Raised interferon β, type 3 interferon and interferon stimulated genes - evidence of innate immune activation in neutrophilic asthma

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Running Title

Raised interferons β and λ in neutrophilic asthma

Abstract

Background: Interferons play an important role in innate immunity. Previous studies report deficiency in virus-induction of interferon (IFN)-α, -β and -λ in bronchial epithelial and bronchial lavage cells in atopic asthmatics. It is now recognized that asthma is a heterogeneous disease comprising different inflammatory phenotypes, some of which may involve innate immune activation in the absence of overt infection.

Objective: The aim of the study was investigate if the severity of asthma or a specific cellular sputum pattern may be linked to evidence of innate immune activation.

Methods: Here we investigate the expression of IFN-β, IFN-λ1 (IL-29), IFN-λ2/3 (IL-28A/B) and the interferon-stimulated genes (ISGs) myxovirus resistance 1 (Mx1), oligoadenylate synthetase (OAS) and viperin in unstimulated sputum cells in 57 asthmatics (including 16 mild, 19 moderate and 22 severe asthma patients) and compared them with 19 healthy subjects.

Results: We observed increased expression of IFN-β, IFN-λ1/IL-29, OAS and viperin in asthmatic compared to healthy subjects while IL-28 was not expressed in any group. The overexpression was restricted to neutrophilic asthmatics (sputum neutrophils ≥ 76%) while eosinophilic asthmatics (sputum eosinophils ≥ 3%) did not differ from healthy subjects or even showed a lower expression of Mx1. No difference in interferon or ISG expression was seen according to clinical asthma severity.

Conclusion and clinical relevance: Neutrophilic, but not eosinophilic, asthmatics display overexpression of IFN−β, IFN−λ1/IL-29 and ISGs in their sputum cells that may reflect ongoing innate immune activation.

Key words: asthma phenotype, neutrophils, eosinophils, interferon β and λ, interferon stimulated genes

Introduction

Interferons beta (IFN-β) and lambda (IFN-λ) play a crucial role in host defense against infectious agents. There is evidence that asthmatics may show impaired innate immunity resulting in inefficient clearing of viruses from epithelial cells [1]. This was shown to be related to a reduced production of IFN-β and IFN-λ from bronchial epithelial cells [2,3] and IFNs -α, -β and −λ from bronchoalveolar lavage cells upon cell infection [2,4]. A recent study has suggested that these deficiencies may be related to asthma severity in children [5] while they were not found in well controlled adult asthmatics [6]. A genome wide transcriptional analysis on primary bronchial epithelial cells from normal and atopic asthmatic donors showed similar response to rhinovirus infection including IFN-β and IFN-λ upregulation [7].

The technique of induced sputum has proved to be useful to investigate the cytokine network in airway cells of asthmatics [8] Measurement of proteins by ELISA in the cell culture supernatant [9,10], sputum supernatant as well as measurement of the gene expression in sputum cells have been used to characterize airway cytokine networks in asthma. A previous study has specifically investigated the expression of IFN-λ in sputum cells from children and adult asthmatics and found a higher expression of IL-28 in asthmatics, particularly in children, which correlated with eosinophil counts while expression of IL-29, which also tended to be higher, was inversely correlated with the symptoms [11]. In another study IFN-λ mRNA was measured in sputum cells and found to be lower at resolution of exacerbation in asthmatics who had the more severe symptoms during viral positive cold [12].

It is now recognized that asthma is a heterogeneous disease featuring several clinical and inflammatory phenotypes [13] with different underlying molecular mechanisms [14]. While asthma is often associated with airway and/or systemic eosinophilic inflammation that makes patients prone to exacerbate [15], a significant proportion of asthmatics display persistent non eosinophilic phenotype [16]. One phenotype suggested by previous workers includes evidence of innate immune

activation in neutrophilic asthma, perhaps as a consequence of airway colonization with microbes in the absence of evidence of overt airway infection [17].

The purpose of this study is to determine whether the severity of asthma or a specific cellular sputum pattern may be linked to evidence of innate immune activation. Here we have examined sputum samples from asthmatics of various severities and sputum cell compositions for the expression of interferons (IFN-β, IFNs-⌊) and interferon stimulated genes (ISGs), such as myxovirus resistance 1 (Mx1) [18], oligoadenylate synthase (OAS) [19] and viperin [20]. Though none of the patients included in our study was in exacerbation, we also investigated the presence of picornaviruses in sputum samples. Our results show that expression of interferons and ISGs in sputum cells is more influenced by the type of airway inflammatory pattern than the clinical disease severity.

Methods

1- Subject characteristics and study design

The study was performed in subjects recruited from the University Asthma Clinic of Liege between June 2011 and September 2013. Asthma was diagnosed on the basis of chronic respiratory symptoms such as cough, breathlessness or dyspnea associated with airway hyper-responsiveness, demonstrated by one or more of the following criteria: increase in forced expiratory volume in one second (FEV_1) of >12% and 200 mL following inhalation of 400μg salbutamol or inhaled concentration of methacholine provoking a 20% fall in FEV_1 of <16 mg/mL. Clinical assessment at the Asthma Clinic was previously reported [21] and included the evaluation of asthma control by the Juniper Asthma Control Questionnaire (ACQ). None of the recruited asthmatics selected for this study was in an exacerbation state requiring initiation or increasing the current dosage of systemic corticosteroids within 4 weeks of sputum samplings. None of them had overt sign of acute pneumonia the day of sputum induction. Atopy was determined by positive skin-prick test to at least one common aeroallergen (cat, dog, house dust mite, grass pollen, tree pollen and a mixture of

moulds). Mild asthmatics were patients with intermittent symptoms, normal baseline lung function (FEV$_1$ > 80% predicted) and not treated by inhaled corticoids (ICS) while moderate asthmatics were receiving maintenance treatment with moderate doses ICS combined to long acting β_2 agonists (LABA) or leukotriene receptor antagonist (LTRA). Refractory asthma was defined according ATS criteria [22], after an extensive evaluation of the patients. These patients had profound lung function impairment despite receiving high dose ICS mostly combined with LABA. Based on sputum cellularity characteristics, patients were classified as eosinophilic phenotype, defined as ≥3% sputum eosinophil count, neutrophilic phenotype, consisted of ≥76% sputum neutrophil counts, mixed granulocytic phenotype, when both neutrophil and eosinophil counts were increased or pauci-granulocytic phenotype, when both cell types were under the percentages described above. The cut-off of 76% for neutrophil counts was chosen from the 90% confidence interval of a sputum neutrophil count observed in a series of 113 healthy subjects recruited in our hospital by advertisement. The mean was 35% and standard deviation was 24%; thus the upper limit of the 90% CI only let 5% of normal subjects above this threshold and can be derived by the formula mean+1.7 SD [23].

Healthy controls were recruited by local advertisement in the hospital. None of them exhibited respiratory symptoms or had airways hyperresponsiveness (provocative concentration of methacholine causing a fall in FEV$_1$ of 20% > 16 mg/mL) and all had normal lung function (FEV$_1$ >80% predicted value).

The study was approved by our local Ethics Committee and subjects signed informed consent.

2. Sputum induction and processing

Sputum was induced and processed as previously reported [23]. Cell viability was checked by trypan blue exclusion and after counting 500 non squamous cells, the differential count was performed on cytospins stained with RAPI DIFF II® stain kit (Atom Scientific, Manchester,UK). When a poor quality sample was detected (squamous cell fraction greater than 30%), the sputum was excluded from the

analyses. Collected cells were centrifuged and the pellet (1 to 2.10^6 cells) was mixed with 400µL of

RNAprotect® cell reagent (Qiagen, Hilden, Germany) and kept at -80°C until RNA extraction.

During the recruitment for this study 8 subjects were excluded because of poor quality of sputum

sample (2 healthy subjects, 2 intermittent asthmatics, 2 moderate and 2 severe asthmatics). Then,

sputum induction was performed in 84 subjects of whom 76 were suitable for gene expression

analysis.

3. RNA extraction/isolation and cDNA synthesis

After removing RNAprotect cell reagent, the pellet was resuspended in 500µL of TriPure isolation

Reagent (Roche Applied Science), to which was added one bead (Stainless Steel Beads, 5mm,

Qiagen). The sample was disrupted and homogenized using a TissueLyser system (TissueLyser II,

Qiagen), for 2 min at 25 Hz. The RNA was separated by phenol-chloroform extraction. The upper

aqueous phase (300 µL) was diluted with equal volume of ethanol and transferred to a NucleoSpin

RNA binding column (Macherey-Nagel). Washing and drying of silica membrane and RNA elution

were executed according to NucleoSpin RNA clean-up protocol (Macherey-Nagel). The residual

genomic DNA was eliminated by a treatment DNAse with TURBO DNA-free™ kit of Ambion (Thermo

Fisher Scientific, Wilmington, USA). The RNA concentration and purity were assessed by Nanodrop

ND-1000 spectrophotometer. The cDNA was prepared from maximum 1µg of RNA using QuantiTect®

Reverse Transcription kit of Qiagen.

4. TaqMan Real Time Polymerase Chain reaction (qPCR)

Quantitative PCR was performed with a 384 well plate (MicroAmp®Optical) on a 7900HT system

(Applied Biosystems) using the QuantiTect® Probe PCR kit (Qiagen). The real-time reaction mixture

was prepared in a total volume of 10µL PCR reaction and 1 µL cDNA. The cycle parameters were as

follows: initial enzyme activation at 95°C for 15 min, followed by 40 cycles of sequential incubations

at 94°C for 15 s and at 60°C for 1 min and one cycle at 40°C for 30 seconds.

Primer pairs and probes, all FAM-TAMRA labelled, were selected according to description of Gielen et al [24] for 18s, OAS, IFN-β, Mx1, viperin, and IL-28. For IL-29, probe: AGTTGCAGCTCTCCTGTCTTCCCCG, forward: CCTTGGAAGAGTCACTCAAGCT and reverse: AGAAGCCTCAGGTCCCAATT, (accession number: NM_172140.1), which were purchased from Eurogentec (Seraing, Belgium). For each sample a supplementary PCR from RNA for IL-28, IL-29 and IFN-β was performed to verify the absence of residual genomic DNA. Copy numbers of each gene were determined via standard curves constructed as dsDNA plasmids, and normalized with the housekeeping gene 18S rRNA. We chose a cut-off of 20 CT for the 18S and excluded samples in which more than 20 CT was needed to pick up 18S (8 out of 84 samples initially tested). The mean CT for 18S was 14.1±1.8 CT in our series of experiments.

5. Measurement of IFN-β and IL-29 proteins in sputum supernatant

A pool of sputum supernatants from healthy subjects and asthmatic patients was spiked with IL-29 (range from 60 to 1000pg/mL) or IFN-β (range from 50 to 2000pg/mL) to construct standard curves. Sputum samples were diluted 1/10 for IL-29 and 1/2 for IFN-β measurements and compared to the standard curves to quantify IFN proteins using commercial kits to IL-29 (Human IL-29/IFN-lambda 1 DuoSet ELISA Kit, R&D Systems) and IFN-β (VeriKine Human IFN Beta ELISA Kit, Pestka Biomedical Laboratories). The recovery in the lower concentrations were 24% for IL-29 and 26% for IFN-β.

6. Detection of virus RNA

Four µl of cDNA was then used to detect picornaviruses in a single round PCR of 32 cycles [25]. Differentiation of rhinoviruses from other picornaviruses was achieved by restriction enzyme digestion of the PCR product [26].

7. Statistical analysis

Data were analyzed using a statistical software package (GraphPad Prism, Version 5). Results were expressed as median (range) for non-parametric data, and as mean (± standard deviation) for parametric data. Frequency was used to summarize categorical data. Groups were compared for qualitative data by Fisher's exact t. For quantitative data groups were compared by the Kruskal-Wallis test and post-hoc Dunn's test. Parametric data were analyzed by ANOVA with post-hoc two sample t-testing with Bonferroni correction. Spearman's Rank correlation coefficient was used to identify relationships between variables that show non-parametric distribution; *p*-values ≤0.05 were considered statistically significant.

Results

Patient characteristics

The patient characteristics according to asthma severity are given in table 1. Refractory asthmatics were patients observed for period of at least 6 months and were characterized by markedly reduced flow rates and FEV_1/FVC ratio accompanied by uncontrolled symptoms reflected by higher ACQ score despite high dose of inhaled corticoids mostly combined with long acting β_2-agonists. The sputum cytology of the four groups is shown in table 2.

Detection of IFNs and ISGs in asthma

Overall IFN-β expression was detected in 23 of 57 asthmatics and in only 1 of 19 healthy subjects (*p*<0.01). IL-29 was detected in 31 of 57 asthmatics and 2 of 19 healthy subjects (*p*<0.001). Viperin expression was increased in asthmatics (*p*<0.01) while that of Mx1 was slightly decreased (*p*=0.05). No difference was seen with respect to OAS (Figure 1). Sputum supernatants from healthy subjects (15 out of 19 samples) and asthmatics (47 out of 57 samples) were analyzed for IL-29 and IFN-β proteins. IL-29 protein was detectable in only one healthy subject (76pg/ml) and 4 asthmatics (range from 74 to 113 pg/ml). Among them 3 samples from asthmatics were also positive for IL-29 mRNA.

As for IFN-β protein, 3 healthy subjects (range from 115 to 187 pg/ml) and 6 asthmatics (range from 115 to 201 pg/ml) had positive samples. Among them 4 samples from asthmatics had IFN-β gene expression detected in sputum cells.

Relationship between IFNs and ISGs and smoking and ICS

Overall IFN-β expression weakly correlated with pack-years (r=0.25, p<0.05). No significant relationship with smoking history was found for IL-29 and the ISGs (p>0.05 for all) even if there was a trend for IL-29 and viperin (r=0.20; p=0.08). There was no difference between asthmatics treated (N=40) and not treated with ICS (N=17) with respect to IFNs and ISGs but only ICS treated (N=40) asthmatics had raised IL-29 (p<0.01), IFN-β (p<0.05) and viperin (p<0.05) compared to healthy subject.

Relationship between IFNs and ISGs and asthma severity

There was no difference in IFN-β and IL-29 expression or in interferon-stimulated genes (ISGs) according to disease severity (Table 3). In addition there was no correlation between asthma control (ACQ) and FEV_1 and any of the interferons and ISGs (Table 4).

Relationship between IFNs and ISGs and sputum cellularity in asthmatics

There were too few asthmatics with mixed granulocytic cellularity (n=5) to make pertinent statistical analyses, thus analyses are restricted to the other three groups. When classified according to their sputum granulocyte cell counts (Table 5), neutrophilic asthmatics were clearly characterized by an increased expression on IFN-β and IL-29 together with interferon-stimulated gene viperin compared to eosinophilic asthmatics and healthy subjects (Figure 2). Neutrophilic asthmatics had also greater IFN-β and IL-29 expression compared to pauci-granulocytic asthmatics. Pauci-granulocytic asthmatics had greater expression of Mx1 compared to eosinophilic asthmatics in whom Mx1 gene expression was even lower than in healthy subjects (Figure 2).

Relationship between IFNs and smoking, ICS and neutrophils.

In a multivariate analysis including smoking status, ICS dosage and sputum neutrophils we found that sputum neutrophils was the dominant factor associated with raised IL-29 and IFN-β in asthmatics (Table 6).

Relationship between IFNs and ISGs and between sputum cellularity and IFNs and ISGs

For the whole asthmatic group there was a strong correlation between gene expression of IFN-β and IL-29 (r=0.75, *p*<0.001) and, to a lesser extent, between IFNs and ISGs (Table 7). The relationship between sputum cell counts and interferon and/or interferon induced genes are given in table 8. IFN-β, IL-29 and viperin correlated with sputum neutrophils while OAS and Mx1 inversely correlated with sputum eosinophils.

Detection of picornavirus in sputum samples

Only 4 subjects out of 68 tested had detectable picornavirus in their sputum sample including 3 healthy subjects (all picornaviruses) and 1 moderate asthmatic in which human rhinovirus was identified. Their sputum neutrophil counts were 87, 88, 60 and 57% respectively. IL-29 was detectable in two of the 4 subjects (1 healthy with picornavirus [256 copies/μL] and 1 asthmatic with rhinovirus [24 copies/μL]) but IFN-β was undetectable in all four. In these 4 subjects ISGs were unremarkable compared to the subjects negative for viruses (data not shown)

Discussion

Our study shows that asthmatics express greater amount of interferon and interferon stimulated genes in unstimulated sputum cells compared to healthy subjects. However, this overexpression is essentially accounted for by the group of neutrophilic asthma while this expression seems to be normal and, even to some extent, reduced in eosinophilic asthma.

Although sputum sampling is a convenient technique to sample the airways the interferon-β and -λ pathways have been poorly investigated so far. One previous study investigating both children and adult stable asthmatics showed increased expression in IL-28 and IL-29 in unstimulated sputum cells from asthmatics and particularly in children [11]. Another recent study has shown a trend for raised expression of IFN-β in sputum cells in adult asthmatics during a viral asthma exacerbation while IFN-λ1 did not change [27]. Our data confirm the increased IL-29 expression in stable asthma and extends this finding by showing increased IFN-β and a corresponding increase in the interferon stimulated gene viperin. Our study has included adult asthmatics encompassing the all disease severity spectra allowing for meaningful evaluation of a link between interferon expression and disease severity. In our study, no convincing relationship was found between disease severity and interferon gene expression. Furthermore gene expression was unrelated to FEV_1 or asthma control. We know that severe asthma is a heterogeneous disease with respect to airway inflammation with half of patients displaying residual eosinophilic inflammation while the other half shows either pure neutrophilic or no clear airway inflammation [28].

Here we bring up new relevant information by showing that the interferon pathway is essentially upregulated in a specific sub-group of asthmatics with elevated sputum neutrophil counts. Our finding is in line with the rise of sputum IFN-β RNA associated with a neutrophil recruitment in the airways during an acute viral exacerbation [27]. Interestingly Simpson et al have recently demonstrated that PBMC from neutrophilic asthmatics released less IFN-α and IFN-β in response to a stimulation with rhinovirus in vitro [29]. This apparent discrepancy may be explained by the different compartments (blood vs airway) that were investigated. Our findings are in airway cells where bacteria/virus presence in the airway might explain high sputum neutrophil counts and basal interferon levels, the same might not be true for the PBMCs that Simpson et al studied as they would not be exposed to the bacteria/virus. Among the ISGs, viperin was the one whose expression best correlated with IFNs -β and -λ, and was the one most markedly increased in neutrophilic asthma.

The results in our study reflect the overall gene expression from a mixture of sputum cells. Therefore we can only speculate about the main cell source of gene expression. However the fact that interferons and ISG are increased in neutrophilic asthma suggests that neutrophils may be a potential source of interferons and ISG gene expression. Alternatively we cannot rule out the fact that increased expression of IFNs and ISGs may favour neutrophilic airway infiltration. The increased cytokine expression in neutrophilic asthma supports the concept of innate immune activation in this phenotype [17]. Whether neutrophilic inflammation and interferon gene expression reflect an infectious process within the airways remains to be demonstrated. Though we did not systematically assess airway microbial status in this study none of the recruited patients had an overt acute lower respiratory tract infection at the time of sputum sampling. Wood et al found only 15% of stable asthmatics to be colonized with potentially pathogen microorganism (PPM) [30]. We also know from personal observation that only a minority of neutrophilic asthmatics show airway bacterial colonization by positive sputum culture. Indeed retrospective analysis from our asthma clinic data base identified 36 patients with sputum neutrophil counts > 76% in whom the clinician had specifically asked for a microbiology analysis for suspicion of infection. Only 14 out of them had PPM at sputum culture. Nevertheless it does not exclude a lower microbial burden that might have been identified through more sensitive technique like gene analysis [31]. Indeed people with asthma (without knowledge of their airway inflammatory cell status) have been recently reported to have an increased burden of potentially pathogenic organisms in their lower airway when stable [32]. In addition, a recent study that used 16S rRNA pyrosequencing for microbial community profiling has shown reduced bacterial diversity and a high prevalence of *Haemophilus influenzae* in neutrophilic asthma [33]. With the advent of sputum cell PCR analysis our current study discards infection with picornavirus, and in particular with rhinovirus, in all but 4 subjects. Among them, rhinovirus was detected in only 1 non-neutrophilic moderate asthmatic. On the other hand it has been established that neutrophils can be recruited by non-specific stimuli such as saline [34], cold air [35] or as a

result of airway irritation by air pollutants [36-38]. Our study cannot clarify whether the interferon pathway is activated here as a consequence of airway infection/colonisation or just airway irritation.

In sharp contrast with what was shown in neutrophilic asthmatics, eosinophilic asthmatics did not show any increase in IFNs or ISGs when compared to healthy subjects and even displayed a reduced expression of Mx1. The striking difference between neutrophilic and eosinophilic asthma with respect to interferon pathway activation is in keeping with previous studies pointing to different molecular mechanisms in neutrophil vs eosinophilic asthma, where cellular pattern rather than clinical severity shows association with molecular pathways [39-42].

Mx1 was different from other ISGs with respect to its expression being possibly reduced when comparing all asthmatics to healthy control subjects, with a P value of borderline significance at P=0.05, while OAS expression was not significantly different between groups, and IL-29, IFN-β and viperin were all increased in asthma. The reduced Mx1 expression in eosinophilic asthma suggests that this asthma phenotype might be more susceptible to viral airway infection, Mx1 represents a first line of defense against viral infection inside the cell and its increased expression may be seen as a sign of ongoing innate immune activation [43], of increased constitutive expression or it could be a spurious finding. We believe these findings should be interpreted with caution due to the relatively low numbers of healthy subjects included, and of asthmatics subjects once divided into sub-groups. A recent study has shown that treatment with inhaled interferon-β may help severe asthmatics to speed up clinical recovery during an exacerbation due to a common cold [44]. However no patient selection according to airway inflammatory pattern was performed before entering the patients in the protocol and it would be of great interest to see whether the size effect could even be stronger in selected eosinophilic asthmatics.

One limitation of our study is the cross sectional design precluding any conclusion on the stability of the inflammatory phenotype over time. Therefore we cannot state that some asthmatics permanently express high or low IFNs and ISGs in their airways. In contrast we believe it is likely to fluctuate according to the airways exposure to offending microbes or particles.

How do our findings relate to many other publications showing that cells from asthmatic airways have reduced type I and type III IFN production in response to rhinovirus infection *ex vivo* [2-5]? We believe the most likely explanation is that our finding of increased IFN and ISG expression in mixed sputum cells with no *ex vivo* infection or stimulation reflects ongoing innate immune stimulation of sputum cells in people with neutrophilic asthma, most likely a consequence of increased bacterial burden in the airways of these subjects. Our processing methods (use of DNAse) did not allow investigation of bacterial burden by analysis of bacterial DNA with molecular techniques in these samples, so we were unable to determine if that hypothesis is true in the present study. Our findings are consistent with the literature reporting deficient/delayed IFN induction in asthma, as current mechanistic explanations for this phenomenon, are that it is at least in part a consequence of increased airway inflammation (in the present study neutrophilic) inducing SOCS1[45], Th2 cytokines [46] and/or TGF-β [47-49], thereby actively suppressing IFN induction in response to virus infection. In conclusion neutrophilic, but not eosinophilic, asthmatics show increased sputum interferon-β and -λ gene expression together with increased ISG(s). Whether this reflects innate immune activation against colonizing microbes or altered microbiome remains to be demonstrated in a further study. The reduced Mx1 expression in eosinophilic asthmatics, if confirmed in future studies, may make them more prone to viral infections*.*

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Table 1: Patient characteristics according to asthma severity

	Healthy subjects	Mild asthmatics	Moderate asthmatics	Refractory asthmatics
	(N = 19)	(N = 16)	(N = 19)	(N = 22)
Age (years)	48 ± 15	53 ± 16	51 ± 17	53 ± 9
Sex (m/f)	10/9	4/12	7/12	6/16
Tobacco status (ns/es/cs)	9/7/3	6/10/0	12/5/2	5/10/7
Pack-year	0.2 (0-42)	17 ᵒᵒᵒ $$ (0-27)	0 * && ᵒ (0-30)	24.8 *** (0-90)

Atopy	7 (37%)	11 (69%)	15 (79%)	12 (57%)
FeNO (ppb)	24.3 (4.1-48.7)	34.3 º (10.2-101)	33.5 º (10-116)	15.7 (4.3-42.5)
IgE	Nd	106 (19-611)	213 (11-4379)	232 (12-1787)
FEV1 (% Pred)	100 ± 13.7	94.5 ± 12.19 ººº	91.37 ± 12.75 ººº	50.59 ± 14.41***
FVC (% Pred)	106.72 ± 12.5	105.69 ± 10.52 ºº	103.53 ± 14.89 ºº	79.48 ± 22.09***
FEV1/FVC (%)	77.64 ± 5.49	74.84 ± 7.58 ººº	73.76 ± 6.51 ººº	53.49 ± 8.79***
Reversibility (%)	Nd	9.46 ± 5.5	6.5 ± 7.29	11.68 ± 15.07
ACQ	Nd	0.97 ± 0.56 ººº	1.42 ± 0.99 ººº	3.56 ± 1.24
PC20M (mg/ml)	>16	1.2 (0.1-15.4)	1.9 (0.2-22)	0.27 and 6.09 [N]
Oral CS	(0%)	0 (0%)	0 (0%)	5 (23%)
Inhaled CS	(0%)	0 (0%)	18 (95%)	22 (100%)
Inhaled CS (eq becl/day)	0	0 ººº $$$	1000 *** (0-2000)	2000 *** (800-4400)
LABA	-	0 (0%)	16 (84%)	22 (100%)
LTRA	-	1 (6%)	8 (42%)	16 (73%)
Theophylline	(0%)	0 (0%)	0 (0%)	4 (18%)

Results are presented as absolute number, absolute number (percentage), mean ± SD or median (range); n: non-smoker; ex: ex-smoker; cs: current smoker; FENO: exhaled nitric oxide; FEV1: forced expiratory volume in 1"; FVC: forced vital capacity; PC20M: metacholine concentration required to provoke a fall of 20% or more in FEV1; ACQ: Asthma Control Questionnaire; LABA: long-acting beta-agonist bronchodilators; LTRA: leukotriene receptor antagonists, nd: not done * p<0.05, ** *p*<0.01, *** *p*<0.001 *versus* healthy subjects; $ *p*<0.05, $$ *p*<0.01 *versus* moderate asthmatics; & *p*<0.05, && *p*<0.01 *versus* mild asthmatics; ° *p*<0.05, ºº *p*<0.01, ººº *p*<0.001 *versus* refractory asthmatics. [N] Note: only for two refractory asthmatics.

Table 2: Sputum cytology according to asthma severity

	Healthy subjects	Mild Asthmatics	Moderate asthmatics	Refractory asthmatics
Sputum weight (g)	4 (1.4-10.3)	3.7 (0.4-11.8)	3.8 (0.9-11.0)	2.2 (0.3-16.6)
Total non-squamous cells (10^6/g)	1.1 (0.1-11.6)	1.7 (0.3-29.0)	1.8 (0.2-17.5)	2.7 * (0.5-37.7)
Squamous cells (%)	13.2 (0-30)	6 (1-30)	14 (0-24)	5 (0-30)
Viability (%)	77 (42.8-92.0)	77.5 (45-96)	78 (50-95)	74.5 (40-97)
Macrophages (%)	37 (6-88)	19.7 (1.6-90.5)	27.3 (1.0-67.4)	9 *** (1.5-98.0)
Macrophages (10^3/g)	418.1 (36.6-2772.0)	392 (23.4-6812.7)	387.4 (31.7-1817.9)	395.3 (14.6-4106.2)
Lymphocytes (%)	2.2 (0-7)	0.9 (0.0-5.8)	1.4 (0.0-6.6)	1.5 (0-7)
Lymphocytes (10^3/g)	21.9 (0.0-417.2)	29.4 (0.0-472.1)	14.8 (0.0-207.9)	18.7 (0.0-589.5)
Neutrophils (%)	55.3 (4-88)	49.2 (3-98)	53 (15.4-98.0)	72.3 (0-98)
Neutrophils (10^3/g)	442.4 (60.4-10036.9)	820.7 (103.8-12730.3)	701.7 (56.8-15627.1)	1568.3 * (0-27734)
Eosinophils (%)	0 (0.0-11.4)	4.9 *** (0.0-70.7)	2 * (0-22)	4 *** (0-92)
Eosinophils (10^3/g)	0 (0-371)	119 *** (0-15365)	36 (0-436)	137 *** (0-34684)
Epithelial cells (%)	2 (0-15)	2 (0-10)	6 (0-33)	4 (0-29)
Epithelial cells (10^3/g)	23 (0-180)	25 (0-2247)	54 (0-726)	127* (0-5175)

Results are presented as median (range); * $p<0.05$, *** $p<0.001$ *versus* healthy subjects.

Table 3: Gene expression of interferons and interferon-induced genes in sputum cells according to

asthma severity

	Healthy subjects	Mild Asthmatics	Moderate asthmatics	Refractory asthmatics
IL-28 (copy/µL)	0 (0-33)	0 (0-0)	0 (0-0)	0 (0-0)
IL-29 (copy/µL)	0 (0-256)	2 * (0-2920)	4 ** (0-1305)	73 ** (0-9712)
IFN-β (copy/µL)	0 (0-2)	0 * (0-7281)	0 * (0-1609)	0 ** (0-5366)
Mx1 (copy/µL)	3855 (744-62906)	2542,4 (93-55944)	2729 (0-29300)	2575 * (161-22792)
OAS (copy/µL)	771 (0-4953)	1084 (35-9205)	724 (191-3138)	1080 (0-16218)
Viperin (copy/µL)	349 (0-1123)	1205 ** (0-20534)	477 (107-16377)	1739 * (0-72222)

Results are presented as median (range); * $p<0.05$, ** $p<0.01$ *versus* healthy subjects.

Table 4: Matrix of correlation between IFNs and ISGs expression and indices of asthma control

	ACQ		FEV1 % pred	
	p value	r	*p* value	r
IL29	0.76	0.04	0.78	0.04
IFNβ	0.68	-0.06	0.21	0.17
Mx1	0.33	-0.14	0.20	0.17
OAS	0.80	0.03	0.68	-0.05
Viperin	0.48	0.10	0.77	-0.04

ACQ: asthma control test. r: Spearman coefficient.

Table 5: Demographic, functional and inflammatory characteristics according to sputum cellular phenotypes

	Healthy subjects	Pauci-granulocytic asthma	Eosinophilic asthma	Neutrophilic asthma
	(N = 19)	(N = 11)	(N = 27)	(N = 14)
Age (yrs)	48 ± 15	45 ± 17	54 ± 9	54 ± 13
Sex (m/f)	10/9	5/6	9/18	2/12
Tobacco status (ns/es/cs)	9/7/3	5/5/1	13/11/3	2/7/5
Pack-year	7 (0-25)	7 (0-30)	0,5 (0-60)	17 (0-40)
Atopy (%)	7 (37%)	6 (55%)	19 (73%)	8 (57%)

NO (ppb)	24 (4-48)	13 (4-70)	35 (6-116)	20 (6-68)
IgE (KU/l)	Nd	60 (11-537)	206 (27-611)	287 (12-4379)
FEV1 (% pred)	100 ± 14	83 ± 23	76 ± 21**	68 ± 29**
FVC (% pred)	107 ± 12	98 ± 22	94 ± 18	93 ± 26
FEV1/FVC (%)	78 ± 5	70 ± 12	66 ± 11**	60 ± 14***
Reversibility (%)	Nd	8 ± 12	11 ± 11	5 ± 8
ACQ	Nd	1.63 ± 1,31	2.16 ± 1,62	2.51 ± 1,3
PC20M (mg/ml)	>16	2.6 (0,2-14,2)	0.9 (0,1-22)	3.1 (0.11-16)
Oral CS	-	1 (9%)	3 (11%)	1 (7%)
Inhaled CS	-	8 (73%)	18 (67%)	12 (86%)
LABA	-	8 (73%)	17 (63%)	12 (86%)
LTRA	-	3 (27%)	13 (48%)	8 (57%)
Theophylline	-	0 (0%)	0 (0%)	4 (29%)
Asthma Severity				
Mild	-	2 (18%)	9 (33%)	2 (14%)
Moderate	-	6 (55%)	8 (30%)	4 (29%)
Refractory	-	3 (27%)	10 (37%)	8 (57%)

Results are presented as absolute number, absolute number (percentage), mean ± SD or median

(range). See table 1 for legends. ** *p*<0.01 and ****p*< 0.001 *versus* healthy subjects.

Table 6: Multivariate analysis on the relationship between and IL-29 (upper table) and IFN-β (lower table) ICS, sputum neutrophils and smoking status in asthmatics

	Univariate Model vs IL-29 (log)					Multivariate Model vs IL-29 (log)			
Factors	β	SE	*p*	R^2		β	SE	*p*	R^2
ICS dose (eq. beclomethasone)	+0.0003	0.0002	0.152	0.037		+0.0001	0.0002	0.484	0.137
Sputum neutrophils (%)	+0.0225	0.008	*0.01*	0.115		+0.019	0.009	*0.038*	
Smoking (ref.			0.282	0.046				0.810	
Never-Smoker)	+0.589	0.531				+0.296	0.530		
Ex-smoker	+1.086	0.723				+0.422	0.787		
Current-smoker									

	Univariate Model vs IFN-β (log)					Multivariate Model vs IFN-β (log)			
Factors	β	SE	*p*	R^2		β	SE	*p*	R^2
ICS dose (eq. beclomethasone)	+0.0002	0.0002	0.346	0.016		-0.000005	0.0002	0.981	0.193
Sputum neutrophils (%)	+0.0276	0.008	*0.002*	0.167		+0.024	0.009	*0.009*	
Smoking (ref.			0.114	0.077				0.453	
Never-Smoker)	+0.896	0.533				+0.578	0.523		
Ex-smoker	+1.340	0.725				+0.797	0.776		
Current-smoker									

Table 7: Matrix of correlations between IFNs and ISGs

	IL-29		IFN-β	
	p-value	r	p-value	r
Mx1	0,6884	0,05379	0,0342	0,2786
OAS	0,0223	0,2997	< 0.0001	0,5261
VIPERIN	0,0087	0,3414	< 0.0001	0,5309

r: Spearman coefficients were calculated for pooled asthmatics and healthy subjects.

Table 8: Relationship between gene expression and percentage of sputum cell counts

Sputum cell counts (%)	IL-29	IFNβ	Viperin	OAS	Mx1
Macrophage	**r = - 0.39**	**r = - 0.37**	**r = - 0.3**	r = 0.11	r = 0.18
	_p_ = 0.0005	**_p_= 0.001**	**_p_ = 0.0092**	_p_ = 0.3347	_p_ = 0.1196
Lymphocyte	**r = - 0.26**	r = - 0.21	r = - 0.08	r = - 0.06	r = 0.05
	_p_ = 0.0263	_p_ = 0.07	_p_ = 0.5193	_p_ = 0.5989	_p_ = 0.6739
Neutrophil	**r = 0.36**	**r = 0.39**	**r = 0.4**	r = 0.07	r = 0.04
	_p_ = 0.0015	**_p_ = 0.0006**	**_p_ = 0.0004**	_p_ = 0.5299	_p_ = 0.7118
Eosinophil	r = - 0.09	r = - 0.09	r = - 0.15	**r = - 0.32**	**r = - 0.49**
	_p_ = 0.4634	_p_ = 0.4618	_p_ = 0.1871	**_p_ = 0.0053**	**_p_ < 0.0001**
Epithelial cell	r = - 0.21	**r = - 0.25**	r = - 0.09	r = - 0.05	r = - 0.03
	_p_ = 0.0771	**_p_ = 0.0339**	_p_ = 0.4226	_p_ = 0.6418	_p_ = 0.7954

Boldface reflects statistically significant correlation.

Figure Legends

Figure 1

Expression of IL-29 and IFN-β on sputum cells in healthy subjects (open circles) and asthmatics

(closed circles) (Upper panel). Expression of myxovirus resistance A (Mx1), oligoadenylate synthase

(OAS) and viperin on sputum cells in healthy subjects (open circles) and asthmatic (closed circles).

The bars represent the median.

Figure 2

Expression of IL-29, IFN-β, Viperin, Mx1 and OAS on sputum cells in asthmatics according to the

inflammatory phenotype. Eos + means sputum sample with sputum eosinophils >3%, Neutro +

means sputum sample with sputum neutrophil > 76%, Pauci means sputum sample with

sputum eosinophil and neutrophil < 3% and 76% respectively. The bars represent the median.
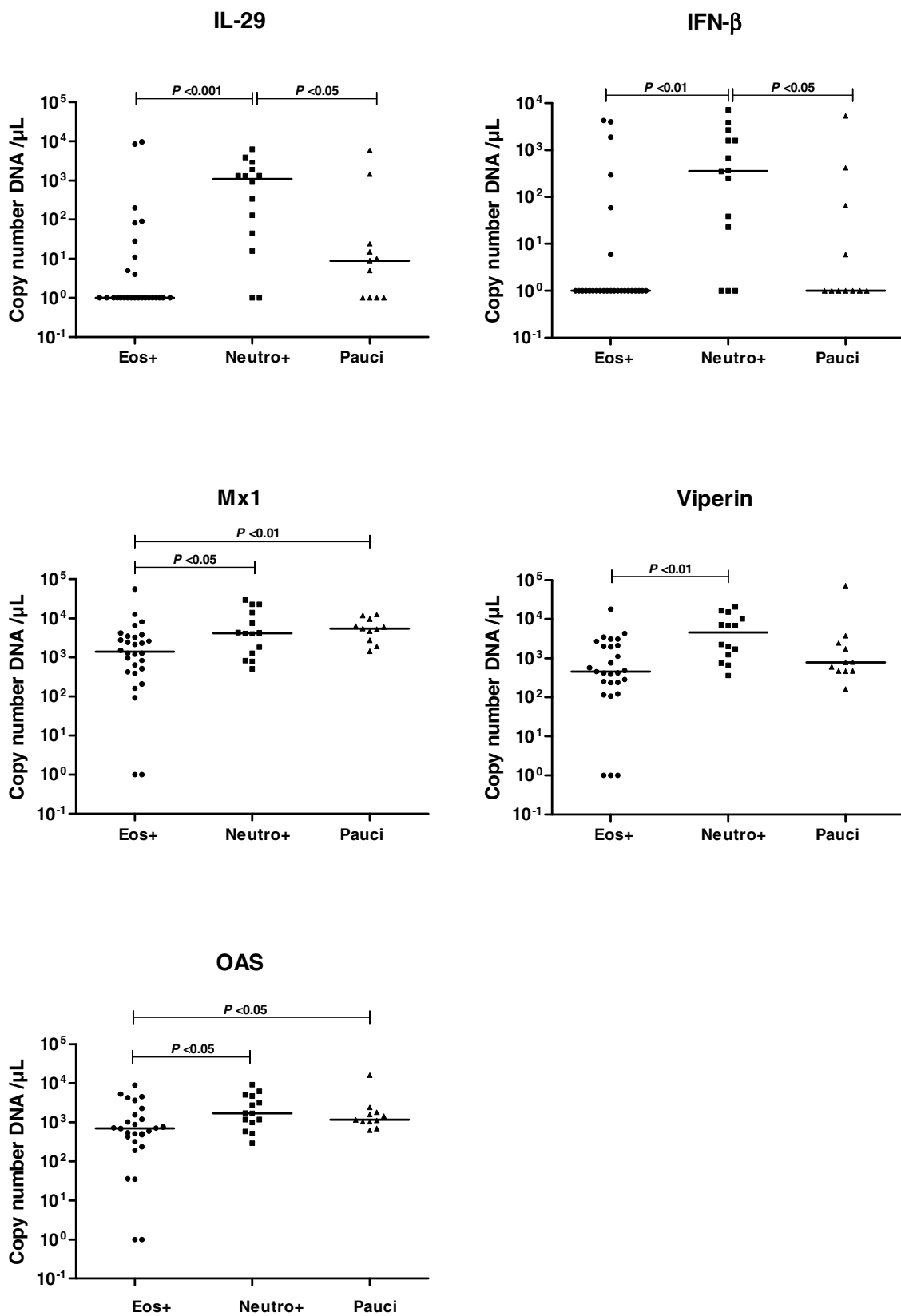
Figure 1

Figure 2