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Distinct epithelial gene expression phenotypes in childhood respiratory allergy

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ABSTRACT: Epithelial cell contribution to the natural history of childhood allergic respiratory disease remains poorly understood. Our aims were to identify epithelial pathways that are dysregulated in different phenotypes of respiratory allergy.

We established gene expression signatures of nasal brushings from children with dust mite-allergic rhinitis, associated or not associated with controlled or uncontrolled asthma. Supervised learning and unsupervised clustering were used to predict the different subgroups of patients and define altered signalling pathways. These profiles were compared with those of primary cultures of human nasal epithelial cells stimulated with either interleukin (IL)-4, IL-13, interferon (IFN)-α, IFN-β or IFN-γ, or during *in vitro* differentiation.

A supervised method discriminated children with allergic rhinitis from healthy controls (prediction accuracy 91%), based on 61 transcripts, including 21 T-helper cell (Th) type 2-responsive genes. This method was then applied to predict children with controlled or uncontrolled asthma (prediction accuracy 75%), based on 41 transcripts: nine of them, which were down-regulated in uncontrolled asthma, are directly linked to IFN. This group also included *GSDML*, which is genetically associated with asthma.

Our data revealed a Th2-driven epithelial phenotype common to all children with dust mite allergic rhinitis. It highlights the influence of epithelially expressed molecules on the control of asthma, in association with atopy and impaired viral response.

KEYWORDS: Allergic rhinitis, asthma, epithelium, microarrays, paediatric asthma, phenotypes of asthma

Asthma is a heterogeneous and complex disease that frequently co-occurs with allergic rhinitis. Classical pathogenic models characterise asthma and allergic rhinitis as inflammatory diseases associated with an increased T-helper cell (Th) type 2 response. Despite chronic inflammatory processes, several additional defects intrinsic to the airway epithelium may contribute to the pathogenesis. The airway remodelling observed in asthma can appear several years before any clear clinical diagnosis, and even affect preschool asthmatic children [1]. Asthma patients appear more susceptible to infections by respiratory viruses [2]. Epithelial cells derived from asthma patients exhibit a reduced capacity to secrete fibronectin, a well-known contributor to repair in damaged airway epithelium, together with defective antioxidant pathways and tight junctions [3–5].

Some studies have attempted to associate specific gene expression profiles with asthma or allergic rhinitis, mostly in adults [3, 6–10]. Information has also been gathered by genome-wide association studies. Single-nucleotide polymorphisms (SNPs) in the *GSDML* locus (near *ORMDL3*), a protein detected in epithelial tissues, are strongly associated with childhood-onset disease [11]. Based on these findings, our rationale was that defects in the airway epithelium could correspond to primary defects or early secondary events, which could favour chronic inflammation and lead to asthma and/or allergic rhinitis. Thus, we determined the transcriptional signatures of nasal brushings collected from paediatric patients with different clinical phenotypes of dust mite respiratory allergy. Data analysis revealed altered behaviour of several signalling pathways that were further analysed in

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an *in vitro* model of differentiated human nasal epithelial cells (HNECs).

MATERIALS AND METHODS

Subjects and samples

Patients were aged 6–17 yrs (n=45). 33 were being followed up for a dust mite-allergic rhinitis. 14 were also asthmatic, with either controlled asthma (group ''a''; n=7) or uncontrolled asthma (group ''A''; n=7). 19 had rhinitis without asthma (group ''R''). The control group (group ''C'') comprised 12 healthy children. Clinical diagnoses and asthma control staging corresponded to the Allergic Rhinitis and its Impact on Asthma and the paediatric US National Asthma Education and Prevention Program Expert Report 3 criteria (see online supplementary file 1 for details). The project received the approval of the Nice University Hospital Ethics Committee (Nice, France) and all of the volunteers' parents gave written, informed consent.

Nasal respiratory epithelium sampling was performed by brushing the inferior turbinates.

Viral respiratory tract infection detection by real-time quantitative PCR (qPCR) was performed using the RealAccurate™ Respiratory Kit according to the manufacturer's instructions (PathoFinder, Maastricht, the Netherlands). Seven children (one asthmatic, five rhinitics without asthma and one control) positive for respiratory viruses were not included in further analyses. The characteristics of the patients and samples are summarised in table 1 and online supplementary table E1.

Isolation and culture of primary HNECs

Primary cultures of HNECs derived from the nasal mucosa of inferior turbinates were performed as described previously [12]. Each experiment was performed using three different donors.

TABLE 1 Subject characteristics

	Group			
	a	A	R	C
Subjects	7	6	14	11
Dust mite-allergic rhinitis	Yes	Yes	Yes	No
Asthma	Controlled	Uncontrolled	No	No
Age yrs	11.5±3.2	9.1±0.6	11.3±2.8	11.5±3.1
Males/females	2/5	4/2	7/7	7/4
Epithelial cells %	85.8±10.4	86.5±6.5	86.9±8.7	86.9±8.5
PMNs %	6.9±6.1	5±3.2	7.2±7.8	6.8±6.1
Lymphocytes %	7.3±10.7	8.4±6	5.5±7.3	6.3±5.6
FEV_1	97.6±13.2	78.2±7.7#	99±9.4	NA
FEV_1/FVC	89.3±5.7	76.5±3.2#	90.4±5.2	NA
$FEF_{25–75\%}$	103.6±17.9	56.2±9.6#	107.3±18.2	NA

Data are presented as n or mean± SD, unless otherwise stated. Statistical comparisons were performed in allergic (groups a, A and R) *versus* healthy control subjects (group C) and in uncontrolled (group A) *versus* controlled (group a) asthmatics using unpaired t-tests and the Chi-squared test. PMN: polymorphonuclear cell; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; $FEF_{25–75\%}$: forced expiratory flow at 25–75% of FVC; NA: not available. #: $p<0.005$ for group A *versus* group a by unpaired t-test.

Before treatment with cytokines, HNECs were cultured for 4 weeks at an air–liquid interface to obtain a well-differentiated epithelium [12]. Tested agents (interleukin (IL)-4 (BD Pharmingen, San Diego, CA, USA) and IL-13 (PeproTech, Rocky Hill, NJ, USA), 10 $ng \cdot mL^{-1}$ for 24 h; interferon (IFN)-αA/D and IFN-β1a (Sigma-Aldrich, Saint-Quentin Fallavier, France), 1,000 $IU \cdot mL^{-1}$ for 6 h; and IFN-γ (BD Pharmigen), 100 $ng \cdot mL^{-1}$ for 6 h) were added on the basolateral side.

Gene expression analyses

Microarray analyses were performed on the GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. Microarray data are archived in the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) under references GSE19190 and GSE22147, and on the MEDIANTE database developed in our laboratory [13, 14]. The qPCR procedure is detailed in online supplementary file 1, with the primers and probes listed in online supplementary table E2.

Biostatistics

Most statistical calculations and prediction analyses were performed using limma and Random Forest, two packages of the R statistical environment [15]. We used the Functional Annotation Clustering report available from the Database for Annotation, Visualization and Integrated Discovery (DAVID) [16]. Hierarchical clustering was performed with the MultiExperiment Viewer program, version 4.3 (TM4, Boston, MA, USA), using a Manhattan distance metric and average linkage [17]. Biological networks were built using Ingenuity Pathway Analysis™ software (Ingenuity Systems Inc., Redwood City, CA, USA). Gene Set Enrichment Analysis (GSEA) was used to determine whether a set of genes defined *a priori* can characterise differences between two biological states [18, 19]. ENCODE (Encyclopedia of DNA Elements) functional genomics data were used to determine potential binding sites for IFN regulatory factors.

Immunohistochemistry

Immunohistochemistry methods are described in online supplementary file 1.

ELISA assay

The level of MUC5AC released by HNECs in response to IL-13 stimulation was measured using ELISA according to the manufacturer's instructions (EIAab and USCNLIFE; Wuhan EIAab Science Co. Ltd, Wuhan, China).

RESULTS

Genes differentially expressed in the nasal epithelial cells of children with dust mite allergy

The transcriptional signature of dust mite-allergic rhinitis children (n=27) was compared with that of the healthy children (n=11). We identified 169 differentially expressed transcripts (adjusted $p<0.05$) (data on request). Among them, 24 transcripts were modulated with an absolute value of $\log_2$(ratio) >1 (table 2). Functional annotation by DAVID is provided in online supplementary table E3. Variation of some genes (*CST1*, *POSTN*, *NTS*, *CD44*, *GSN* and *ALOX15*) was confirmed by qPCR (online supplementary fig. E1).

Supervised clustering was used to predict a status for each of the 38 patients (allergic or healthy). 35 out of the 38 patients were correctly predicted (prediction accuracy of 91%) (fig. 1a).

TABLE 2 Genes differentially expressed in nasal epithelial brushings from dust mite-allergic rhinitis patients *versus* healthy subjects (microarray analysis)

Probe set identifier	mRNA accession number	UniGene identifier	Gene	Gene product	Cytoband	log_2(signal)	log_2(allergic/healthy)	p-value
8065412	**NM_001898**	**Hs.123114**	***CST1***	**Cystatin SN**	**20p11.21**	**9.65**	**7.15**	**4.95×10^{-12}**
7971077	**NM_006475**	**Hs.136348/ Hs.664318**	***POSTN***	**Periostin, osteoblast-specific factor**	**13q13.3**	**7.12**	**3.56**	**2.66×10^{-3}**
8083260	NM_001870	Hs.646	*CPA3*	Carboxypeptidase A3 (mast cell)	3q21–q25	6.17	2.51	5.20×10^{-5}
7921690	NM_017625	Hs.50813	*ITLN1*	Intelectin 1 (galactofuranose binding)	1q22–q23.5	5.83	2.45	3.87×10^{-2}
8065416	NM_001322	Hs.669305	*CST2*	Cystatin SA	20p11.21	7.97	1.87	1.06×10^{-3}
8084657	**NM_014375**	**Hs.81073**	***FETUB***	**Fetuin B**	**3q27**	**5.78**	**1.77**	**3.63×10^{-3}**
8021635	**NM_002575**	**Hs.594481**	***SERPINB2***	**Serpin peptidase inhibitor, clade B (ovalbumin), member 2**	**18q21.3**	**9.20**	**1.64**	**6.97×10^{-3}**
8056222	**NM_001935**	**Hs.368912**	***DPP4***	**Dipeptidyl-peptidase 4 (CD26)**	**2q24.3**	**6.44**	**1.56**	**6.18×10^{-3}**
7921900	**NM_053282**	**Hs.350581**	***SH2D1B***	**SH2 domain-containing 1B**	**1q21**	**5.91**	**1.55**	**3.05×10^{-7}**
8036755	NM_001828	Hs.889	*CLC*	Charcot-Leyden crystal protein	19q13.1	4.57	1.50	2.73×10^{-2}
7984001	NM_004751	Hs.194710	*GCNT3*	Glucosaminyl (*N*-acetyl) transferase 3, mucin type	15q21.3	8.73	1.39	8.15×10^{-5}
8063761	**NM_177980**	**Hs.54973**	***CDH26***	**Cadherin-like 26**	**20q13.2–q13.33**	**8.67**	**1.38**	**6.84×10^{-3}**
8089568	NM_138806	Hs.309158	*CD200R1*	CD200 receptor 1	3q13.2	8.90	1.35	3.90×10^{-5}
8112668	**NM_016591**	**Hs.272404**	***GCNT4***	**Glucosaminyl (*N*-acetyl) transferase 4, core 2 (β-1,6-*N*-acetylglucosaminyltransferase)**	**5q12**	**5.85**	**1.29**	**4.05×10^{-2}**
8070567	NM_003226	Hs.82961	*TFF3*	Trefoil factor 3 (intestinal)	21q22.3	9.86	1.26	2.11×10^{-2}
7942135	**NM_018043**	**Hs.503074**	***TMEM16A***	**Transmembrane protein 16A**	**11q13.3**	**7.41**	**1.23**	**6.48×10^{-3}**
8154233	**NM_014143**	**Hs.521989**	***CD274***	**CD274 molecule**	**9p24**	**6.75**	**1.23**	**3.84×10^{-5}**
7957458	NM_006183	Hs.80962	*NTS*	Neurotensin	12q21	9.14	1.16	4.79×10^{-2}
8023688	**NM_002974**	**Hs.123035**	***SERPINB4***	**Serpin peptidase inhibitor, clade B (ovalbumin), member 4**	**18q21.3**	**8.67**	**1.15**	**3.40×10^{-3}**
8147132	NM_000067	Hs.155097	*CA2*	Carbonic anhydrase II	8q22	6.44	1.14	3.87×10^{-2}
7909946	GENSCA-N00000026059	NA	NA	NA	NA	6.01	1.14	1.05×10^{-2}
8156134	NM_006180	Hs.494312// Hs.653428	*NTRK2*	Neurotrophic tyrosine kinase receptor, type 2	9q22.1	5.63	1.10	5.93×10^{-3}
8102050	NM_178833	Hs.546482	*NHEDC2*	Na^+/H^+ exchanger domain-containing 2	4q24	6.02	1.02	6.84×10^{-3}
8089851	NM_000187	Hs.368254	*HGD*	Homogentisate 1,2-dioxygenase (homogentisate oxidase)	3q13.33	5.71	-1.22	2.50×10^{-2}

Genes were ranked according to decreasing log_2(allergic/healthy). Only genes with a log_2(ratio) >1 and a significant p-value ($p<0.05$) after a Benjamini–Hochberg correction for multiple tests were selected. Bold indicates genes induced *in vitro* with a value of log_2(ratio) >1 by both interleukin (IL)-4 and IL-13 in human nasal epithelial cells. A more detailed table is provided as online supplementary table E3. NA: not available.

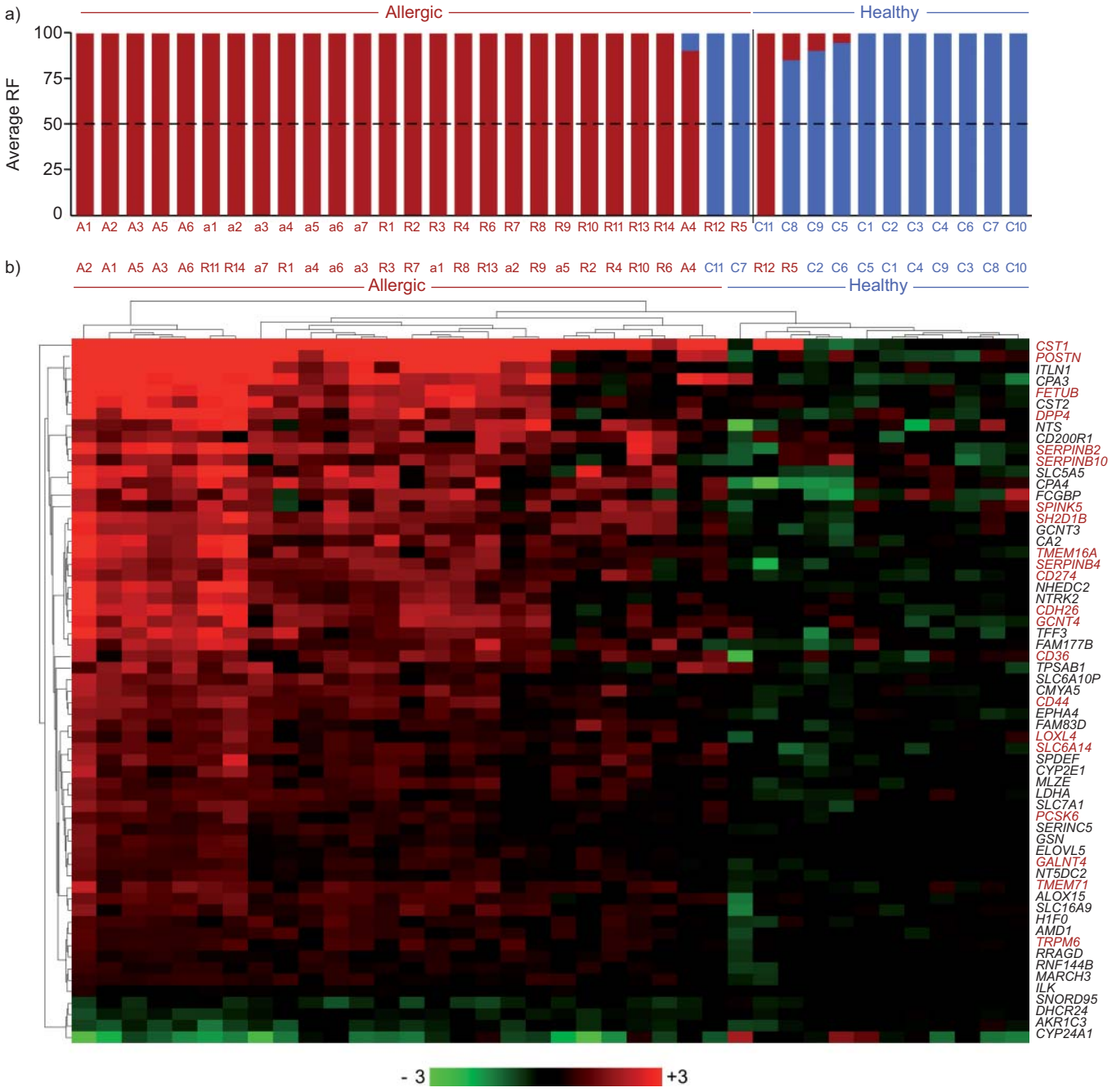

FIGURE 1. Discrimination by Random Forest (RF) of children with dust mite allergic rhinitis from healthy children and heat-map representation of the 61 most discriminant genes. a) Histogram showing, for each patient, the percentage of classification as a dust mite-allergic rhinitis (red) or healthy (blue) child. b) Nonsupervised, hierarchical clustering of the same patients using the set of 61 genes common to all Random Forest classifiers (see details in the Materials and methods section). Each square represents the expression level of a given gene in a given sample relative to the average expression level in controls. A red to green colour scale indicates gene expression levels above (red) or below (green) the average level of expression in healthy subjects for the same transcript. Clustering was performed using an average linkage method, using a Manhattan distance. The red colour on gene names (right) indicates genes induced *in vitro* by interleukin (IL)-4 and IL-13.

Specificity and sensitivity are summarised in a receiver operating characteristic (ROC) curve in online supplementary figure E2A, showing an area under the ROC curve of 0.88. The Random Forest approach that was used allowed the identification of the most influential genes for the prediction, leading to a set of 61 transcripts that was common to 20 independent cross-validations. Most genes were up-regulated in the allergic conditions (fig. 1b). 16 of them had previously been linked to asthma or allergic rhinitis by gene expression profiling (online supplementary table E4) [3, 6–10]. The signature was linked to allergic status but independent of asthma status. Indeed, supervised clustering was unable to discriminate the 13 asthmatic patients from the 14 allergic rhinitis patients without asthma. No differential expression was detected between these two groups of patients (data not shown).

Epithelial Th2 signature discriminates allergic children from healthy controls

We observed that several transcripts dysregulated in patients allergic to dust mites were also linked in the literature to IL-4 or IL-13 [20]. We mimicked this situation *in vitro* by stimulation of primary HNECs with IL-4 or IL-13. With this model, we confirmed that IL-13 stimulation increased the expression (online supplementary fig. E3A) and secretion of MUC5AC, a goblet cell marker (online supplementary fig. E3B). We also observed, in parallel, the decreased expression of FoxJ1, a ciliated cell marker (online supplementary fig. E3A). 46 airway epithelial transcripts were selected, based on their high response to Th2 cytokines and their detection in highly differentiated airway epithelial cells (online supplementary table E5). 21 transcripts of this ''Th2 signature'' were among the 61 transcripts predicting allergic or healthy children (fig. 1). The importance of Th2 regulation was assessed by a normalised enrichment score >1 (1.61, with nominal p-value and false discovery rate q-value of <0.001), as determined by GSEA [18, 19]. Interestingly, we noticed that the transcripts associated with the Th2 signature displayed a larger $\log_2$(ratio) in uncontrolled (group A) than in controlled (group a) asthma (fig. 1). This led us to investigate the existence of a specific expression profile associated with uncontrolled asthma.

Control of asthma affects gene expression signature of nasal epithelial cells

The next analysis compared the two groups of subjects with uncontrolled or controlled asthma. Table 1 describes the characteristics of these patients. The expression of 160 genes differed significantly between the two groups (online supplementary table E6). Functional annotation is reported in online supplementary table E7. The Random Forest approach correctly predicted the class for 12 out of 14 samples, with a 75.4% prediction accuracy (fig. 2a). Specificity and sensitivity are summarised in a ROC curve in online supplementary figure E2B, showing an area under the ROC curve of 0.833. It also identified a minimal set of the 41 most influential genes for the prediction (fig. 2b). Up-regulation of *POSTN*, *FETUB* or *DPP4* (also belonging to Th2 signature) (online supplementary table E5) in the uncontrolled group is coherent with the larger $\log_2$(ratio) observed for Th2 responding transcripts in figures 1 and 2. We also noticed a down-regulation of several IFN-stimulated genes (ISGs), such as *CXCL10*, *MX2*, *IFIT2*, *DUOX2*, *GBP2* and *GBP5*, among a larger cluster of down-regulated genes (fig. 2). This was illustrated by Ingenuity Pathways Analyses (online supplementary fig. E4). Our cluster of down-regulated genes also included *GSDML*, a gene located in a region of chromosome 17q21, which has recently been linked to juvenile asthma in several genome-wide association studies [11]. The differential expression of *CXCL10*, *MX2*, *IFIT2*, *DUOX2* and *GSDML* was confirmed by qPCR (online supplementary fig. E1). *GSDML* and *DUOX2* proteins were immunodetected by histochemistry in nasal and bronchial airway epithelia (fig. 3).

IFN response analysis in patients and cultured HNECs

IFN regulation was explored *in vitro* by stimulating primary HNECs with IFN-α, IFN-β or IFN-γ. We selected IFN-responsive transcripts (online supplementary table E8). The GSEA approach revealed significant normalised enrichment scores for all subtypes of IFNs in the uncontrolled/controlled asthma comparison (1.7 for IFN-α, 1.64 for IFN-β and 1.56 for IFN-γ). The capacity of IFNs to induce the expression of *MX1*, *ST8SIA4* and *GSDML* was confirmed by qPCR on primary cultures of HNECs. A peak of *GSDML* induction was observed after 12 h of IFN-α stimulation, while the peak of induction for *MX1* and *ST8SIA4* was reached at 6 h (fig. 4a). A survey of the genomic context for *MX2*, *IFIT2*, *CXCL10*, *GSDML*, *ST8SIA4* and *DUOX2* revealed the presence of several consensus binding sequences for IFN regulatory factors (full data on request) (online supplementary fig. E5). Altogether, these observations suggest a direct regulation of these different genes by IFN. Importantly, the transcript levels of IFN-β, IFN-λ1, IFN-λ2/3, IFN-α1 and IFN-α2 were decreased in uncontrolled asthmatics compared with either controlled asthmatics or healthy subjects (fig. 4b). Endogenous production of epithelial IFNs, rather than defective IFN signalling pathways, is therefore probably involved in these processes.

Airway epithelial regeneration and severity of allergic diseases

Impaired capacity of repair of the airway epithelium is a hallmark of allergic airway diseases [5]. We have recently established gene expression signatures during *in vitro* HNEC regeneration and differentiation [12]. The ''terminal differentiation'' signature was used to enrich the annotation derived from the expression profiles obtained with Th2 cytokines and IFN experiments. As expected, the classification of the patients was principally linked to the effects of Th2 cytokines and IFNs. However, several transcripts associated with the disease were also linked to the terminal differentiation signature (online supplementary table E9). A first group of genes, including *PLK4*, *CDC20B*, *GSDML* and *CYFIP2*, was clearly induced during differentiation while being down-regulated in patients with uncontrolled asthma. This group puts together important markers of multiciliogenesis (*PLK4* and *CDC20B*) [12] and molecules associated with asthma in genome-wide analyses (*GSDML* and *CYFIP2*). A second group, which includes *POSTN*, *SPINK5*, *CD44*, *TFF3*, *ITLN1*, *FOXA3* and *SPDEF*, was clearly associated with basal or mucous cells [21]. Most of them were decreased during *in vitro* differentiation while all were up-regulated in patients with uncontrolled asthma.

DISCUSSION

The airway epithelium is a complex physical barrier, playing an intrinsic role in innate and adaptive immunity, through the production of numerous cytokines and chemokines. Our work highlights the importance of IFN- and Th2-driven epithelial responses in the pathogenesis of allergic respiratory diseases. Our findings were based on biostatistical approaches, such as Random Forest, Ingenuity and GSEA, which take into account not only the number of patients, but also the size of gene clusters that can be regulated by these cytokines. Importantly, these approaches increase the statistical significance of our observations.

The choice to perform our investigations on upper, rather than on lower, airway epithelium was motivated by its noninvasive sampling, which is easily applicable in young children. It allows the recruitment of true controls exempt of pulmonary disease, a situation which is clearly not possible in studies using bronchoscopic sampling [1]. It also allows investigation of patients with severe or uncontrolled asthma phenotypes without stopping their long-term inhaled corticosteroid treatment for asthma, as nasal deposition can be considered as negligible with the devices used in this study [22]. Interestingly, we observed a good overlap

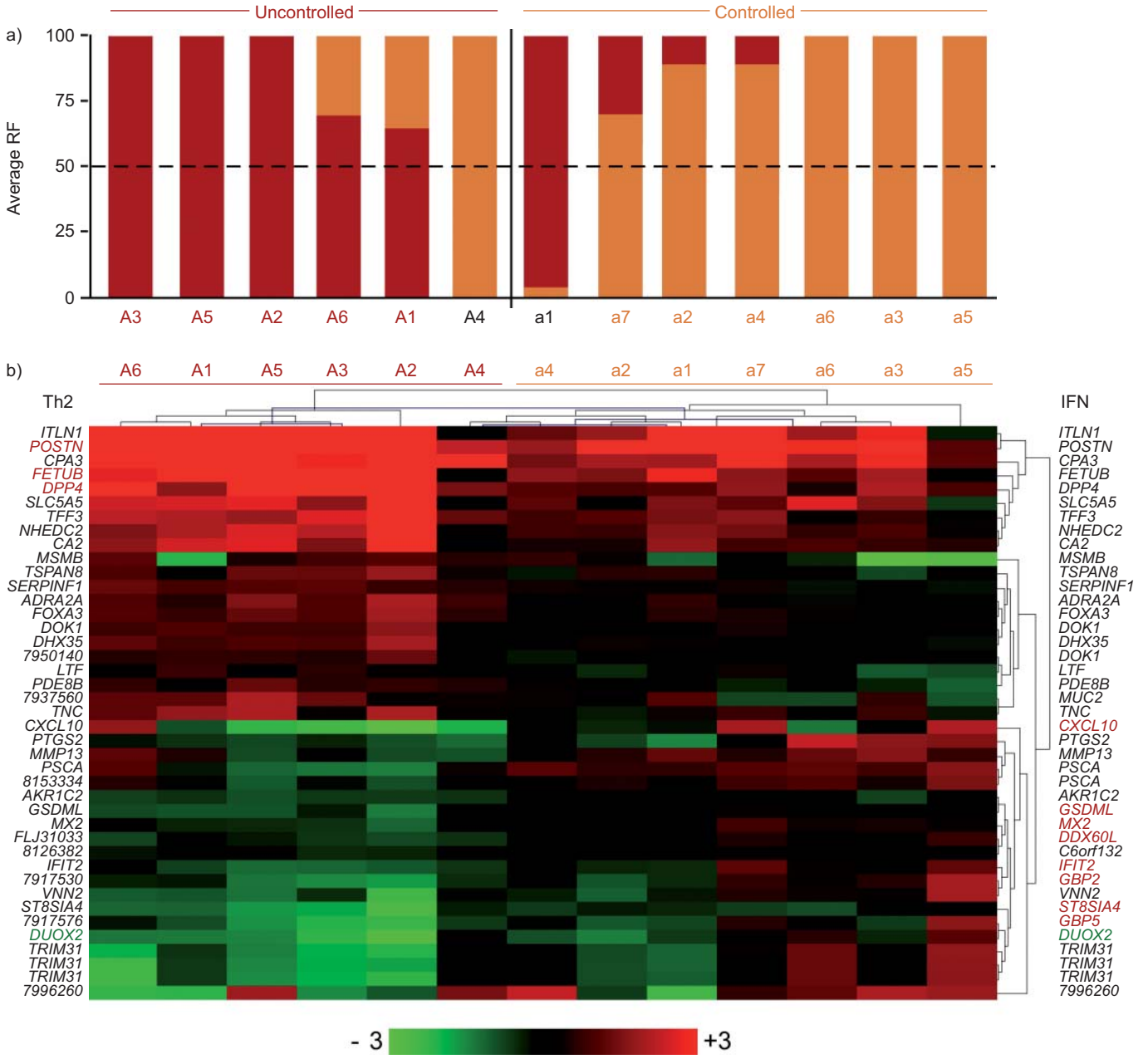

FIGURE 2. Discrimination by Random Forest (RF) of children with uncontrolled *versus* controlled asthma and heat-map representation of the 41 most discriminant genes. a) Histogram showing, for each patient, the percentage of classification as uncontrolled asthma (red) and controlled asthma (orange). b) Nonsupervised, hierarchical clustering of the same patients using the 41 probes common to all Random Forest classifiers (see Materials and methods section for details). Each square represents the expression level of a given gene in a given sample. A red to green colour scale indicates gene expression levels above (red) or below (green) the average level of the healthy controls for the same transcript. Clustering was performed using an average linkage method, using a Manhattan distance. Red-coloured gene names indicate transcripts induced by interleukin (IL)-4 and IL-13 (left) or by interferons (IFNs) (right) (corresponding to a $\log_2$(ratio) >1 in human nasal epithelial cells (HNECs)). Gene names coloured in green indicate transcripts down-regulated by IL-4 and IL-13 (left) ($\log_2$(ratio) >-1 in HNECs).

between our ''nasal epithelial'' gene lists and several gene expression profiles in bronchial epithelium, notwithstanding differences between nasal and bronchial epithelial cells [23, 24], such as the near absence of remodelling in the nasal epithelium [25]. In this context, the use of alternative techniques to harvest lower airway cells during general anaesthesia could be worth investigating [26]. Our inability to identify *ad hoc* biomarkers for discriminating asthmatic and rhinitis patients could be due to the choice of sampling material. If this is true, a comprehensive comparison of bronchial and nasal epithelial cells from the same patients would help explore this issue. This was unfortunately not planned in the present study. The relatively small size of the study (45 samples) could also be limiting. A third explanation could be that the major differences discriminating asthma from allergic rhinitis would, rather, depend on other cell types (dendritic or mesenchymal cells, *etc.*). From that perspective,

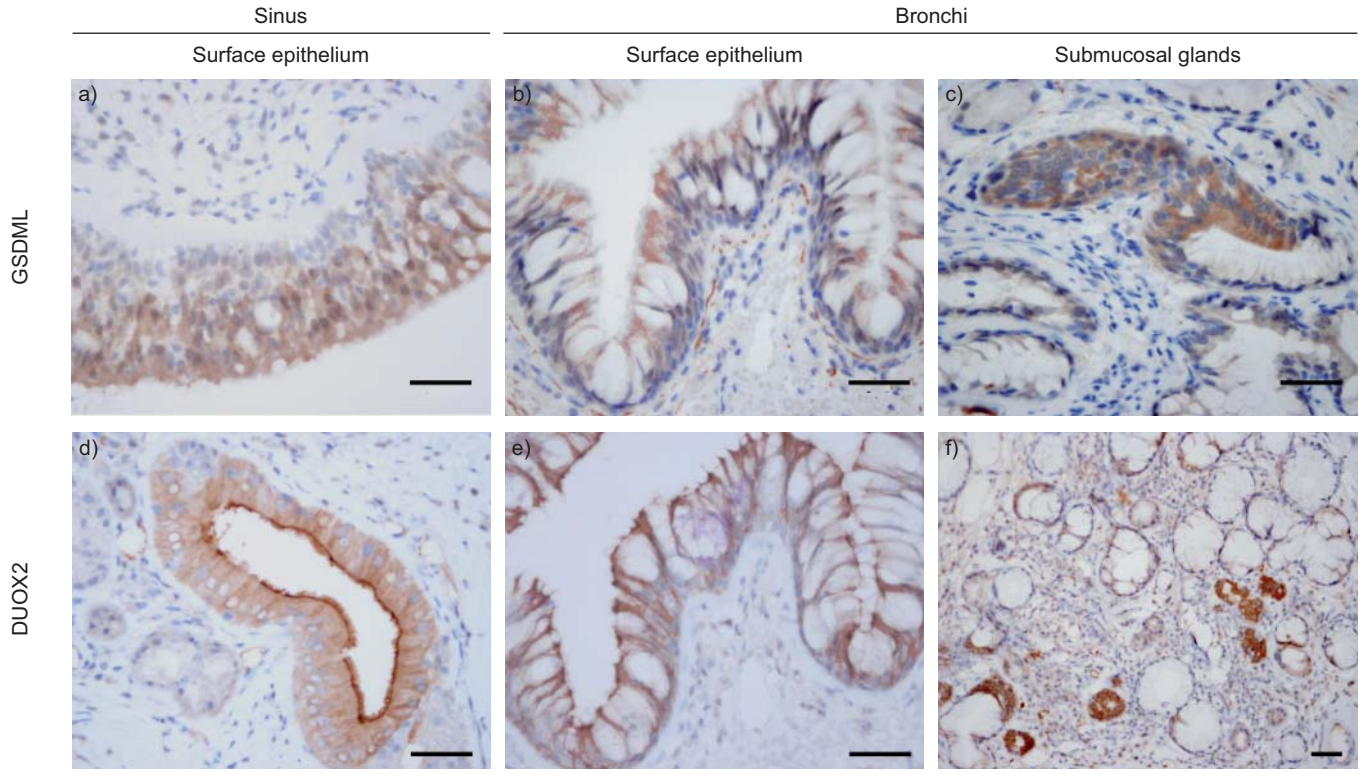

FIGURE 3. Immunohistochemical staining of sinus and bronchial biopsies. Representative immunostaining of a–c) GSDML and d–f) DUOX2 in surface epithelium and submucosal glands for healthy adults are shown. Positive immunostaining was observed for the two proteins in sinus and bronchial sections. Strong signals were also detected in submucosal glands for the different markers. Scale bars=100 μm.

progression from allergic rhinitis to asthma could not be detected in the epithelium *per se*.

Our study establishes epithelial activation by Th2 cytokines as a central trait of airway allergic respiratory disease. This is in line with the observation that IL-13 can recapitulate most of the characteristics of asthma in several experimental models of the disease [27]. Our clinical and *in vitro* results clearly demonstrate the major impact of Th2 cytokines on allergic subjects, reflecting the strength of the atopic status. Several Th2-induced genes, such as *POSTN*, *ITLN1*, *ALOX15*, *CD44* and *SERPINB4*, have previously been linked to asthma. Others could represent additional relevant biomarkers, due to their large and consistent variations in the allergic group. For example, *CST1*, which encodes a cysteine protease inhibitor, is already associated with pulmonary fibrosis in systemic sclerosis patients [28]. Another example is *NTS*, which has been implicated in wound healing in chronic colitis [29].

An enhanced up-regulation of Th2-responsive genes (characterised by larger $\log_2$(ratio) values) was observed in uncontrolled asthma. In fact, asthma severity in children has already been associated with atopy in epidemiological studies [30]. This association seems specific for childhood asthma, as an inverse relationship has been reported in adults [31]. Interestingly, IL-13 has been shown to induce a profibrotic bronchial phenotype *via* a continuous secretion of transforming growth factor-β2 [32]. Strikingly, the first gene ontology theme in the comparison between uncontrolled and controlled asthma corresponds to the term ''structural constituents of extracellular matrix'', with enhanced expression of *POSTN*, *TNC*, *FBN2*, *FBLN2*, *FMOD* and *COL4A6*. It is tempting to speculate that this dysregulation could be directly related to the structural modifications and remodelling of the epithelium particularly observed in severe asthma [33]. At the same time, *POSTN* has been reported to induce re-entry of differentiated mammalian cardiomyocytes into the cell cycle, in connection with tissue repair [34]. Mucus hypersecretion and goblet cell metaplasia are important traits of asthma, also linked with IL-13 dysregulation. We also noticed that *TFF3*, which is expressed in mucous cells and can promote the differentiation of ciliated human airway epithelial cells [21], was up-regulated in allergic patients. This illustrates the complexity of the mechanisms leading to abnormal repair and remodelling.

The major impact of Th2 cytokines probably masks weaker, intrinsic epithelial features. We believe that this represents a confounding factor when analysing epithelial susceptibility with the microarray technique. In order to dampen the impact of the Th2 response, we have compared subgroups of patients with uncontrolled or controlled asthma.

Another observation of our study is the existence of an altered IFN response in the nasal epithelium of uncontrolled asthmatic patients; this trait may be more directly related to an intrinsic epithelial susceptibility. The IFN response was reduced in the uncontrolled asthma group relative to the controlled asthma and healthy groups. We excluded the possibility that these effects were caused by viral infections: 1) a viral PCR assay, able to detect 90% of respiratory tract viral infections, eliminated cases of viral infection; and 2) a careful evaluation of CXCL10 transcript levels in ''uninfected'' patients identified additional infected samples.

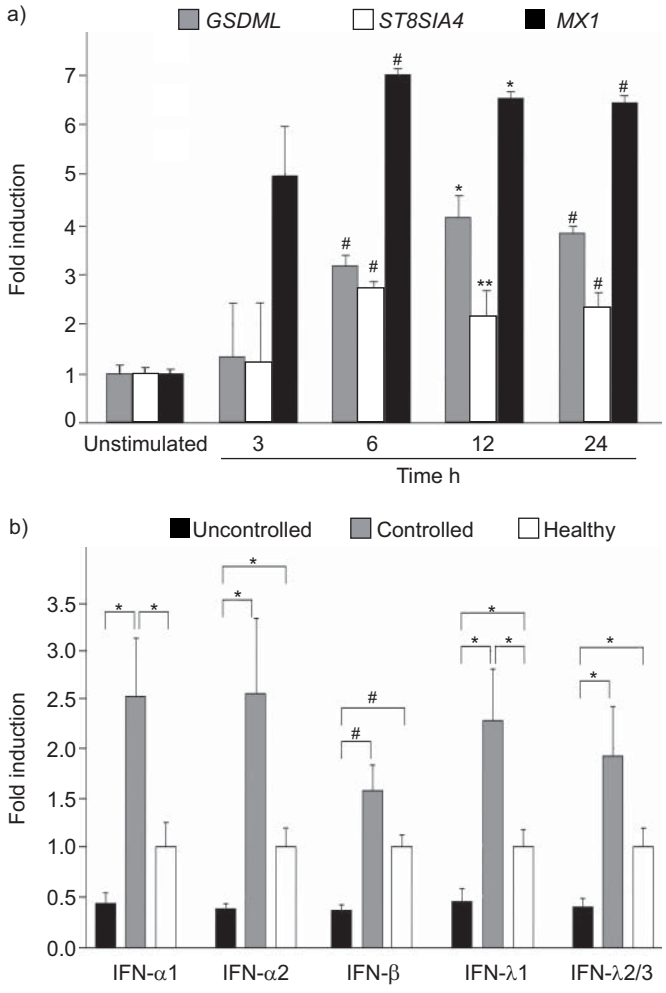

FIGURE 4. Characterisation of the interferon (IFN) response. a) Measurement by real-time quantitative PCR (qPCR) of the induction of *GSDML*, *ST8SIA4* and *MX1* by IFN-α in human nasal epithelial cells (HNECs). Levels of *GSDML*, *ST8SIA4* and *MX1* were assessed in differentiated HNECs 3, 6, 12 and 24 h after stimulation with IFN-α. Measurements were performed using SYBR Green. Results are expressed as fold-change relative to the unstimulated HNECs. b) Measurement of IFNs by qPCR in patients. Levels of IFN-α1, IFN-α2, IFN-β, IFN-λ1 and IFN-λ2/3 transcripts in patients with uncontrolled asthma, patients with controlled asthma and healthy controls. Data are expressed relative to an average of 11 healthy controls. Measurements were performed using Taqman probes. Results are expressed as fold-change relative to the healthy control group. Data are presented as mean±SEM. #: $p<0.005$ by unpaired t-test; *: $p<0.05$ by unpaired t-test; **: $p<0.01$ by unpaired t-test.

ISGs that are decreased in patients with uncontrolled asthma represent a quarter of the genes identified by our second supervised analysis. Unexpectedly, we identified *GSDML* in that cluster. Our study reports several additional observations suggesting a direct association of this gene with the abnormalities affecting the paediatric asthmatic epithelium: first, the existence of several response elements for IFN regulatory factors in its promoter region; secondly, its transcriptional induction by epithelial IFN in HNECs; and thirdly, its protein expression in airway epithelial cells. This is in line with the fact that one of its SNPs (rs7216389) has been associated with early onset of asthma, acute severe exacerbations (80% of which are virus-induced in children) and asthma severity [11, 35, 36]. MOFFATT *et al*. [37] initially noted an association of childhood asthma risk with modified expression of *ORDML3*, located in the immediate vicinity of *GSDML*. The identification of a variant in a splice site for *GSDML* in strong linkage disequilibrium with *ORMDL3* has recently suggested that *GSDML* would be the causative gene associated with asthma [38].

Decreased levels of IFN-α, IFN-β, IFN-λ1 and IFN-λ2/3 paralleled the down-regulation of IFN-stimulated genes observed in patients with uncontrolled asthma. This confirms and extends findings by DE BLIC *et al*. [39] showing a correlation between asthma symptoms and the IFN response in a cohort of 28 children with difficult-to-treat asthma. In their study, lower levels of IFN-γ were associated with persistent symptoms [39]. Deficient epithelial cell IFN-β and IFN-λ production during rhinovirus-induced asthma exacerbations was associated with increased viral load, defective apoptosis and cell death bias [2]. Epidemiological studies have associated the onset of asthma with reduced IFN-γ production by peripheral blood mononuclear cells at 9 months of age, while a normalisation of IFN-γ production was observed in children showing a resolution of asthma symptoms [40, 41].

Regardless of the exact molecular mechanisms leading to impaired IFN production, from our results, one could anticipate some benefits of IFN treatments in patients with uncontrolled asthma, as suggested by recent *in vitro* experiments with IFN-β [42]. As the association between defective epithelial cell IFN pathways and the lack of asthma control in patients could be specific to paediatric asthma, careful evaluation of therapeutic options based on the development of innate immunity would require the organisation of clinical trials addressing this specific issue in a paediatric population. Finally, our work illustrates some specificities of severe paediatric asthma, which not only results from the development of T-cell-mediated biased adaptive responses, but also from an inefficient epithelial innate immunity.

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CLINICAL TRIAL

This study is registered as www.clinicaltrials.gov with identifier number NCT00569361.

STATEMENT OF INTEREST

A statement of interest for the study itself can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

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