Supplementary material

Bronchial inflammation and bacterial load in stable COPD is associated with TLR4 overexpression

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Methods

Subjects

All COPD patients and control subjects who underwent bronchoscopy and bronchial biopsies were recruited from the Respiratory Medicine Unit of the Fondazione Salvatore Maugeri, Institute of Veruno (Veruno, Italy). In COPD patients, the severity of the airflow obstruction was graded using current GOLD criteria [http://goldcopd.org/]. All former smokers had stopped smoking for at least one year. COPD and chronic bronchitis were defined according to international guidelines, [http://goldcopd.org/]. COPD severity was graded according to international guidelines, [http://goldcopd.org/]. All COPD patients were stable with no previous exacerbation in the six months before bronchoscopy. None of the subjects were treated with theophylline, antibiotics, antioxidants, mucolytics, and/or glucocorticoids in the month prior bronchoscopy. COPD patients were using short-acting inhaled β2-agonists (SABA) or short-acting inhaled antimuscarinics (SAMA) prn or regular long-acting inhaled β2- agonists (LABA) and/or regular inhaled anticholinergics, including SAMA or long-acting inhaled antimuscarinics (LAMA) at the dosage recommended in current COPD guidelines [http://goldcopd.org/] at the time of their recruitment. The study conformed to the Declaration of Helsinki and was approved by the ethics committees of the (formerly) Fondazione Salvatore Maugeri, at present: Istituti Clinici Scientifici Maugeri SpA, Società Benefit [Veruno (Novara), Italy, the University Hospital of Ferrara, Italy. Written informed consent was obtained from each subject and bronchial biopsies were performed according to the local ethic committee guidelines.

Lung function tests and volumes

Pulmonary function tests were performed as previously described (**E1**) according to guidelines recommendations. Pulmonary function tests included measurements of FEV_1 and FEV_1/FVC under baseline conditions in all the subjects examined (6200 Autobox Pulmonary Function Laboratory; Sensormedics Corp., Yorba Linda, CA, USA). In order to assess the reversibility of airflow obstruction and post bronchodilator functional values, the FEV_1 and FEV_1/FVC% measurements in the groups of subjects with FEV_1/FVC% $\leq$70% pre-bronchodilator was repeated 20 min after the inhalation of 0.4 mg of salbutamol.

Fiberoptic bronchoscopy and bronchial biopsy processing

Fiberoptic bronchoscopy, collection and processing of bronchial biopsies were performed as previously described (**E1**). In brief, four bronchial biopsy specimens were taken from segmental and subsegmental airways of the right lower and upper lobes using size 19 cupped forceps. Bronchial biopsies for immunohistochemistry were gently extracted from the forceps and processed for light microscopy as previously described (**E1**). At least two samples were embedded in Tissue Tek II OCT (Miles Scientific, Naperville, IL, USA), frozen within 15 min in isopentane pre-cooled in liquid nitrogen, and stored at –80°C. The best frozen sample was then oriented and 6μm thick cryostat sections were cut for immunohistochemical light microscopy analysis and processed as described below.

Immunohistochemistry analysis of TLRs and NLRs signaling pathways on bronchial biopsies

One cryostat section from each biopsy was stained with one each of a panel of primary antibodies specific for the inflammatory cells or signaling molecules studied (**Table E1**). Briefly, after blocking non-specific binding sites, primary antibody was applied at optimal dilutions and incubated for 1h at room temperature. Antibody binding was revealed with secondary anti-mouse (Vector, BA 2000), anti-rabbit (Vector, BA 1000) or anti-goat (Vector, BA 5000) antibodies followed by ABC kit AP AK5000, Vectastain and fast-red substrate (red color) or ABC kit HRP Elite, PK6100, Vectastain and diaminobenzidine (DAB) substrate (brown color). Human tonsil or nasal polyp were used as positive controls. For the negative controls, normal mouse, rabbit or goat non-specific immunoglobulins (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at the same protein concentration as the primary antibody. Double staining for identification of CD8+, macrophages (CD68+) and endothelial cells (CD31+) co-expressing TLR4 and NOD1 was also performed; briefly, mouse monoclonal antibodies used for identification of CD8+,

CD68+ and CD31+ cells were revealed using the horse anti-mouse (Vector, BA 2000) followed by ABC kit AP AK 5000, Vectastain and fast-red substrate (red color). Rabbit or goat primary antibodies for identification of NOD1 (rabbit) and TLR4 (goat) were revealed using a goat anti rabbit (Vector, BA 1000) and a rabbit anti goat (Vector, BA 5000) antibodies followed by ABC kit HRP Elite, PK 6100, Vectastain and DAB substrate (brown color). For the negative controls, normal mouse, rabbit or goat non-specific immunoglobulins (Santa Cruz Biotechnology) were used at the same combination and protein concentration as the primary specific antibodies.

Scoring system for immunohistochemistry

Morphometric measurements were performed with a light microscope (Leitz Biomed, Leica Cambridge, UK) connected to a video recorder linked to a computerized image system (Quantimet 500 Image Processing and Analysis System, Software Qwin V0200B, Leica). Light-microscopic analysis was performed at a magnification of 630x. The immunostaining for all the antigens studied was scored (range: 0 = absence of immunostaining to 3 = extensive intense immunostaining) in the intact (columnar and basal epithelial cells) bronchial epithelium, as previously described (**E1**). The final result was expressed as the average of all scored fields obtained from each biopsy. A mean±SD of 0.70±0.26 millimeters of epithelium was analyzed in COPD patients and control subjects. A minimum length of 450 microns of intact epithelium was studied for each patient.

Immunostained cells in bronchial submucosa (lamina propria) were quantified 100μm beneath the epithelial basement membrane in several non-overlapping high-power fields until the whole specimen was examined. The final result, expressed as the number of positive cells per square millimeter, was calculated as the average of all the cellular counts performed in each biopsy.

Quantification of the bacterial load in the bronchial biopsies

qRT-PCR was used to quantify the 16S ribosomal subunit (total bacterial load) and the genome copy number (copies/ml) per mm^2 of bronchial tissue examined of *Pseudomonas aeruginosa (P. aeruginosa), Haemophilus influenza (H. influenza), Moraxella catarrhalis (M. catarrhalis)* and *Streptococcus pneumonia (S. pneumoniae).* In brief, total bacterial DNA was extracted under sterile conditions from 30μm cryostat sections of bronchial biopsies using the QIAmp DNA Mini Kit (Cat. # 56304, Qiagen) following the manufacturer's instructions and re-suspended in 100μl nuclease-free water. DNA was

stored at −20°C before amplification. DNA standards for qRT-PCR were prepared from pure DNA cultures of *Escherichia coli, H. influenzae, M. catarrhalis, P. aeruginosa, and S. pneumoniae* and were used to generate standard curves. The standard curves were always performed in triplicate with regression coefficients close to 1 (range of R^2 values: 0.984-0.994 for *E. coli*, 0.986-0.999 for *H. influenzae*, 0.990-0.998 for *M. catarrhalis*, 0.987-0.999 for *P. aeruginosa* and 0.984-0.997 for *S. pneumoniae*) and showed a linear increase within the range of DNA concentration utilized.

Primers for the specific amplification of *H. influenzae, M. catarrhalis, P. aeruginosa,* and *S. pneumoniae* were synthesized by Life Technologies (Milan, Italy) and are shown in **Table E2**. DNA amplification and detection were performed in a Rotor Gene Q system (Qiagen) using the QuantiFastTM SYBER Green PCR Kit (Cat. # 204054, Qiagen). RT-PCR cycling conditions were: 95°C for 5 min (PCR initial activation step); 40 amplification cycles of 95°C for 5 s (denaturation) and 60°C for 10 s (combined annealing/extension), followed by melting curve analysis to ensure the specificity of the PCR amplification. For each reaction, negative controls were run in triplicate, consisting of primers, PCR Mastermix and sterile water instead of DNA template. Amplification, data acquisition, and cycle threshold (CT) values analysis were performed using the Rotor Gene Q software (Rotor-Gene Q Series Software 2.0.2). For each patient and control subject, an adjacent 6µm cryostat section, stained with H&E, was used for measurement of the sub-epithelial basement membrane length. All data were expressed as number of bacterial DNA copies/ml normalized for the sub-epithelial basement membrane length multiplied by the cryostat section thickness (30µm), corresponding to the square millimeters (mm^2) of the more superficial layer of bronchial tissue examined for each subject.

Cell Culture and Treatments

The 16HBE human epithelial cell line was grown in Dulbecco's modified Minimum Essential Medium (DMEM), supplemented with 10% v/v fetal bovine serum (FBS), 50 IU/ml penicillin, 50µg/ml streptomycin, 1x non-essential amino acids, 1mM sodium pyruvate and 2mM glutamine (37°C, 5% CO_2). When cells were at 60-70% confluent, the medium was replaced with supplemented DMEM without FBS for starvation time (24h), followed by supplemented DMEM plus 1% FBS in the absence or presence of H_2O_2 (100µM) for 2, 4 ,8, 16 and 24 hours. All experiments were performed at least four times (in quadruplicate).

Extraction and Quantification of RNA and qRT-PCR from 16HBE

Total cellular RNA from treated and non-treated cultures was purified and isolated, using RNAspin Mini RNA Isolation kit (GE Healthcare, Buckinghamshire, UK) following the manufacturer's instructions. Total RNA was re-suspended in 100µl nuclease-free water and RNA concentration determined by spectroscopy (λ260/280 nm, Eppendorf BioPhotometer plus), and stored at -80°C until use. Gene expression was measured using the Syber green for qRT-PCR in a Rotor Gene Q (Qiagen) system. One-step real-time PCR was carried out by amplifying mRNA using the QuantiFastTM SYBER Green RT-PCR Kit (Qiagen), according to the manufacturer's instructions, and gene specific primers (Qiagen) for TLR4 (Cat. # QT01670123, Qiagen) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cat # QT01192646). Cycle threshold (CT) values were determined using the Rotor Gene Q software (Rotor-Gene Q Series Software 2.0.2). The expression levels of all genes studied were normalized to GAPDH levels in each sample to determine the expression between treated and non-treated cells using the $2^{-\Delta\Delta Ct}$ method (**E2**).

Statistical analysis

Group data were expressed as mean (standard deviation) for functional data and median (range) or interquartile range (IQR) for morphologic data. Differences between groups were analyzed using analysis of variance (ANOVA) for functional data. The ANOVA test was followed by the unpaired t-test for comparison between groups. The Kruskal-Wallis test applied for morphologic data was followed by the Mann-Whitney U test for comparison between groups. Correlation coefficients were calculated using the Spearman rank method. Probability values of $p<0.05$ were considered significant. Data analysis was performed using the Stat View SE Graphics program (Abacus Concepts Inc., Berkeley, CA, USA).

Table E1 Primary antibodies and immunohistochemical conditions used for identification of innate immune proteins, cytokines and inflammatory cells.

Target	Supplier	Cat.#[a]	Source	Dilution	Positive control
TLR2	Serotec	AHP1424	rabbit	1:300	Nasal polyp, tonsil
TLR4	R&D	AF1478	goat	1:80	Nasal polyp, tonsil
TLR9	Serotec	AHP1823	goat	1:150	Nasal polyp, tonsil
CD14	Sigma	C7673	mouse	1:40	Nasal polyp, tonsil
NOD1	Novus Biol.	NBP131349	rabbit	1:300	Nasal polyp, tonsil
NOD2	Santa Cruz	Sc-56168	mouse	1:25	Nasal polyp, tonsil
MYD88	Santa Cruz	Sc-11356	rabbit	1:150	Nasal polyp, tonsil
TIRAP	Serotec	AHP866T	rabbit	1:300	Nasal polyp, tonsil
Phospho-IRAK1	Santa Cruz	Sc-130197	rabbit	1:50	Nasal polyp, tonsil
IRAK4	R&D	AF3919	goat	1:300	Nasal polyp, tonsil
CD4	Dako	M716	Mouse	1:100	tonsil
CD8	Dako	M7103	Mouse	1:200	tonsil
CD68	Dako	M814	Mouse	1:200	tonsil
CD31	Dako	M823	Mouse	1:40	Nasal polyp
Neutrophil elastase	Dako	M752	Mouse	1:100	Nasal polyp

[a]Cat#, catalogue number; (p): paraffin embedded peripheral lung tissue pretreated with citrate buffer (pH 6) and microwave exposure. See methods section for details

Table E2. List of primers used for qRT-PCR of bacteria in the study of bronchial biopsies

Primer type	Primer	Sequence (5' to 3')	Reference
16S rRNA	*Eub-F*	*F: 5'-TCCTACGGGAGGCAGCAGT-3'*	*Nadkarni, 2002*[a]
	Eub-R	*R:5'-GGACTACCAGGGTATCTAATCCTGTT-3'*	*Nadkarni, 2002*[a]
Escherichia coli	*E.coli F*	*F: 5'-CATGCCGCGTGTATGAAGAA-3'*	*Huijsdens, 2002*[b]
	E.coli R	*R: 5'-CGGGTAACGTCAATGAGCAAA-3'*	*Huijsdens, 2002*[b]
Haemophilus influenzae	*HelS-F*	*F: 5'-CCGGGTGCGGTAGAATTTAATAA-3'*	*Rogers GB, 2014*[c]
	Eub-R	*R: 5'-CTGATTTTTCAGTGCTGTCTTTGC-3'*	*Rogers GB, 2014*[c]
Moraxella catarrhalis	*copB-F*	*F: 5'-GTGAGTGCCGCTTTTACAACC-3'*	*Sethi S, 1997*[d]
	copB-R	*R: 5'-TGTATCGCCTGCCAAGACAA-3'*	*Sethi S,1997*[d]
Pseudomonas aeruginosa	*gyrB-F*	*F: 5'-CCTGACCATCCGTCGCCACAAC-3'*	*Qin X, 2003*[e]
	gyrB-R	*R: 5'-CGCAGCAGGATGCCGACGCC-3'*	*Qin X, 2003*[e]
Streptococcus pneumoniae	*Spn9802-F*	*F: 5'-AGTCGTTCCAAGGTAACAAGTCT-3'*	*Abdeldaim, 2008*[f]
	Spn9802-R	*R: 5'-ACCAACTCGACCACCTCTTT-3'*	*Abdeldaim, 2008*[f]

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E-References

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E2. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25:402-408.