

Supplementary file 1

Materials and Methods

Subjects and samples

The study included 45 children aged 6 to 17 years old. Thirty-three patients were followed for an allergic rhinitis to dust mite. Fourteen of them were also asthmatic, with controlled asthma (group “a”, n = 7) or uncontrolled asthma (group "A", n = 7). Nineteen had rhinitis without asthma (group “R”, n=19). The control group (group "C") comprised 12 children. This project received the approval of the Nice University Hospital Ethics Committee and all volunteers' parents gave written, informed consent.

The diagnosis of dust-mite-allergic rhinitis was based on clinical criteria (ARIA: rhinorrhea, nasal obstruction, nasal itching and sneezing) and positive prick-tests with *Dermatophagoïdes pteronyssinus* and *Dermatophagoïdes farinae*. Prick-tests used 9% phosphate codeine and 10 mg/mL histamine as positive controls and 50% glycerol solution as a negative control. The test was positive when the diameter of the papule was greater than 3 mm or greater than 50% of the largest papule observed with the 2 positive controls. All allergic children presented a perennial rhinitis with mild or moderate-severe form. Standard spirometry was performed on all patients. None were being treated with nasal local glucocorticosteroids for at least 1 month at time of recruitment.

Asthma was defined by the following clinical criteria (NAEPP/Expert Panel Report 3 (EPR3) 2007): recurring episodes of wheezing, dyspnea, cough, and obstructive syndrome reversible under beta2-mimetic. This last criterion was detected by spirometry.

Asthma control classification

Asthma severity and control were assessed following NAEPP/EPR3 criteria. We only considered two statuses: “well-controlled class” (controlled) and “not-well-controlled or poorly-controlled class” (uncontrolled). Long-term inhaled treatments (glucocorticosteroids +/- long-acting β 2-agonists) of asthmatic subjects were not stopped.

Subjects, aged 6-11 years of age, were defined with (1) controlled asthma if all the following points were present during the preceding three months: no more than two days/wk with trouble breathing, no more than one night-time awakening, no interference with normal activity, use of short-acting beta agonist ≤ 2 days/wk, FEV1 $>80\%$ predicted, FEV1/FVC $>80\%$ and no more than one exacerbation requiring oral systemic corticosteroids during the last year, (2) uncontrolled asthma if one or more of these features was absent.

Subjects, aged ≥ 12 years of age, were defined with (1) controlled asthma if all the following points were present during the preceding three months: no more than two days/wk with trouble breathing, no more than two night-time awakenings, no interference with normal activity, use of short-acting beta agonist ≤ 2 days/wk, FEV1 $>80\%$ predicted, ACT ≥ 20 , and no more than one exacerbation requiring oral systemic corticosteroids during the last year, (2) uncontrolled asthma if one or more of these features was absent.

Control subject selection was based on the absence of any personal or family history of atopy, rhinitis or asthma. We relied on the clinical definition of asthma and no spirometry measurements were performed in the healthy donor group.

Before inclusion, all subjects underwent a clinical examination. Subjects with symptoms of infectious rhinitis or bronchitis in the previous 15 days were excluded.

Nasal respiratory epithelium sampling was performed by brushing the inferior turbinates and the adjacent nasal wall using cytological brushes (Ventimed, France). Brushings were recovered in 5 ml culture medium Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA 92008, United States). For anatomo-pathological analysis, 200 μ l of the sample

were centrifuged on cytospin in order to perform cell counting and identification according to morphological criteria, after staining by the Papanicolaou stain method. The cell pellet was immediately lysed with 100 µl Absolutely RNA® Microprep Kit lysis buffer (Stratagene, La Jolla, CA 92037, United States), mixed with 0.7 µl β-mercaptoethanol 14.2 M and stored at -70°C.

Viral respiratory tract infection detection by Real-time quantitative PCR (qPCR)

The RealAccurate™ Respiratory Kit (PathoFinder, 6229 EV Maastricht, The Netherlands) was used according to the manufacturer's instructions, for each subject, to detect the possibility of viral respiratory tract infection. The kit contained optimized sets of primers and probes for the detection of 13 respiratory viruses (influenza types A and B, respiratory syncytial virus (RSV) types A and B, parainfluenza (PIV) types 1,2,3,4, coronavirus types 229E and OC43, rhinovirus, human metapneumovirus, adenovirus). All probes were 5'-labeled with 6-carboxyfluorescein (FAM) and 3'-labeled with tetramethylrhodamine (TAMRA), used as quencher. The amplicons were detected by measuring the FAM fluorescence. The RT-PCR reaction was performed with 10 ng total subject RNA isolated as described above. A positive RNA control, an internal control and a negative control (dilution buffer) were included in each test performed. Using the LightCycler 480 Real-Time PCR System (Roche, Indianapolis, IN 46250, United States), the following real-time cycler conditions were used: 30 min at 50°C for reverse transcription, 15 min at 97°C for Taq DNA polymerase activation and inactivation of the reverse transcriptase, then 42 cycles ((a) denaturation at 94°C for 15 s, (b) annealing/extension at 55°C for 60 s). A strong positive sample was characterized by a Ct (cycle threshold) value of < 30 (between 25 - 30), a positive sample with a value of 30 < Ct < 35, whereas a value of 35 < Ct < 40 indicated a sample showing low levels of expression.

Seven children (1 asthmatic, 5 rhinitis without asthma, 1 control) were positive for respiratory viruses. Five were positive for rhinovirus and 2 for parainfluenza virus type 1 (PIV1). These children were not included in subsequent analyses. Characteristics of the patients and samples (sex, age, ARIA score, asthma control, treatment and respiratory parameters, percentage of epithelial cells) are summarized in Table E1. These values do not significantly differ among experimental groups except for respiratory parameters of patients with uncontrolled asthma compared with the other groups (Table 1).

Isolation and culture of primary human nasal epithelial cells (HNECs)

Inferior turbinates were collected after surgical turbinectomy. Cystic fibrosis, asthmatic or allergic patients were excluded from the study. All procedures were approved by the Nice University Hospital Ethics Committee. The primary cultures of human epithelial cells derived from nasal mucosa of inferior turbinates were performed as described elsewhere (1). Briefly, after excision, the tissue was immediately immersed in HBSS (Ca²⁺/Mg²⁺-free; Invitrogen)-HEPES (25 mM, Gibco product, Invitrogen) culture medium containing 100 U/ml penicillin (Gibco, Invitrogen), 100 mg/ml streptomycin (Gibco, Invitrogen), 100 mg/ml gentamicin sulfate (Gibco, Invitrogen), and 2.5 mg/ml amphotericin B (Gibco, Invitrogen). Tissue was then digested with 0.1% Pronase (Sigma-Aldrich, St. Louis, MO 63103, USA) at 4°C overnight. Cells were gently removed from the digestion medium and HNECs were detached from the stroma by gentle agitation with a medium containing 10% fetal calf serum (FCS). The cell suspension was centrifuged (150 g, 10 min, 4°C) and the pellet was re-suspended in 10% FCS-HBSS-HEPES medium. After a second centrifugation, the second cell pellet was then suspended in the 10% FCS-Dulbecco's modified Eagle's (DMEM) medium (Invitrogen). Cells were gently dissociated mechanically by several passages through a 0.8 mm diameter needle using a 10 ml syringe. The cells were counted and then plated at a density of 3.10^4

cells/cm² on type IV collagen (Sigma-Aldrich)-coated Transwell permeable supports (6.5 or 12 mm diameter; 0.4 mm pore size; Sigma-Aldrich). They were incubated overnight in a humidified atmosphere of 5% CO₂ at 37°C. The next day, the culture medium was replaced by a proliferating Bronchial Epithelial Growth Medium (BEGM), reconstituted from Bronchial Epithelial Basal Medium (BEBM, Lonza, Basel, Switzerland), and supplemented with insulin, apo-transferrin, epidermal growth factor, epinephrine, hydroxycortisone, 3,30,5-triiodothyronine, endothelial cell growth supplement, retinoic acid (low concentration ~10 nM), amphotericin B (2.5 mg/ml), streptomycin (100 mg/ml), penicillin (100 U/ml), gentamicin sulfate (50 mg/ml) and L-glutamine (2 mM), according to the manufacturer's recommendation. Confluent cell monolayers were obtained after 7 days. Apical medium was then removed so that cells were cultured at an air-liquid interface (ALI). Basolateral medium was replaced by a differentiating medium, corresponding to an equal mix of BEBM and DMEM high glucose (Invitrogen). The differentiating medium was supplemented with the same growth factors as above except for retinoic acid which was added at a final concentration of 300 nM, in order to induce differentiation. Differentiation was assessed during 21 days of culture at an air-liquid interface (ALI). This protocol allows the recapitulation of the different steps of the regeneration, including proliferation (ALI D0), polarization (ALI D7) and differentiation (early=ALI D14, full=ALI D21). Cells were cultured until ALI D 25-28 before treatment with cytokines. All cultures used in this study had a transepithelial resistance greater than 500 Ω/cm². Each experiment was replicated using samples from three different donors. Tested agents: IL-4 (BD Pharmingen, San Diego, CA 92121, United States), IL-13 (PeproTech, Rocky Hill, NJ 08553, United States), IFN-αA/D (Sigma-Aldrich), IFN-β1a (Sigma-Aldrich), were added on the basolateral side at each time and concentration indicated in the text.

Microarray analysis

RNA extraction

Total RNA was isolated from nasal brushings using the Absolutely RNA® Microprep Kit (including the optional RNase-free DNase I digestion of contaminating DNA), and from primary HNECs using Trizol (Invitrogen), RNEasy kit (Qiagen, France) and RQ1 RNase-free DNase (Promega), Madison, WI 53711, United States), according to the manufacturers' instructions. The amount of RNA was estimated by spectrophotometry with a Nanodrop ND-1000 UV spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA quality was verified by electrophoresis on a Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA 95051, USA) with RNA Pico LabChip® (Agilent Technologies, Santa Clara, CA 95051, USA).

GeneChip Human Gene 1.0 ST Array

cDNA was generated from 300 ng total RNA using the GeneChip WT cDNA Synthesis and Amplification Kit (Affymetrix, Santa Clara, CA 95051, USA), according to the manufacturer's instructions. cDNA was fragmented and end-labeled using the GeneChip WT Terminal Labeling Kit (Affymetrix). Labeled DNA targets were hybridized to the Affymetrix GeneChip Human Gene 1.0 ST Array at 45°C for 17 h, according to the manufacturer's recommendation. At the end of the hybridization, microarrays were washed and stained on a GeneChip Fluidics Station 450 and scanned on a GeneChip Scanner 3000 7G (Affymetrix). Gene-expression levels were summarized using the Gene Level-RMA sketch method available in the Expression Console software (Affymetrix). Normalized data were analyzed using R with packages from Bioconductor (Biobase, limma, randomForest, bioDist).

Data storage

Microarray data are archived in GEO under reference GSE19190 and GSE22147. Experimental design, Tiff images and files resulting from the quantification stage were

initially stored in the MEDIANTE database developed by the laboratory and are available at: <http://www.microarray.fr:8080/merge/index> (2, 3).

Bioanalysis programs and statistical analyses

Most calculations were performed using limma and Random Forest, two packages of the R statistical program (4). The sizepower package was used to evaluate *a priori* the minimal size of our groups. With a \log_2 fold change superior to 1 and a false discovery rate of 5%, groups of 6-7 patients appeared sufficient to reach a power of 90%. Differentially-expressed genes in each comparison group were identified using linear modeling associated with Benjamini-Hochberg correction of p-value for multiple tests. For each gene, we define an A-value, which is the average \log_2 expression level for each gene across all microarrays, and M-values, which are the \log_2 fold-change for the comparisons that are considered. Prediction analysis was performed using the Random Forest (RF) approach developed by Breiman (5), according to the specifications made by Diaz-Uriarte (5, 6). This classification algorithm has comparable performance to other robust classification methods such as SVM in multi-class problems with group sizes similar to the one in the present study. Moreover, the gene selection procedure yields very small sets of genes while preserving predictive accuracy (6). Independent cross-validations were performed in order to control the robustness of the classifiers. In order to limit over-fitting, a partition of the datasets in two independent sets (i.e. training and test) was carried out. Predictions were based on the data obtained for the test groups only, and did not take into account the results obtained with the training groups. A 10-fold partition was performed in the Allergic versus Healthy comparison, including 38 patients. The training set varied from 34 to 35 subjects and the test set from 3 to 4 subjects. A 5-fold cross partition was performed in the uncontrolled versus controlled asthmatics comparison, including 13 patients. The training set varied from 10 to 11 subjects and the test set from 2 to 3 subjects in this latter case. The number of feature sets was reduced in the training group with limma before running

the Random Forest in order to perform the random forest on the most relevant probes. We performed 20 independent cross-validations. Every cross-validation was associated with a set of genes, which corresponded to the most influential variables contributing to determine the class of the sample. A gene set was defined as the group of genes that belonged to all twenty cross-validations. Prediction accuracy was based on the averaged results obtained at the end of the 20 cross validations. Random Forest classified each patient into one of the following categories: false positive (FP), true positive (TP), false negative (FN) and true negative (TN). The prediction accuracy (ACC) for each cross validation is given by:

$$ACC = \frac{TP + TN}{TP + TN + FP + FN} \times 100\%$$

We presented in the text the prediction accuracy based on the averaged results obtained at the end of 20 independent cross validations for the patients belonging to the “test” groups.

So the numerator was replaced by: $\sum(\%TP + \%TN)$ for each patient among the 20 cross validations.

Ontologies attached to each gene were then used to classify relevant genes according to main biological themes. We used for this the Functional Annotation Clustering report available from DAVID on <http://david.abcc.ncifcrf.gov/home.jsp> (7). The underlying grouping algorithm is based on the hypothesis that similar annotations should have similar gene members. It uses Kappa statistics to measure the relationships. Hierarchical clusterings were performed using the TIGR MultiExperiment Viewer (MeV) program, version 4.3 available at <http://www.tm4.org/mev.html> (Manhattan distance metric and average linkage) (8) or using R statistical environment.

Biological networks were built using Ingenuity Pathway Analysis software (<http://www.ingenuity.com/>). We presented the networks with the highest “network score” in the comparison. The network score was calculated with a right-tailed Fisher’s exact test. Gene Set Enrichment Analysis (GSEA) was used to determine whether an *a-priori* defined set of

genes can characterize differences between two biological states (e.g. phenotypes) <http://www.broad.mit.edu/gsea/index.jsp> (9, 10). Four key values were generated at the end of the gene set enrichment analysis: (1) the enrichment score (ES) reflected the extent of the over-representation of a gene set at the top or the bottom of a ranked list of genes; (2) the normalized enrichment score (NES) took into account differences in gene set size and in correlations between gene sets and the expression data set; (3) the false discovery rate (FDR) was the estimated probability that a gene set with a given NES represented a false-positive finding; (4) the nominal p-value estimated the statistical significance of the enrichment score for a single gene set.

ENCODE functional genomics data were used to determine potential binding sites for interferon regulatory factors. Genomic regions and Encyclopedia of DNA Elements (ENCODE) functional genomics data were retrieved and displayed using the UCSC Genome Browser on Human March 2006 (NCBI36/hg18) Assembly (<http://genome.ucsc.edu/>) (11, 12). H3K4Me1 histone marks (monomethylated histone H3 at lysine 4) are associated with enhancer and promoter regions. ENCODE information about histones was derived from 8 cell lines. Histone H3K4Me3 (trimethylated histone H3 at lysine 4) binding was associated with promoter regions. ENCODE Histone Mark H3K4Me3 was derived from 9 cell lines. Hypersensitivity to DNase I treatment is associated with accessible chromatin regions. DNase I hypersensitivity clusters are represented as gray boxes where the darker values represent the highest sensitivity. ENCODE Transcription Factor ChIP-seq (chromatin immunoprecipitation followed by deep-sequencing) data include IRF4 binding site experimental analysis on the GM12878 cell line. IRF4 binding sites are represented as gray boxes where the darker values represent the highest binding signal. Predicted IRF sites were retrieved from HMR conserved transcription factor binding sites. Mammalian conservation within 28 species is shown in grayscale where darker values indicate the highest conservation

according to phastCons score (PHYlogenetic Analysis with Space/Time Conservation), a phylo-HMM-based method for detecting conserved regions in multiple sequence alignments.

Real-time quantitative PCR (qPCR)

First-strand cDNA was synthesized from 500 ng total RNA using 1 μ L ImProm-II™ Reverse Transcriptase (Promega) in a final volume of 20 μ l with 500 ng oligo-dT (Proligo, Sigma-Aldrich). Quantitative PCR was carried out in 96-well plates using the LightCycler 480 Real-Time PCR System (Roche Applied Science, Indianapolis, IN46250, USA) with the qPCR Master Mix Plus for SYBRs Green I (Roche). All reactions were performed in a total volume of 20 μ l and contained 5 ng reverse-transcribed RNA (based on the initial RNA concentration) and 500 nM of each primer set. The primer sets were designed using the Primer Express software from Applied Biosystems (Carlsbad, California 92008, United States) for the following human transcripts: CST1 (NM_001898), POSTN (NM_006475), NTS (NM_006183), CD44 (NM_000610), GSN (NM_000177), ALOX15 (NM_001140), GSDML (NM_001042471), MX2 (NM_002463), IFIT2 (NM_001547), DUOX2 (NM_014080), CXCL10 (NM_001565), GSDML (NM_001042471), ST8SIA4 (NM_005668), MX1 (NM_002462) and housekeeping genes GAPDH (NM_002046), ACTB (NM_001101), RPL13A (NM_012423), TTC1 (NM_003314) (Supplementary Table 1). Primer sets were usually designed to span an intron in order to avoid amplification from potential traces of genomic DNA in the total RNA preparations, except for NTS, MX2, IFIT2, DUOX2 and GAPDH. Additional negative controls corresponded to the same samples reverse transcribed in the absence of reverse transcriptase. The efficiency and specificity of each primer set were validated using serial dilutions. Thermal cycling was performed at 95°C for 5 min, followed by 40 cycles comprising each a denaturation step at 95°C for 10 s and an annealing/extension step at 60°C for 10 s, then, finally, 72°C for 10 s. Amplification of the

appropriate product was verified by analyzing the dissociation curve that was obtained after PCR with the following steps: 5 s at 95°C, 1 min at 65°C, and then a slow ramp from 65°C to 97°C. The purity of the amplified product was determined as a single peak of dissociation curve. The SYBR Green dye was measured at 530 nm during the extension phase. The threshold cycle (Ct) value was determined as the cycle number at which the fluorescence generated within a reaction crosses a given threshold. The relative mRNA amount in each sample was calculated from the Ct compared with the Ct of the two most stable housekeeping genes. These latter were chosen by the software geNorm from a set of four tested candidate reference genes (GAPDH, ACTB, RPL13A, TTC1) (13). A gene expression normalization factor was calculated for each sample based on the geometric mean of the selected reference genes. Each sample was measured in duplicate and the mean value was calculated. Results were calculated with the delta-Ct method, relating the Ct value of our genes (either target or reference) to a control/calibrator (i.e. the sample with the highest expression, characterized by the lowest Ct value):

$$\frac{Efficiency^{(lowest\ Ct\ value - gene\ Ct\ value)}}{Normalization\ factor}$$

For detecting IFN- α , IFN- β , IFN- λ 1 and IFN- λ 2/3 transcripts, we used Taqman® probes, primers (Integrated DNA Technologies, Leuven, Belgium) previously described elsewhere (14). Briefly, quantitative PCR was carried out in 96-well plates using the LightCycler 480 Real-Time PCR System (Roche Applied Science) with the Quantitect Probe One-step RT-PCR kit (Qiagen). All reactions were performed in a total volume of 20 μ l and contained 100 ng total RNA, 200 nM Taqman® probe and 1 μ M of each primer set. The primer and probe sets corresponded to those used by Khaitov and col.: IFN- α 1, IFN- α 2, IFN- β , IFN- λ 1 and IFN- λ 2/3 (14). We used as a housekeeping gene, GAPDH (NM_002046), designed using Integrated DNA Technologies online-available software. Real-time cycler conditions consisted of an initial reverse transcription step of 30

min at 50°C, then a two-step cycling was performed at 95°C for 15 min, followed by 40 cycles comprising each a denaturation step at 94°C for 15 s, and an annealing/extension step at 60°C for 60 s. Results were normalized with respect to GAPDH:

$$2^{-(\text{GAPDH Ct value} - \text{gene Ct value})}$$

For MUC5AC and FOXJ1 transcripts, real-time PCR was performed using Taqman gene expression Assay (Applied Biosystems) following manufacturer's instructions.

Immunohistochemistry

Four-micrometer sections from formalin-fixed and paraffin-embedded normal human bronchial, nasal and sinus mucosa were mounted on glass slides (SuperFrost Plus; Menzel, Braunschweig, Germany), then air-dried overnight at 57°C. After rehydration, antigens were retrieved with the CC1 protocol for the anti-GSDML antibody, and with the CC2 protocol for anti-Duox1/ThOx1, as developed by Ventana (Ventana Medical Systems, Roche Group, Inc., Tucson, AR). Intrinsic peroxidase activity was blocked by 3% hydrogen peroxide for 20 min. Non-specific antibody binding was blocked with normal goat serum (5%; Sigma). The anti-GSDML rabbit polyclonal antibody (Sigma-Aldrich, St. Louis, MO) was used at a 1:200 dilution. Anti-Duox1/Duox2 rabbit polyclonal antibody (kindly provided by Professor Françoise Miot, Université Libre de Bruxelles, Belgium) was used at a 1:600 dilution (15). Staining procedures were performed on a Benchmark immunohistochemistry staining system (Benchmark XT, Ventana Medical Systems). The primary antibodies were incubated for 32 min. Slides were washed in TBST (50 mM Tris pH 7.6, 300 mM NaCl, 0.1% Tween 20), before incubation with labeled polymer-HRP anti-rabbit or anti-mouse secondary antibody for 30 min at room temperature. For visualization, the EnVision FLEX kit (Dako) was used according to the manufacturer's instructions.

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