Online data supplement

Differential switching to IgG and IgA in active smoking COPD patients and healthy controls

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Materials and Methods

Subjects

For the isolation of leukocytes from blood, 23 COPD patients and 36 healthy individuals were included in the study. COPD patients had to have a clinical diagnosis of COPD, post bronchodilator $FEV_1/FVC < 70\%$, post bronchodilator $FEV_1 < 80\%$ predicted, and no exacerbation in the 6 weeks preceding the study. Healthy individuals had to have no signs and symptoms of pulmonary disease, and $FEV_1/FVC > 70\%$, and $FEV_1 > 90\%$ predicted. All participants were older than 40 years, had no major co morbidities and a negative skin prick test or Phadiatop for the most common aeroallergens. The use of (inhaled or systemic) corticosteroids in the 6 weeks preceding the study was not allowed. To avoid the effect of gender only males were included in the study. Smokers and ex-smokers had to have a smoking history of at least 10 packyears and ex-smokers had to have quit smoking for a least one year. Approval was obtained from the medical ethics committee of the University Medical Center Groningen and all participants gave their written informed consent.

For the isolation of leukocytes from lung tissue, 14 COPD patients and 9 non-COPD controls were included in the study. COPD lung tissue was derived from COPD patients undergoing surgery for lung transplantation (Stage IV, n=9) or surgery for pulmonary carcinoma (Stage

II-III, n=5). COPD patients did not show clinical signs of chronic bronchitis and were not α1-antitrypsin deficient. COPD stage IV patients who underwent lung transplantation had quitted smoking for at least 1 year before surgery. Non-COPD control lung tissue was derived from non-involved lung tissue from patients undergoing surgery for pulmonary carcinoma (n=7) or from donor lungs (n=2). Lung tissue derived from tumor resections was always taken as far as possible from the tumor, or from a non-involved lobe. The study protocol was consistent with national ethical and professional guidelines ("Code of Conduct; Dutch Federation of Biomedical Scientific Societies"; http://www.federa.org).

Cell isolation from peripheral blood

Participants donated 20 ml of peripheral blood. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque plus (GE Healthcare, UK) density gradient centrifugation. Total isolated cells were counted using a Sysmex pocH-100i cell counter (Sysmex, Roche, Germany). PBMCs were used for flow cytometry analysis, immunocytochemical staining on cytospins, and stimulation experiments. A schematic overview of the study design is depicted in Figure E1.

Lung cell isolation

Single cell leukocytes suspensions were freshly isolated from lung tissue. For the lung suspensions we adapted and optimized our protocol for leukocyte isolation from mouse lungs (E1;E2). A piece of lung (ranging from about 4-8cm^3) was minced with a razor blade and incubated in a shaking water bath for 45 min at 37°C in digestion buffer containing RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), Penicillin-Streptomycin (all from Lonza, Verviers, Belgium), DNAse I (100 U/ml; Boehringer, Mannheim, Germany), and collagenase I (250 U/ml; Sigma–Aldrich). The digested lung tissue was passed through a 70

µm cell strainer (BD Bioscience, San Jose, USA) and subsequently centrifuged for 10 minutes at 1800rpm and 4°C. The pellet was resuspended in RPMI and leukocytes were isolated from this fraction using ficoll-paque plus density gradient centrifugation. To remove adherent cells and macrophages, which cause a lot of auto fluorescence on the flowcytometer, cells were resuspended in 1 ml RPMI and plated in a 6 wells plate (Corning, Amsterdam, the Netherlands) for 1 hour at 37°C. The non adherent cells were removed, counted using a Sysmex PocH-100i cell counter and used for flow cytometry analysis.

Flow cytometry analysis

Before staining the surface markers, $1*10^6$ PBMCs or lung leukocytes were incubated in a 96-wells plate with cold 0.5% human serum (Sigma-Aldrich, Zwijndrecht, the Netherlands) for 15 minutes on ice to block non-specific binding sites. Plates were centrifuged and cells were subsequently incubated with the antibody cocktail for 30 minutes on ice, protected from light. The antibody cocktail consisted of CD20-Alexa fluor 700 (eBioscience, San Diego, USA), CD27-APCCy7 (Biolegend, San Diego, USA), CD38-PECy7 (eBioscience), CD138-APC (IQ Products, Groningen, the Netherlands), IgM-Biotin (BD Bioscience), IgG-PECy5 (eBioscience), CD3-FITC (BD Bioscience), CD14-FITC (IQ Products) and CD16-FITC (IQ Products). After washing the cells with phosphate buffered saline solution (PBS) containing 2% bovine serum albumine (BSA, Serva, Heidelberg, Germany), the cells were incubated for 15 minutes with Streptavidin-PE (BD Bioscience). The cells were washed again three times with PBS/2%BSA, resuspended in FACS lysing solution (BD Bioscience), and kept in the dark on ice until flow cytometry analysis. The fluorescent staining of the cells was measured on an LSR-II flowcytometer (BD Biosciences) and data were analyzed using FlowJo Software (Tree Star, Ashland, USA). First, lymphocytes were gated based on cell size (forward scatter) and density (side scatter). This population was used as input population for the analyses.

Subsequently, cells with expression of CD3, CD14 and CD16 were excluded from the analysis to leave out T cells, monocytes, macrophages and granulocytes. Next, the different B-cell subsets were distinguished based on the expression of CD20, CD27, IgM and IgG. Total B cells were analyzed based on CD20 expression, and total memory B cells were analyzed based on co-expression of CD20 and CD27 (Figure E2A). Within the total CD20 population, class-switched memory B cells were classified as CD27 positive and IgM negative (Figure E2B). Within the memory B-cell population, IgG positive memory B cells, IgM positive memory B cells and memory B cells negative for IgG and IgM could be distinguished (Figure E2C).

PBMC stimulation

To asses the responsiveness to lung specific antigens, freshly isolated PBMCs from COPD patients and healthy controls were stimulated with extracellular matrix proteins lung elastin, lung collagen (both 1µg/ml, Elastin Products, Owensville, USA) and recombinant human decorin (1µg/ml, R&D Systems, Minneapolis, USA) in combination with a mixture of IL-10 (50ng/ml), IL-4 (10ng/ml, both Peprotech, Rocky Hill, USA), IL-2 (1.25 pg/ml, R&D Systems) and anti-CD40 (5µg/ml, eBioscience)(cytokine mix) to stimulate plasma cell differentiation and Ig production. Two million cells in 1 ml of RPMI containing 10% FCS and Penicillin-Streptomycin were plated into 24-wells plates. Cells were stimulated for 6 days at 37°C and 5% CO_2 with 1; the mixture of extracellular matrix proteins (ECM) and cytokine mix, 2; a double dose of cytokine mix (positive control) and 3; no stimulants (negative control). After 6 days of culture, cells and supernatants were harvested. Supernatants were stored at -20°C and used for the analysis of IgG, IgM and IgA production. The cells were used for ELISPOT analysis and immunocytochemical staining on cytospins.

Immunocytochemistry

The presence of B cells and plasma cells expressing IgA, IgG and IgE was assessed using immunocytochemical staining of cytospins from freshly isolated PBMCs or cytospins from stimulated cells. IgA, IgG and IgE expression was demonstrated by an anti-IgA (Dako, Heverlee, Belgium) antibody followed by a biotin labelled rabbit-anti-mouse secondary antibody (Dako) and AB complex, an anti-IgG (BD) antibody followed by peroxidase labelled rat-anti-mouse (Dako) and goat-anti-rabbit (Dako) secondary and tertiary antibodies respectively, and a rabbit-anti IgE antibody (Dako) followed by peroxidase labelled goat-anti-rabbit (Dako) and rabbit-anti-goat (Dako) secondary and tertiary antibodies respectively. Positive B cells or plasma cells could be distinguished based on their morphology. B cells are relatively small, round cells, with very little cytoplasm and a dense nucleus that show a clear membranous staining. Plasma cells are large cells, with a very strong staining in the cytoplasm and often an eccentric nucleus. Per cytospin, 1000 cells were counted and expressed as percentage positive cells.

IgG, IgA and IgM ELISA

IgA levels were determined using a commercially available ELISA kit (Bethyl laboratories, Montgomery, TX, USA). For detection of IgM and IgG, plates were coated overnight at room temperature (RT) with anti-human Ig (SBA, 1:500 in carbonate buffer). Plates were washed 5 times with PBS containing 0.05% Tween and blocked with PBS containing 1% BSA for 1 hour at RT. After washing 5 times, the supernatants diluted in PBS containing 1% BSA were added to the plate and incubated for 2 hours at RT. Plates were washed 5 times and alkaline phosphatase (AF) conjugated anti-human IgG or IgM (both from Sigma, 1:500 in PBS/1%BSA) was added and incubated for 1 hour at RT. Plates were washed 5 times and developed using p-nitrophenyl phosphate substrate (1 tablet Phosphate substrate from Sigma

in 40ml dietholaminebuffer). The reaction was stopped using 5N NaOH and the absorbances were read at 405 nm. Human reference serum (Bethyl laboratories) was titrated in serial dilutions to obtain standard curves for each ELISA, which was used to calculate antibody concentrations and to correct for plate to plate variability.

Antigen specific ELISPOT analysis

Ninety six wells filter plates (Millipore, Billerica, USA) were coated with 10µg/ml lung elastin, lung collagen or recombinant human decorin for 3 hours at 37°C. After washing the plate with PBS containing 0.05% Tween, the plate was incubated for 1 hour at 37 °C with RPMI containing 10% FCS to block a-specific binding sites. After washing the plate three times with PBS/Tween and two times with PBS, $0.5*10^6$ stimulated cells were added per well and incubated overnight at 37°C and 5% CO_2. The next day, the plates were washed vigorously with PBS/Tween and incubated with rabbit-anti-human IgG-biotin (SBA) for 2 hours at 37°C followed by a wash step and incubation with peroxidase labeled streptavidin (Dako) for 2 hours at 37°C. Spots were visualized with AEC substrate and counted using an automated reader (AELVIS GmbH, Hannover, Germany).

Statistical analyses

A multiple linear regression model was used to determine whether having COPD, being a current smoker or their combination affected the different parameters from the PBMC experiments. This method disentangles the separate effects of COPD and current smoking and their interaction. First, the effects of COPD and current smoking were tested together with the interaction between COPD and current smoking as independent variables. When the interaction between COPD and current smoking was not significant, the effects of COPD and current smoking were tested again without the interaction term. The normal distribution of the

residuals was analyzed with a Kolmogorov-Smirnov test and when needed and possible the data were log-transformed to normalize distributions. Only when significant effects of COPD or current smoking were found or a significant interaction was present between COPD and current smoking, additional Mann Whitney U (MWU) tests were used for specific post-hoc analyses. In case of an effect of current smoking, MWU tests were used to compare COPD smokers with COPD ex-smokers and healthy smokers with healthy ex-smokers and never smokers to investigate whether the effect of current smoking was present in both COPD patients and healthy controls or in patients or controls only. In case of an effect of COPD, MWU tests were used to compare COPD smokers to healthy smokers and COPD ex-smokers to healthy ex-smokers to investigate whether the effect of COPD was present in both current smokers and ex-smokers or in current smokers or ex-smokers only. For the lung tissue experiments, MWU tests were used to compare COPD patients with non-COPD controls and current smokers with ex- and never smokers. A value of $p<0.05$ was considered significant.

References online repository

(E1) Brandsma CA, Hylkema MN, van der Strate BW, Slebos DJ, Luinge MA, Geerlings M, Timens W, Postma DS, Kerstjens HA. Heme oxygenase-1 prevents smoke induced B-cell infiltrates: a role for regulatory T cells? Respir Res 2008;9:17.

(E2) Brandsma CA, Timens W, Geerlings M, Jekel H, Postma DS, Hylkema MN, Kerstjens HAM. Induction of autoantibodies against lung matrix proteins and smoke-induced inflammation in mice. BMC Pulmonary Medicine 2010;10:64.

Figure legends online repository

Figure E1; Study design

A schematic overview of the study design is depicted. PBMCs = peripheral blood mononuclear cells, ICC = immunocytochemistry, ELISPOT = Enzyme-linked immunosorbent spot, ELISA = Enzyme-linked immunosorbent assay.

Figure E2. Flow cytometry plots of B cell subsets in peripheral blood

A) the gates for total B cells (CD20 positive) and memory B cells (CD20 and CD27 positive), B) the gate for class-switched memory B cells (CD27 positive and IgM negative) within the total B cell population, and C) the gates for IgG positive memory B cells, IgM positive memory B cells and memory B cells negative for IgG and IgM within the memory B cell population are shown.

Figure E3. B cell subsets in peripheral blood

Percentages of total B cells, memory B cells, and class switched memory B cells in peripheral blood of COPD patients (closed symbols) and healthy individuals (open symbols) are shown. COPD patients had lower percentages of total B cells (COPD effect p=0.009) and memory B cells (COPD effect p= 0.029) than healthy individuals. Current smokers had higher percentages of memory B cells (smoke effect p=0.029) and class switched memory B cells (smoke effect p=0.001) than ex- and never smokers. The results of the Mann Whitney U tests are depicted in the figures. * indicates that $p < 0.05$.

Figure E4. IgE positive cells in peripheral blood

Percentages of IgE positive cells in peripheral blood of COPD patients (closed symbols) and healthy individuals (open symbols) are shown. COPD patients had lower percentages of IgE positive B cells than healthy individuals (COPD effect p=0.001).

Figure E5. IgG and IgM antibody levels in supernatant

Total IgG (A) and IgM (B) antibody levels in supernatants from PBMCs derived from COPD patients (closed symbols) and healthy individuals (open symbols), which were cultured for 6 days without stimulation (left), with ECM + cytokine mix stimulation (middle) or cytokine mix stimulation (right) are shown. The results of the Mann Whitney U tests are depicted in the figures. * indicates that $p < 0.05$.

Figure E6. IgA and IgG plasma cells

Percentages of IgA and IgG plasma cells in cytospins from PBMCs derived from COPD patients (closed symbols) and healthy individuals (open symbols), which were cultured for 6 days with ECM + cytokine mix stimulation (left) or cytokine mix stimulation (right) are shown. The results of the Mann Whitney U tests are depicted in the figures. * indicates that $p < 0.05$.