

Inhibition of p38 Mitogen-Activated-Protein-Kinase controls airway inflammation in cystic fibrosis

Valeria Raia, Luigi Maiuri, Carolina Ciacci, Ida Ricciarelli, Loredana Vacca, Salvatore Auricchio, Mariano Cimmino, Matteo Cavaliere, Massimiliano Nardone, Antonio Cesaro, Jennifer Malcolm, Sonia Quaratino, and Marco Londei.

On-line only and supplemental material

Ex-vivo tissue cultures of nasal polyp mucosal explants

Just after surgical removal nasal biopsies were cut under stereomicroscopic observation in several fragments of similar size and weight. Mucosal samples were placed on a stainless steel mesh positioned over the central well of an organ culture dish with the epithelium of the biopsy uppermost and the well was then filled with culture medium at 37°C so as just to reach the cut surface of the biopsies. In this way the surface which is normally exposed to air in the respiratory tract is feed by capillarity and retains the normal polarity thus allowing a better model for the leukocyte (neutrophil) migration/redistribution studies. The ex-vivo challenge took place as previously described.[E1][E2][E3] Briefly, 10 ml of culture medium consist of Trowell's T8 medium (6.5 ml), NCTC 135 medium (2 ml), fetal calf serum (1.5 ml), penicillin 50000 IU and streptomycin 5000 IU.

Biopsy specimens were cultured in the presence of medium alone or medium added with 1 µg/ml Lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* (*PA*) (*PA*-LPS) (Sigma Chem Co, St Louis, MO, USA,) or *PA*-LPS added with selective p38-MAP-k inhibitor SB203580 [E4] (Calbiochem, San Diego, CA) at a final concentration of 1 µM, and then stored at -80°C. *PA*-LPS was also tested at lower concentrations (0.5 µg/ml) with similar results.

COX-2 and IL-8 mRNA detection in tissue samples

RNA was extracted from frozen tissue belonging to 5 CF and 5 control samples cultured for 4 hours as previously described, using TRIZol method, (Invitrogen Ltd. Paisley, UK) then cleaned up using RNeasy kit (Qiagen Ltd. West Sussex, UK.).[E3] Samples were loaded into Total RNA Nano chip (Agilent Technologies UK Ltd. West Lothian) and tested on Agilent Technologies 2100 Bioanalyzer for RNA quality and concentration. cDNA was made using 1 µg of total RNA, oligo dT (Roche Diagnostics Ltd. East Sussex, UK) RNase inhibitor Super-ase in (Ambion Europe Cambridgeshire, UK) and Omniscript RT Kit (Qiagen Ltd. West Sussex, UK.) Real time PCR was performed with Quantitect SYBR green PCR kit (Qiagen Ltd. West Sussex, UK.) 95°C 5 mins, (95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec) for 45 cycles. GAPDH and IL-8 PCR as above, but use 57°C annealing temperature. **COX-2** forward primers 5'-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3', reverse 5'-AGA TCA TCT CTG CCT GAG TAT CTT-3',[E5] **GAPDH** forward primer 5'-AGC CAC ATC GTC CAG ACA C-3', reverse 5'-GAG GCA TTG CTG ATG ATC TTG-3'.[E6] **IL-8** forward primers ATG ACT TCC AAG CTG GCC GTG GCT, reverse TCT CAG CCC TCT TCA AAA ACT TCT C [E7] were used on a Machine Opticon Monitor (MJ Research Inc. NV, USA). Standard curve constructed of triplicates and all results were analysed using $2^{-\Delta\Delta C_T}$ Method.[E8] The level of COX-2 and IL-8 was calculated as the ratio to the expression of GAPDH in each sample.

Antibodies, antigen detection and morphometric analysis in tissue samples

Cryostat tissue sections (5 µm) were air dried at room temperature and then fixed in acetone for 10 minutes except for NF-kappaB detection in which unfixed sections were incubated with primary mAb. All the sections were repeatedly washed at room temperature in Tris-buffered saline (TBS) and then individually incubated with the antibodies listed in Table E1 for 2 hours at room temperature. The antigen expression was visualized by direct immunofluorescence (COX-2) or indirect immunofluorescence (NF-kappaB-p65, PY-99, phospho-p38-MAP-k, pan-cytokeratin) or peroxidase staining (Cathepsin G positive polymorphonuclear (PMN) leukocytes), as previously described,[E1] [E3] and as detailed in Table E1. All the staining procedures were carefully controlled by incubating tissue sections with non-immune Igs of the same species and isotype of the tested antibody (mouse or rabbit Ig) or with isotype-matched antibodies against inappropriate blood group antigens (Dako) or mouse monoclonal antibodies against human lactase or human sucrase (5 µg/ml), kind gift of Dr D Swallow, UCL, London.[E9] At least 5 sections of each sample were tested with each primary antibody and the appropriate control. All the staining control procedures have been detailed in our previous papers.[E1] [E10]

Two colour immunofluorescence [E3] with FITC mAb anti-COX-2 and R-phycoerithrin(PE)-conjugated mAbs anti-CD68 (mouse PE-IgG_{2b}, clone Y1/82A, BD Biosciences, USA) or CD3 (mouse PE-IgG_{1k}, clone UCHT1, Dako, 1:20) was performed to detect the phenotype of COX-2 positive MNC. The tissue distribution of neutrophils was analyzed by counting Cathepsin G positive PMN leukocytes in different mucosal compartments per standard reference areas, as detailed in the legend of Figure 4: in the deeper mucosal layer (**dl**, from bottom of the mucosa to 100 μm below the epithelial basal membrane) (per reference area of $5 \times 10^4 \mu\text{m}^2$), in the subepithelial region (**sec**, 100 μm thick below the epithelial basal membrane) (per reference area of $5 \times 10^4 \mu\text{m}^2$), within the epithelium (**iec**) (per mm of epithelium) or in the area outside the epithelial apical membrane (**lumen**, 50 μm thick just above the epithelial surface) (per reference area of $2.5 \times 10^4 \mu\text{m}^2$,). The analysis of the redistribution of different cell types within mucosal biopsies upon antigen stimulation has been already performed in our previous papers.[E1] [E10]

Epithelial cells with nuclear expression of NF-kappaB-p65 were numbered per 100 epithelial cells. At least 500 epithelial cells were numbered in each slide and the expression of NFkappaB-p65 within the nucleus was calculated. At least 4 slides for each sample belonging to each patient or control were blindly evaluated by two different observers and the analysis was simultaneously performed in each set of experiments (cultures with medium, *PA*-LPS, *PA*-LPS added with SB203580, DMSO alone at different times of incubation). Control experiments of specificity were simultaneously analyzed by a similar procedure.

The fluorescence of PY-99+ and phospho-p38+ epithelial cells was graded using a Zeiss Axioskop 2 Plus microscope equipped with AxioCam HR color and Axiovision KS300 for quantitative photometry (Zeiss, Jena, Germany). Slides were analyzed under fluorescence examination according to the manufacturer's suggestions. Briefly the images were acquired as black and white at high resolution. The black area within the slide outside the section was scored as 0, whereas a defined white area of a reference white filter was scored as 100. The system was calibrated, as above mentioned, before the image acquisition of each set of experiments. All the procedures of the image acquisition were randomly repeated to avoid any variability.

Five slides for each sample and for each marker were subjected to image analysis. At least 5 different areas with 50 epithelial cells on each slide were measured and individually scored according to the above mentioned procedures by the Zeiss Axiovision KS300 software. The great majority of epithelial cells showed a normalized optical density above 60 or below 20 of the score values and only a marginal spectrum of values was observed, thus allowing a good discrimination between cells with strong expression and cells with faint to negligible expression of the tested marker. Epithelial cells with optical density higher than 60 were numbered per 100 epithelial cells. The epithelial cells were clearly identified by the morphological appearance as well as by specific staining of section with pan-cytokeratin mAbs (Table E1), as described in our previous paper.[E11] During the morphometric evaluation the more external parts of the section were ruled out, since the edge of the specimens might be damaged during the culture procedures. Moreover in the great majority of the tissue area no damage was observed even after 24 hours of culture after incubation with medium alone. As clearly shown in Figure 4 the stimulation of CF, but not control, samples with *PA*-LPS for 24 hours led to a patchy lesion in those areas in which neutrophil infiltration was particularly evident and the treatment with SB203580 clearly prevented such *PA*-LPS induced patchy mucosal lesion. No lesion was observed in CF samples after 20 minutes or 4 hours of incubation even in the presence of *PA*-LPS in the culture medium. Pan-cytokeratin (5/6/8/18) mouse Ig, clone 5D3 and LP34, Novocastra, Newcastle, UK, followed by 1:40 FITC rabbit anti-mouse Ig F(ab)₂ (1:20, Dako) was used for the definition of epithelial cells.

Western blot

Western Blot was performed on protein extracted from nasal polyp explants from 2 patients (n° 6 and n°7 of Table 1) after 4 hours incubation with medium alone, *PA*-LPS or *PA*-LPS added with SB203580, as previously detailed.

Protein was extracted by homogenizing polyps in cells disruption buffer (PARIS kit, Ambion catalog number 1921) followed by incubation for 10 min all on ice. After performing a protein assay, 50µg of each sample were loaded on a 10% SDS PAGE gel and run on Biorad Mini Protean apparatus. The gel was then transferred on to PVDF membrane and then blocked with 5% skimmed milk in PBS containing 0.1% Tween 20 (PBST 0.1%) for 1 hour. The membrane was then incubated with anti-MAPKAP-k-2 [pT³³⁴] (Biosource Europe, catalog number 44-516G) 1:800 in 5% skimmed milk in PBST 0.1% overnight at 4°C, then washed 3x 5 min with PBST 0.1%. Then the secondary antibody, Swine anti Rabbit HRP (Dako, catalog number P0399) 1:2000 in 5% skimmed milk in PBST 0.1% for 2 hours at room temperature was applied. Following additional washes with PBST 0.1% the enzymatic development was performed by ECL system (Amersham Pharmacia Biotech, catalog number RPN2106). The blot was then re-probed with mouse anti Actin (Chemicon, catalog number MAB1501) 1:2500 in 5% skimmed milk in PBST 0.1% for 1 hour at room temperature; the blot was then washed 3x 5 min with PBST 0.1%. The secondary antibody goat anti mouse HRP (Dako, catalogue number P0447) 1:2000 in 5% skimmed milk in PBST 0.1% was then applied for 1 hour at room temperature. Following additional washes with PBST 0.1%, the enzymatic development was performed by ECL system (Amersham Pharmacia Biotech).

Figure Legends for on line only figures

Extrafigure 1: p65 nuclear localization in the airway epithelium of nasal polyp explants from a CF patient upon 4 h PA-LPS challenge.

Two colour immunofluorescence, NF-kappaB-p65 (green), nuclear counterstaining with propidium iodide (red). Note p65 nuclear localization (yellow colour) in many epithelial cells.

Original magnification, x600

Extrafigure 2: Cytokeratin expression in nasal polyp mucosa from control (A) and CF (B) patients, as revealed by anti-Pan-Cytokeratin Ab.

Two colour immunofluorescence, Cytokeratin (green), nuclear counterstaining with DAPI (blue). The morphology of nasal polyp epithelium is similar in control and CF patients.

Original magnification, x600.

Table E1: Antibodies and antigen detection

Antigen	Antibodies	Detection
COX-2	Fluorescein isothiocyanate (FITC)- mouse IgG1 (1:30) Cayman Ann Arbor, MI	Direct immunofluorescence
NF-kappaB	mouse IgG3, p65 subunit, Chemicon Int, CA, 1:200	FITC rabbit anti-mouse Ig F(ab) ₂ , (1:20), Dako, Copenhagen, Denmark
Phospho-tyrosine PY99	mouse IgG2b, Santa Cruz Biotechnology, CA, 1:80	FITC rabbit anti-mouse Ig F(ab) ₂ , (1:20), Dako
Phospo- p38-MAP-k	Ab#9211 rabbit polyclonal (1:600) Cell Signaling Technology, Beverly, MA	biotinylated goat anti-rabbit Ig (1:200), Dako, followed by FITC-conjugated streptavidin (1:50), Dako.
Cathepsin G (neutrophils)	mouse Ig, clone AHN-11, Pharmingen, USA, 5 µg/ml	anti-mouse Ig peroxidase labelled (1:100), Dako
Pan-cytokeratin (5/6/8/18)	mouse Ig, clone 5D3 and LP34, Novocastra, Newcastle, UK, 1:40	FITC rabbit anti-mouse Ig F(ab) ₂ , (1:20), Dako

Extra-References for *online supplemental material*

- E1. Maiuri L, Picarelli A, Boirivant M, *et al.* Definition of the initial immunologic modifications upon in vitro gliadin challenge in the small intestine of celiac patients. *Gastroenterology* 1996;**110**:1368-78.
- E2. Maiuri L, Ciacci C, Auricchio S, *et al.* Interleukin 15 mediates epithelial changes in celiac disease. *Gastroenterology* 2000;**119**:996-1006.
- E3. Maiuri L, Ciacci C, Ricciardelli I, *et al.* Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. *Lancet* 2003;**362**:30-7.
- E4. Lee J C, Kumar S, Griswold D E, Underwood D C, *et al.* Inhibition of p38 MAP kinase as a therapeutic strategy. *Immunopharmacology* 2000;**47**:185-201.
- E5. Badawi A F, Habib S L, Mohammed M A, *et al.* Influence of cigarette smoking on prostaglandin synthesis and cyclooxygenase-2 gene expression in human urinary bladder cancer. *Cancer Invest* 2002;**20**:651-6.
- E6. Lehmann M H, Weber J, Gastmann O, *et al.* Pseudogene-free amplification of human GAPDH cDNA. *Biotechniques* 2002;**33**:766-70.
- E7. MacQuillan G C, Mamotte C, Reed W D, *et al.* Upregulation of endogenous intrahepatic interferon stimulated genes during chronic hepatitis C virus infection. *J.Med.Virol.* 2003;**70**:219-27.
- E8. Livak K J and Schmittgen T D. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. *Methods* 2001;**25**:402-8.
- E9. Maiuri L, Rossi M, Raia V, *et al.* Mosaic regulation of lactase in human adult-type hypolactasia. *Gastroenterology* 1994;**107**:54-60.
- E10. Maiuri L, Ciacci C, Vacca L, *et al.* IL-15 drives the specific migration of CD94+ and TCR-gammadelta+ intraepithelial lymphocytes in organ cultures of treated celiac patients. *Am.J.Gastroenterol.* 2001;**96**:150-6.
- E11. Quaratino S, Badami E, Pang Y Y, *et al.* Degenerate self-reactive human T-cell receptor causes spontaneous autoimmune disease in mice. *Nat.Med.* 2004;**10**:920-6.



