for five minutes at RT. The supernatant was discarded and the cells were resuspended in 4 ml RPMI medium, 20% FBS, 10x penicillin/streptomycin, followed by cell count in an improved Neubauer haemocytometer. BALF sample cells were observed after one hour using an inverted microscope, to verify adherence of macrophages.

Equal amounts of BALF sample cells were loaded onto six-well plates, using RPMI supplemented with 10% heat-inactivated FBS as culture medium.

BALF samples were analysed as previously described [5].

TLR4 stimulation with LPS (MERCK, Lipopolysaccharide, *E. coli* O111, cat#437627-5MG) (250 pg/ml, incubated for 2hrs at a 37^{o}C/5% CO_2 humidified incubator) was followed by NLRP3-inflammasome activation with a 5mM ATP pulse (Sigma Aldrich, Adenosine 5′-triphosphate disodium salt hydrate cat# A2383) for 20min at 37^{o}C/5% CO_2, in the presence or absence of caspase-1 inhibitor (Calbiochem, Caspase-1 Inhibitor I cat#400010-5MG) (Supplementary Figure 1), as

previously described [6]. Cells were harvested by centrifugation at 1000 rpm at 4˚C. Supernatants were collected and cells were lysed in RIPA buffer (Sigma Aldrich cat#R0278), according to the manufacturer's instructions. Supernatants and cell extracts were stored at -80˚C.

Total RNA extraction was performed with Tri Reagent, MBL followed by Nucleospin RNA II kit, Macherey Nagel. cDNA synthesis was performed with Maxima First Strand cDNA Synthesis Kit, Thermo Scientific. qPCR Master Mix, Thermo Scientific, using the MxPro 3000P, Agilent Technologies for the evaluation of: *NLRP3, Caspase-1*. GAPDH was used as housekeeping gene.

qRTPCR was utilised to determine the expression levels of the following genes: *NLRP3(*F5'GATCTTCGCTGCGATCAACAG-3', R 5'CGTGCATTATCTGAACCCCAC3'), *Caspase-1* (F 5'-CAGAGCTGTGCAGATGAGT-3', R 5'- CTGCAGCCACTGGTTCTGT-3'). GAPDH (F 5'-GGAAGGTGAAGGTCGGAGTCA-3', R5'-GTCATTGATGGCAACAATATCCACT-3') was used as normalization-housekeeping gene. qRT-PCR was performed in duplicates using Maxima SYBR Green qPCR Master Mix, Thermo Scientific, using the MxPro 3000P, Agilent Technologies.

All reactions were run in duplicates and transcript levels were calculated and normalized to GAPDH as well as the appropriate calibrators, using the Pfaffl method for relative quantification. Normalized values were calculated using the following equation: Fold change=eff goi^(Ct goi calibrator-Ct goi test sample)/eff ref^(Ct ref calibrator-Ct ref test sample) [7].

Intracellular IL-1β protein levels (normalized to beta actin) were assessed by immunoblotting, as previously described [4]. In detail, BALF macrophages from 16 IPF patients, 8 RA-UIP patients and 9 control subjects were homogenised in order to obtain the corresponding protein extracts. The protein lysate was added to 1/3 volume of SDS-preparation buffer (NuPAGE LDS 4X LDS Sample Buffer, Invitrogen Corp., USA). Sample preparations of each BALF protein sample (50 ng) were separated by 12.5% SDS-polyacrylamide gel electrophoresis. The proteins were then transferred

electrophoretically from the gels to a nitrocellulose membrane. Membranes were incubated with rabbit anti-mature IL-1β polyclonal antibody (17kDa protein, Cell Signalling Technology cat#2022). After applying a secondary antibody, immunodetection was performed with enhanced chemiluminescence, detected on X-ray films (Fuji films). Mouse anti-actin antibody (MAB 1501, Chemicon, Temecula, CA) was used in order to normalize IL-1β expression. Films were scanned and the protein lanes were quantified using the Photoshop CS2 image analysis software (Adobe Systems Inc., CA).

Supplementary Results

Smoking status matching

While comparing the results based on smoking status, we aimed to compare results from matching smoking history subjects between groups. This comparison did not alter the results.

TNF-a secretion

We analysed the secretion levels of TNF-α in BAL fluid (baseline) and in the supernatants of untreated macrophage cultures from all groups. A trend favouring the RA-UIP group at baseline was observed, while the untreated RA-UIP macrophages secreted significantly higher amounts of TNF-α than IPF macrophages (Supplementary Figure 2).

Intracellular IL-1β

Aiming to investigate the IL-1β protein expression profile in the macrophages population, we further analyzed the protein cell extracts at baseline, inflammasome activation via LPS plus ATP and lastly LPS plus ATP plus Caspase-1 inhibitor conditions.

Results showed significantly higher levels of intracellular IL-1β in controls when compared to all other groups, in all conditions (Supplementary Figure 3).

Moreover, the RA-UIP patient group showed significantly higher levels of intracellular IL-1β than IPF at baseline and at the inflammasome activation via LPS plus ATP condition.

References

1. Raghu G. Idiopathic pulmonary fibrosis: guidelines for diagnosis and clinical management have advanced from consensus-based in 2000 to evidence-based in 2011. *The European respiratory journal* 2011: 37(4): 743-746.
2. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis and rheumatism* 1988: 31(3): 315-324.
3. Kim EJ, Elicker BM, Maldonado F, Webb WR, Ryu JH, Van Uden JH, Lee JS, King TE, Jr., Collard HR. Usual interstitial pneumonia in rheumatoid arthritis-associated interstitial lung disease. *The European respiratory journal* 2010: 35(6): 1322-1328.
4. Economidou F, Antoniou KM, Soufla G, Lasithiotaki I, Karagiannis K, Lymbouridou R, Proklou A, Spandidos DA, Siafakas NM. Role of VEGF-stromal cell-derived factor-1alpha/CXCL12 axis in pleural effusion of lung cancer. *J Recept Signal Transduct Res* 2010: 30(3): 154-160.
5. Margaritopoulos GA, Antoniou KM, Karagiannis K, Samara KD, Lasithiotaki I, Vassalou E, Lymbouridou R, Koutala H, Siafakas NM. Investigation of Toll-like receptors in the pathogenesis of fibrotic and granulomatous disorders: a bronchoalveolar lavage study. *Fibrogenesis Tissue Repair* 2010: 3: 20.
6. Choulaki C, Papadaki G, Repa A, Kampouraki E, Kambas K, Ritis K, Bertsias G, Boumpas DT, Sidiropoulos P. Enhanced activity of NLRP3 inflammasome in peripheral blood cells of patients with active rheumatoid arthritis. *Arthritis Res Ther* 2015: 17: 257.
7. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001: 29(9): e45.