

Supplementary material

COPD-specific gene expression signatures of alveolar macrophages and also peripheral blood monocytes overlap and correlate with lung function

Szilard Poliska^{1,4}, Eszter Csanky³, Attila Szanto¹, Istvan Szatmari¹, Bertalan Meskó^{1,4}
Lajos Szeles^{1,2}, Balazs Dezso⁵, Beata Scholtz^{1,4}, Janos Podani⁶, Iain Kilty⁷, Laszlo
Takacs⁸ and Laszlo Nagy^{1, 2, 4*}

¹ Department of Biochemistry and Molecular Biology and ² Apoptosis and Genomics Research Group of the Hungarian Academy of Sciences, Research Center for Molecular Medicine, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary;

³ Department of Pulmonology, Semmelweis Health Care Center of Miskolc (previous Department of Pulmonology, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary)

⁴ Clinical Genomics Center, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary,

⁵ Department of Pathology, University of Debrecen, Medical and Health Science Center, Debrecen, Hungary

⁶ Biological Institute, Eötvös University, H-1117 Budapest, Hungary.

⁷ Pfizer Global Research and Development, Sandwich, United Kingdom

⁸ Biosystems International SAS, Evry, France

Running title: COPD specific gene expression in alveolar macrophages and monocytes

*Corresponding author: Laszlo Nagy
Tel.: +36 52 416432
Fax: +36 52 314989
e-mail: nagyl@med.unideb.hu

I. Detailed Materials and Method:

Study population

Informed consent

The Research Ethics Committee of University of Debrecen Medical and Health Science Center approved the clinical protocol and the study. Written informed consent was obtained before the subjects enter the study. The investigator explained the nature, purpose and risk of the study and provided the subject with a copy of the information sheet. The subjects were then be given time to consider the study's implication before deciding to participate.

Inclusion/exclusion criteria

Before starting sample collection we defined the inclusion and exclusion criteria of diseased and healthy patients. All patients were recruited from Eastern Hungary with Caucasian origin.

Inclusion criteria for COPD subjects were age 40 to 65 years old. Patients must have predicted value of forced expiratory volume at 1 second (FEV_1) $<80\%$ and $FEV_1/FVC\% < 70\%$ (stage 2 and 3 according to GOLD criteria). Patients must show $<15\%$ reversibility of airway obstruction to a standard inhaled dose of salbutamol (Ventoline 2 puffs of $100\mu\text{g}$) (www.goldcopd.com). Patients were excluded from the study who had unstable COPD, such as exacerbations in the 3 previous month (using physician's diagnosis) or reversibility $> 15\%$ of airway obstruction to a standard inhaled dose of salbutamol. Inclusion criteria for control patients were age between 40 and 65

years, must have normal spirometry, $FEV_1 \geq 80\%$ (predicted value) and $FEV_1/FVC\% \geq 70\%$. All patients must be current or ex-smoker (minimum 15 pack years).

Exclusion criteria for all patients:

1. Plasma IgE level $> 120U/ml$,
2. Alpha1-antitrypsin deficiency,
3. Evidence of asthma,
4. Atopic disease
5. History of lung disorders such as childhood lung disease, cystic fibrosis, bronchiectasis, tuberculosis and occupational lung disease
6. History of respiratory infection in the past 3 months,
7. Other inflammatory diseases such as inflammatory bowel disease (Crohn's disease and ulcerative colitis), rheumatoid arthritis, osteoarthritis, psoriasis, eczema etc
8. History of autoimmune disease such as systemic connective tissue disease Sjögren's disease, Lupus, systemic sclerosis, etc.
9. History of other clinically significant disease such as on-stable congestive heart failure, recent myocardial infarction (in the previous 12 months) unstable angina, diabetes, renal disease, cancer
10. Positive plasma test for HIV, Hepatitis B or Hepatitis C.

Medication:

1. History of oral, inhaled intranasal or topical corticosteroid or immunosuppressive drug treatment in the previous 3 months

2. History chromoglycate treatment in the previous 8 weeks
3. History of leukotriene modulator treatment in the previous 8 weeks
4. History of antibiotic treatment in the previous month
5. History of theophyllin treatment in the previous 3 days (short-acting) or 1 week (long-acting)
6. History of anticholinergic treatment in the previous 8 hours (short acting) or 1 week (long acting)
7. History of beta2-agonist treatment in the previous 8 hours (short acting) or 12 hours (long acting)
8. Vaccination against Influenza or *S. pneumonia* in the last two weeks

BAL procedure

After pre-medication with atropin (0.5 mg i.m.) local anaesthesia of nostrils mouth and throat was achieved with lidocaine spray. Fiber-optic bronchoscope (Olympus) passed through the nose, pharynx, large airways and the tip of a total 300 ml 0.9% physiological saline solution sterile warm (37 Celsius degree), in 50 ml aliquots. For intermediate aspiration using same 50 ml syringe. Each aliquot transferred into plastic tubes and placed immediately on melting ice.

Alveolar macrophage separation

BALF samples volumes were 100-150 ml and samples were filtered with sterile gauze to eliminate mucus and centrifuged at 1500 RPM for 10 minutes at 4-Celsius

degree to collect the cells. After resuspending in 1 ml PBS alveolar macrophages (AM) were separated by Percoll (Amersham Biosciences) gradient centrifugation. Percoll was pre-centrifuged at 15000 RPM for 20 minutes at 4-Celsius degree and then cell suspension was layered on it. Then we centrifuged at 3000 RPM for 30 minutes at 4-Celsius degree. During the centrifugation AM cells formed a cell ring in Percoll phase. We collected alveolar macrophages from there, washed in 10 ml PBS. Total cell number was determined by counting in hemocytometer. We carried out differential cell count on hematoxilin-eosin stained cytopsin slides before and after the gradient separation. Cell viability was checked by Trypan blue exclusion. After the separation >95% AM purity was reached. (AM 95-98% $96.6 \pm 1.3\%$, lymphocytes 0.5-4% $1.7 \pm 1\%$, neutrophils 1-2.4% $0.8 \pm 0.6\%$)

Peripheral blood monocyte separation

50 ml heparin treated venous blood was collected from healthy and diseased patients. Blood samples centrifuged at 2500 RPM for 20 minutes, then the supernatant (plasma) was removed and all of the cells were resuspended in 20 ml of physiological saline and layered on Ficoll, then centrifuged at 1700 RPM for 15 minutes to separate peripheral blood mononuclear cells (PBMC). Layer of PBMCs were collected and washed in 20 ml of physiological saline, centrifugation step was repeated to collect the cells. Cell number was determined and based on it, accurate volume of buffer and CD14 magnetic beads were added. After 15 minutes incubation at 4-Celsius degree monocytes were separated in magnetic field (>98% MO, 98-99% MO) (VarioMACS, Miltenyi Biotec.).

RNA processing

Total RNA from alveolar macrophages and peripheral blood monocytes were purified using RNeasy Mini Kit (Qiagen). RNA quality was determined Agilent Bioanalyser 2100 (Agilent Technologies), RNA sample with >9.0 RIN number and 28S/18S ratio=1.6-2.0 was used for microarray analysis. RNA concentration was measured on NanoDrop ND-1000 instrument (UV spectrophotometer). Affymetrix HG-U133A arrays (Affymetrix) were used to perform gene expression analysis. In BALF collection we used every sample separately, but we made a control and COPD RNA mix from 5-5 patients in the case of monocytes.

Complementary DNA (cDNA) was generated from 5 µg total RNA, using SuperScript choice system (Invitrogen) Double stranded cDNA was purified by phenol chloroform extraction and precipitation. This material was used for in vitro transcription (IVT). We used BioArray™ HighYield™ RNA Transcript Labeling Kit (T7) (ENZO Life Sciences) to synthesize biotine labeled cRNA. We used the RNeasy Mini kit to purify our cRNA samples. We fragmented 20 µg labeled cRNA by 5x Fragmentation Buffer (200 mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc). During the process we checked the quality of cDNA, cRNA and fragmented cRNA on agarose gel. We sent our fragmented samples to hybridize and scan to EMBL Genomics Core Facility (Heidelberg).

TaqMan RT-QPCR

In order to validate the microarray results, we used TaqMan Low Density Arrays (TLDA) and individual gene expression assays (Applied Biosystems). To confirm AM and MO common genes we used Roche Universal Probe Library system (UPL probes). TLDA contained 96 genes. 4 of them were housekeeping genes: beta-actin (ACTNB), glyceraldehydes-3-phosphate dehydrogenase (GAPDH), peptidyl-prolyl isomerase A (PPIA), ribosomal phosphoprotein large P0 (RPLP0). 69 of the 92 genes were chosen from our microarray analysis. Probe sets that did not show an at least 1.7 fold change when the COPD group was compared to the control group were excluded from the assay. Expressed sequence tags, hypothetical protein coding probes were removed and only one probe set per gene was retained and genes with low expression levels (raw values < 50) were filtered out. Finally 69 genes were selected to which we have added another 23 candidate genes, which were known from previous COPD microarray studies, or expressed in macrophages.

First strand cDNA was generated from 5 ug total RNA using cDNA Archive Kit (Applied Biosystems). For RT-QPCR reaction 200 ng cDNA/sample and 2X TaqMan PCR mix (Applied Biosystems) was used. TLDA were run in ABI Prism HT 7900 machine. Two samples could be loaded on this TLDA format.

Independent real-time QPCR assays were carried out and analyzed using the $\Delta\Delta$ Ct method. Housekeeping genes were tested by geNorm software (<http://www.wzw.tum.de/gene-quantification>). According to our data, GAPDH is the least affected housekeeping gene in this system, therefore only GAPDH was used for normalize RT-QPCR data. Normalized results were statistically analysed in GraphPad

Prism 5.0 software (<http://www.graphpad.com/>), using non-parametric Mann-Whitney U-test.

Canonical variates analysis (CVA)

Separation between predefined groups of objects is best revealed by CVA, which avoids the problem of accumulating Type I errors in simultaneous individual univariate tests. It was used to determine whether the groups of healthy and COPD patients are separable in the multidimensional space spanned by the genetic variables, and if so, which gene subsets have the best discriminatory power. The results of CVA are the so-called canonical scores obtained from the canonical functions derived through eigenanalysis, which serve as coordinates of observations in the canonical space. Due to the fact that the maximum number of canonical axes is one less than the number of groups, in the present study CVA did not allow graphical display, and separation of diseased and non-diseased patients is expressed merely by a list of scores for observations on a single canonical axis. If the observations are taken at random, variances are homogeneous and the variables satisfy multivariate normality, then statistical procedures are available to test the significance of group separation (chi-square test). Wilks lambda is another useful statistic to measure goodness of separation: the closer its value is to 0, the better the separation of groups. Nevertheless, if these criteria are not met, examination of the two groups whether they overlap on the canonical axis or not, provides equally meaningful information. A partial limitation of CVA is that the number of variables (genes) cannot exceed the number of observations (patients).

Computations were performed by the SYN-TAX 2000 package (Podani, J, 2001, SYN-TAX 2000. Computer programs for data analysis in ecology and systematics. User's Manual. Scientia, Budapest)