

Reduced Antiviral Interferon Production in Poorly Controlled Asthma Is Associated With Neutrophilic Inflammation and High-Dose Inhaled Corticosteroids



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BACKGROUND: Asthma is a heterogeneous chronic inflammatory disease in which host defense against respiratory viruses such as human rhinovirus (HRV) may be abnormal. This is a matter of some controversy, with some investigators reporting reduced type I interferon (IFN) synthesis and others suggesting that type I IFN synthesis is relatively normal in asthma.

OBJECTIVE: The objective of this study was to examine the responsiveness of circulating mononuclear cells to HRV in a large cohort of participants with poorly controlled asthma and determine whether IFN- α and IFN- β synthesis varies across different inflammatory phenotypes.

METHODS: Eligible adults with asthma ($n = 86$) underwent clinical assessment, sputum induction, and blood sampling. Asthma inflammatory subtypes were defined by sputum cell count, and supernatant assessed for IL-1 β . Peripheral blood mononuclear cells (PBMCs) were exposed to HRV serotype 1b, and IFN- α and IFN- β release was measured by enzyme-linked immunosorbent assay.

RESULTS: Participants (mean age, 59 years; atopy, 76%) had suboptimal asthma control (mean asthma control questionnaire 6, 1.7). In those with neutrophilic asthma ($n = 12$), HRV1b-stimulated PBMCs produced significantly less IFN- α than PBMCs from participants with eosinophilic ($n = 35$) and paucigranulocytic asthma ($n = 35$). Sputum neutrophil proportion and the dose of inhaled corticosteroids were independent predictors of reduced IFN- α production after HRV1b exposure.

CONCLUSIONS: Antiviral type I IFN production is impaired in those with neutrophilic airway inflammation and in those prescribed high doses of inhaled corticosteroids. Our study is an important step toward identifying those with poorly controlled asthma who might respond best to inhaled IFN therapy during exacerbations. CHEST 2016; 149(3):704-713

KEY WORDS: asthma; inhaled corticosteroids; interferon; neutrophil; rhinovirus

ABBREVIATIONS: ACQ6 = asthma control questionnaire 6; CXCL = C-X-C motif ligand; ELISA = enzyme-linked immunosorbent assay; HRV = human rhinovirus; ICS = inhaled corticosteroid; IFN = interferon; PBMC = peripheral blood mononuclear cell

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Asthma remains common and continues to cause significant morbidity and mortality even in countries with ready access to the best available therapies such as inhaled corticosteroids (ICS) and long-acting β_2 agonists. Acute exacerbations are major contributors to the personal and societal burden of asthma, with most exacerbations linked to acute infections with human rhinoviruses (HRVs) and other respiratory viruses.¹

Evidence suggests that host defense against respiratory viruses is abnormal in asthma, or at least in a subset of people with asthma.^{2,3} Many investigators report abnormal airway and systemic immune responses to HRVs in asthma; this is thought to facilitate exaggerated lower airway inflammation in response to otherwise minor respiratory viral infections. Viral replication is higher in HRV-infected airway epithelial cells from patients with asthma compared with healthy control subjects, in association with deficient interferon (IFN)- β and IFN- λ synthesis, a finding independent of ICS treatment.^{3,4} HRV-activated alveolar macrophages also produce less IFN- λ than those from healthy individuals.⁴ Similarly, circulating leukocyte populations from patients with asthma are less responsive to RNA viruses and viral nucleic acids than cells from healthy control subjects,^{2,5} producing less IFN- α and exhibiting reduced expression of multiple antiviral signaling pathways.⁶

The concept of abnormal viral defenses in asthma remains controversial, with reports that airway epithelial cells from patients with asthma and healthy individuals produce equivalent IFN- β ,^{7,8} especially in well-controlled asthma.⁹ One study reported that blood mononuclear cells from patients with asthma exhibit normal IFN synthesis *in vitro*,⁹ although Durrani et al¹⁰ demonstrated significant reduction in virus-stimulated

IFN production by blood mononuclear cells isolated from children with asthma. Moreover, this deficient antiviral response was amplified by cross-linking of the high-affinity IgE receptor (Fc ϵ RI).

It is possible therefore that deficient antiviral IFN production is confined to a subset of patients with asthma, perhaps those with the most severe disease,¹¹ or highly sensitized individuals with recent allergen exposure. Such patients are the most prone to acute exacerbations. Asthma is a heterogeneous inflammatory disease that can be categorized into a number of inflammatory subtypes based on the presence or absence of sputum neutrophils and eosinophils, which vary in their inflammatory and immunological profiles and their responsiveness to corticosteroid treatment.¹²⁻¹⁴ This inflammatory response is orchestrated by a plethora of inflammatory mediators, including IL-6 and IL-1 β , which have been described both in asthma in general¹⁵⁻¹⁷ and, more specifically, in neutrophilic asthma.¹⁸⁻²² Little progress has been made in assessing the relationship between these inflammatory phenotypes and antiviral IFN production. Inhaled IFN is currently being developed to ameliorate virus-induced asthma exacerbations, an approach that appears most effective in a subset of patients with difficult-to-treat asthma.²³ There is a need to identify which patients exhibit deficient antiviral IFN production, as they should belong to the specific subgroup that might respond best to inhaled IFN.

The aim of this study was to examine systemic antiviral IFN production in a large group of patients with exacerbation-prone, poorly controlled asthma. Our hypothesis was that HRV-induced IFN- α and IFN- β production would vary across different inflammatory phenotypes.

Methods

Study Design and Participants

This cross-sectional study assessed eligible adults with asthma ($n = 86$) before commencement of study medication as part of a larger multicenter randomized controlled trial. The diagnosis of asthma was established using the American Thoracic Society guidelines as

outlined in the [supplementary material](#). Participants with asthma were stable but symptomatic, despite being prescribed maintenance ICS treatment with an Asthma Control Questionnaire 6 (ACQ6) score >0.75 .²⁴⁻²⁶ Participants had no reported exacerbations or alterations in respiratory medications in the previous 4 weeks. Exclusion criteria and patient assessment details can be found in the [supplementary material](#). Local institutional review boards approved the protocol (detail in [supplementary material](#)), and written informed consent was obtained from all patients.

Sputum Induction and Analysis

Spirometry (CPFS/D USB Spirometer, BreezeSuite v7.1, MGC Diagnostics) was performed, venous blood samples were collected, and sputum was induced by hypertonic saline (4.5%) inhalation as described by Gibson et al.²⁷

Sputum was processed as described,²⁷ with further detail in the [e-Appendix 1](#). Asthma subtypes were defined using sputum

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eosinophils and neutrophils as previously described with the cut point for eosinophilic asthma being a sputum eosinophil count $\geq 3\%$ and the cut point for neutrophilic asthma being a sputum neutrophil count $>61\%$.²²

Isolation and Culture of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by density gradient centrifugation as described⁵; see [Supplementary Methods](#) for further details. All cultures were performed in RPMI 1640 medium (Life Technologies) with 5% autologous plasma and penicillin/streptomycin/glutamine (Life Technologies). PBMCs were cultured with either medium alone or live HRV1b (multiplicity of infection, 0.1) for 24 hours. Supernatant was harvested and assayed by enzyme-linked immunosorbent assay (ELISA). C-X-C motif ligand (CXCL) 10 (also known as IFN- γ -induced protein 10 [IP-10]), CXCL8, and IL-6 were measured by sandwich ELISA using paired antibody sets (BD Biosciences). The limit of detection was 3.9 pg/mL. Commercially available sandwich ELISA kits were used to assay IFN- α (PBL Assay Science) and IFN- β ([elisakit.com](#)). The limits of detection were 12.5 pg/mL and 1.9 pg/mL, respectively. In samples in which the cytokine concentration was below the lower limit of detection for that assay, it was not possible to

determine whether the true cytokine was zero, so the concentration was arbitrarily assigned to be half the lower limit of detection.

Rhinovirus Generation and Titration

Rhinovirus was propagated by passage in Ohio HeLa cells as outlined previously,²⁸ followed by purification with iodixanol solution (Sigma-Aldrich). The 50% tissue culture infective dose was determined by titration in Ohio HeLa cells as described by Carroll et al.²⁹

Effects of IL-1 β and IL-6 on Rhinovirus-Stimulated Interferon Production

PBMCs from 9 healthy volunteers (median age, 35 years; range, 25–61 years) were incubated with IL-6 or IL-1 β (BD Biosciences) for 4 hours before addition of HRV1b (multiplicity of infection, 0.1). After 24 hours, supernatant was harvested and assayed by ELISA.

Statistical Analyses

The results are expressed as mean $\pm$ SD for continuous variables and median with interquartile range when data were not normally distributed, with further detail on the analysis methods described in the [Supplementary Methods](#).

Results

Study participants comprised 86 adult patients with asthma (mean age, 59 years) who were predominantly atopic (76%) with a mean ACQ6 score of 1.7 ([Table 1](#)). All except 3 participants were taking ICS in combination with long-acting β_2 agonists.

Based on induced sputum cell count, 35 participants (41%) each had an eosinophilic asthma phenotype, 35 (41%) had paucigranulocytic, 12 (14%) had a neutrophilic phenotype, and 4 (5%) had a mixed eosinophilic/neutrophilic phenotype. Because of the small number of participants with mixed granulocytic asthma, they were not included in this analysis. The clinical characteristics along with the peripheral blood lymphocyte and monocyte counts according to inflammatory phenotype are shown in [e-Table 1](#). Participants with neutrophilic asthma had a significantly lower FEV₁ % predicted and few peripheral blood lymphocytes compared with participants with eosinophilic asthma. As expected, participants with neutrophilic asthma had a significantly higher total number of sputum leukocytes and proportions of neutrophils ([Table 2](#)). There was no difference in smoking status or smoking pack-years between inflammatory phenotypes ($P > .05$, data not shown). Sputum IL-1 β was assessed in 80 samples (when sufficient supernatant was available) and was significantly higher in participants with neutrophilic asthma than in those with eosinophilic and paucigranulocytic asthma. Serum IL-6 was assessed in 85 participants; results varied significantly across the four inflammatory phenotypes,

with the highest concentrations observed in the neutrophilic and mixed granulocytic asthma subtypes ([Table 2](#)).

TABLE 1] Participant Characteristics

Characteristic	Result (n = 86)
Age, mean (range), y	59 (20–80)
Sex, female (%)	54 (63)
Atopy, n (%)	65 (76)
Ex-smoker, n (%)	43 (52)
Pack-years	20 (6, 40)
FEV ₁ , mean (SD), % predicted	79.8 (17.7)
FEV ₁ /FVC, mean (SD), %	70.9 (10.2)
ACQ6, mean (SD)	1.7 (0.8)
ICS dose, μ g/d	1,180 (800, 2,000)
Courses of oral corticosteroids for an asthma exacerbation in the past year	1 (0, 2)
Serum IL-6, pg/mL	1.9 (1.3, 2.7)
Sputum inflammatory cell counts	
Total cell count, $\times 10^6$ /mL	4.0 (2.6, 9.0)
Viability, %	81 (70, 85)
Neutrophils, %	38.4 (21.0, 53.3)
Eosinophils, %	1.5 (0.3, 9.0)
Macrophages, %	44.5 (31.0, 60.5)
Lymphocytes, %	0.5 (0.0, 1.0)
Columnar epithelial cells, %	1.6 (0.5, 4.8)
Squamous, %	4.9 (2.0, 9.5)
Sputum IL-1 β , pg/mL (n = 80)	13.5 (5.8, 81.1)

Data are presented as median (q1, q3), unless indicated otherwise. ACQ6 = asthma control questionnaire 6; ICS = inhaled corticosteroid.

TABLE 2] Inflammatory Cell Counts and Mediators by Asthma Inflammatory Phenotype

Variable	Eosinophilic (n = 35)	Paucigranulocytic (n = 35)	Neutrophilic (n = 12)	Mixed Granulocytic (n = 4)	P Value
Total cell count, $\times 10^6/\text{mL}$	3.4 (2.6, 7.7)	3.3 (2.1, 9.8)	17.8 (4.2, 28.3) ^{a,b}	7.4 (4.3, 8.7)	.001
Viability, %	78 (70, 85)	80 (470, 83)	88 (80, 97)	74 (66, 79)	.065
Neutrophils, %	30.4 (20.5, 42.5)	32.5 (16.3, 49.3)	85.4 (68.6, 90.9) ^{a,b}	65 (61.5, 82.0) ^{a,b}	<.001
Eosinophils, %	9.3 (4.0, 34.0)	0.3 (0.0, 1.0) ^a	0.6 (0.3, 1.0) ^a	4.5 (3.3, 13.3) ^b	<.001
Macrophages, %	41.0 (30.8, 58.8)	58.0 (47.5, 78.8)	12.8 (6.3, 28.4) ^{a,b}	17.7 (3.4, 30.3) ^b	<.001
Lymphocytes, %	0.5 (0.0, 1.0)	0.25 (0.0, 1.0)	0.5 (0.0, 1.5)	0.3 (0.1, 0.5)	.906
Columnar epithelial cells, %	1.8 (0.3, 5.5)	2.0 (1.0, 8.0)	0.0 (0.0, 1.2)	0.3 (0.3, 0.3)	.070
Squamous, %	5.0 (2.2, 9.5)	5.0 (2.9, 13.6)	1.0 (0.4, 9.3)	3.8 (0.5, 21.8)	.163
Sputum IL-1 β , pg/mL (n = 80)	10.3 (5.6, 26.1)	11.9 (4.7, 58.4)	163.4 (82.0, 1,090) ^{a,b}	200, 430 (n = 2)	<.001
Serum IL-6, pg/mL (n = 85)	1.9 (1.3, 2.4)	1.8 (1.3, 2.7)	2.6 (1.9, 3.8)	2.7 (2.5, 2.9)	.038

Data are median (q1, q3).

^a $P < .008$ vs eosinophilic asthma.

^b $P < .008$ vs paucigranulocytic asthma.

Response of Peripheral Blood Mononuclear Cells to Human Rhinovirus 1b Exposure

The cytokine release from all participants is shown in [e-Figure 1](#). PBMCs from all participants responded to HRV1b exposure, releasing IFN- α and CXCL10 in excess of media alone. HRV1b-activated PBMCs released IFN- β in 90% of participants, IL-6 in 71%, IL-1 β in 60%, and CXCL8 in 46%. These percentages were similar across the different inflammatory phenotypes. The median values and first and third quartiles for PBMCs incubated with medium alone are shown in [e-Table 2](#).

[Figure 1](#) shows the cytokine levels released in response to HRV1b stimulation, corrected for baseline (unstimulated) cytokine values. This was calculated by subtracting the baseline cytokine result from the HRV1b-stimulated result. Therefore a zero data point indicates that no additional release of cytokine was observed above the unstimulated levels measured from the control wells. Further details of the data are shown in [e-Figure 1](#). HRV1b-activated PBMCs from participants with neutrophilic asthma released significantly less IFN- α and IFN- β than PBMCs from those with eosinophilic and paucigranulocytic asthma (post-hoc rank-sum $P = .002$ and $P = .022$, respectively). Although HRV1b-activated PBMCs from participants with neutrophilic asthma also tended to release less CXCL10, CXCL8, and IL-6 than PBMCs from participants with other inflammatory subtypes, these differences were not statistically significant.

Clinical and Inflammatory Associations With Human Rhinovirus 1b-Induced Interferon- α Release

IFN- α release from HRV1b-activated PBMCs was significantly inversely related to the dose of ICS ([Fig 2A](#)), asthma control (as assessed by ACQ6 score, [Fig 2B](#)), proportion of sputum neutrophils ([Fig 2C](#)), serum IL-6 ([Fig 2D](#)), and sputum IL-1 β ([Fig 2E](#)) and was positively associated with atopy (as assessed by allergy skin test [[Fig 2F](#)]) and the number of peripheral blood lymphocytes ([Fig 2G](#)) before randomization. There was no significant association with age, sex, or smoking pack-years.

Using stepwise multiple linear regression, the proportion of sputum neutrophils and the dose of ICS were the only significant independent predictors of IFN- α release after correction for age and sex ([Table 3](#)). The model included 79 data points and explained 27% of the variance in released IFN- α with $P < .0001$.

Effect of IL-1 β on the Response of Peripheral Blood Mononuclear Cells to Human Rhinovirus 1b

Because neutrophilic asthma has been linked to overexpression of the IL-1 β pathway,²² and because we observed that serum IL-6 ([Fig 2](#)) and sputum IL-1 β were inversely associated with HRV-induced IFN- α in the current study, we next explored the potential impact of IL-1 β on the ability of HRV1b-activated PBMCs to synthesize type I IFN in vitro. Preexposure of PBMCs

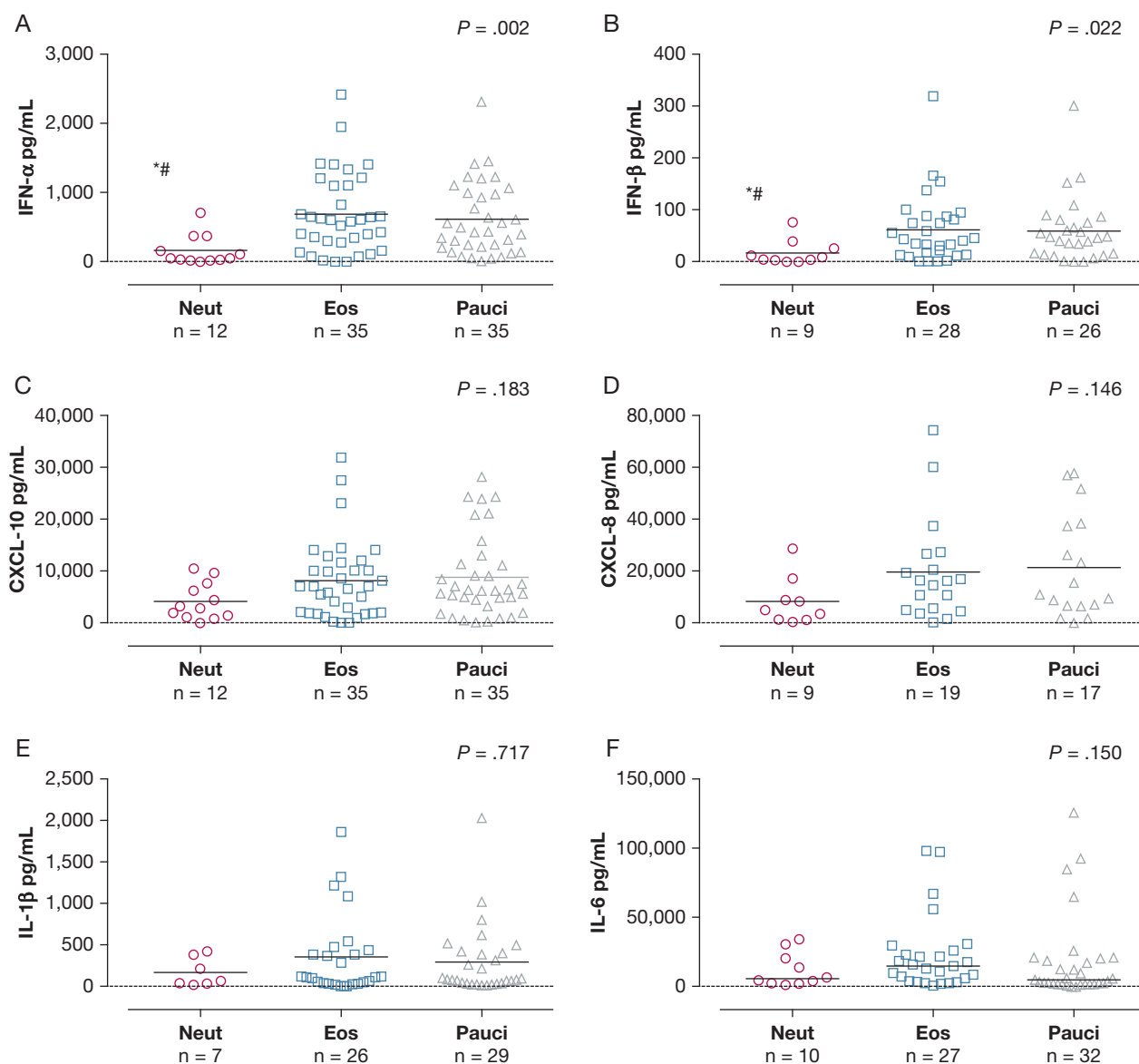


Figure 1 – Inflammatory cytokine levels in supernatant from human rhinovirus 1b-activated, cultured peripheral blood mononuclear cells are shown (data corrected for baseline unstimulated levels). Bars represent median levels for IFN- α (A), IFN- β (B), CXCL10 (C), CXCL8 (D), IL-1 β (E), and IL-6 (F). The number of participants for each group is below the inflammatory subtype in each panel; the dotted line shows the zero level for each cytokine. Results for all three groups were compared by Kruskal-Wallis test and indicated by the probability value in each panel. Significant differences between any two groups ($P < .05$ by post-hoc rank-sum test) are indicated by * compared with eosinophilic asthma and # compared with paucigranulocytic asthma. CXCL = C-X-C motif ligand; Eos = eosinophilic asthma; IFN = interferon; Neut = neutrophilic asthma; Pauci = paucigranulocytic asthma.

from healthy donors to IL-1 β significantly reduced IFN- α and IFN- β release after HRV exposure (Fig 3). In contrast, IL-6 had no effect on HRV-stimulated IFN- α and IFN- β release (data not shown).

Discussion

The key finding to emerge from the present study is that HRV1b-activated PBMCs from adults with neutrophilic asthma have a significantly lower capacity to produce both IFN- α and IFN- β than PBMCs from adults with

eosinophilic and paucigranulocytic asthma. Using stepwise multiple linear regressions, we identified both sputum neutrophil proportion and ICS dose as independent predictors of type I IFN production.

To the best of our knowledge this is the largest study to date of antiviral IFN production in poorly controlled asthma and the first to systematically examine the relationship between type I IFN production and asthma inflammatory phenotypes. IFN- α and IFN- β release was lowest in those participants with neutrophilic asthma,

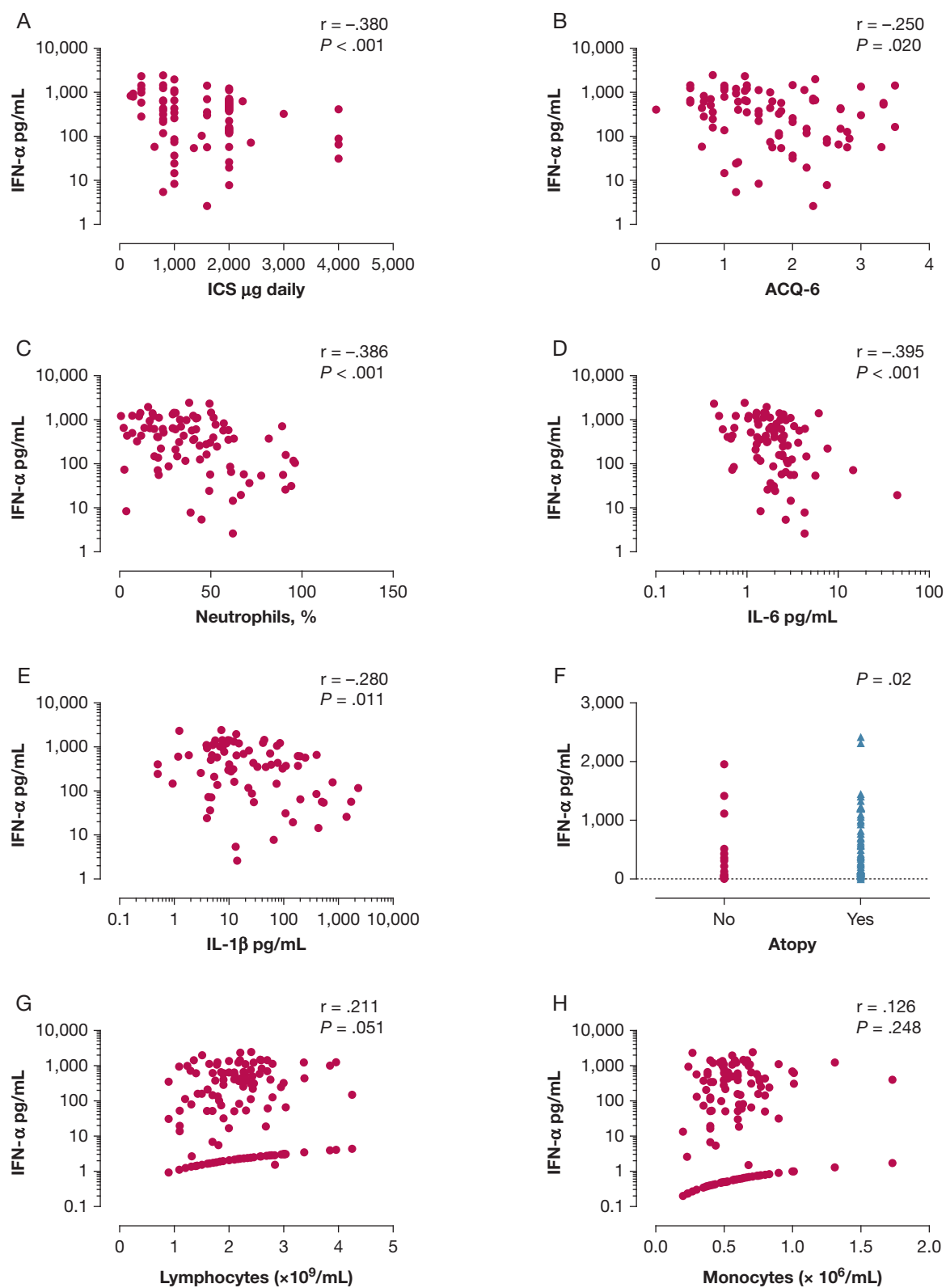


Figure 2 – Scatter plots of IFN production stimulated by human rhinovirus 1b selected for ICS total daily dose (A), mean ACQ-6 score (B), sputum neutrophil % (C), serum IL-6 (D), sputum IL-1 β (E), atopy (F), blood lymphocytes (G), and blood monocytes (H). Spearman correlation coefficient (r) and probability values are as indicated. The comparison between atopic and nonatopic participants was assessed using Mann-Whitney test. ACQ-6 = asthma control questionnaire 6; ICS = inhaled corticosteroid. See [Figure 1](#) legend for expansion of other abbreviation.

TABLE 3] Univariate and Stepwise Multivariate Regression Analysis

Variable	Coefficient	SE	P Value	95% CI	
Univariate					
Age	−0.104	0.087	.239	−0.277	0.070
Sex	2.880	2.603	.272	−2.296	8.056
Atopy	6.347	2.939	.034	0.501	12.193
ACQ6	−3.222	1.541	.040	−6.286	−0.158
ICS dose	−0.050	0.001	<.001	−0.008	−0.002
Neutrophils, %	−0.177	0.049	<.001	−0.274	−0.081
Blood lymphocytes, × 10 ⁹ /mL ^a	3.503	1.903	.069	−2.810	7.286
Serum IL-6, pg/mL	−0.573	0.253	.026	−1.075	−0.070
Sputum IL-1β, pg/mL	−0.010	0.003	.005	−0.017	−0.003
Multivariate					
Age	−0.015	0.081	.857	−0.177	0.147
Sex	1.749	2.367	.462	−2.968	6.466
ICS dose	−0.005	0.001	.001	−0.007	−0.002
Neutrophils, %	−0.170	0.048	.001	−0.266	−0.075
Atopy	4.698	2.816	.100	−0.915	10.310
Coefficient	28.904	7.848	<.001	13.263	44.545

The model included 79 data points and explained 27% of the variance in IFN-β levels and was highly significant at $P < .0001$. ACQ6 = asthma control questionnaire 6; ICS = inhaled corticosteroid.

^aPeripheral blood lymphocytes were assessed 2–4 weeks before randomization.

a phenotype that made up 14% of this cohort. These participants were characterized by an elevated total number of sputum leukocytes, increased sputum neutrophil proportions, and significantly elevated sputum IL-1β. Release of several other proinflammatory chemokines and cytokines by HRV-activated PBMC also tended to be lower in participants with neutrophilic asthma, although the differences did not reach statistical significance. Serum IL-6 as a marker of systemic inflammation was readily detectable across all

participants, regardless of inflammatory phenotypes, but was highest in those with elevated sputum neutrophils.

IFN-α and IFN-β form part of the first line of defense against viral infections. These IFNs are produced both by airway resident cells and by circulating, bone marrow-derived cells that provide a reservoir that can be rapidly recruited to the lung during viral infection. HRV-stimulated PBMCs readily produce IFN-α and IFN-β, the vast majority of which arises from plasmacytoid

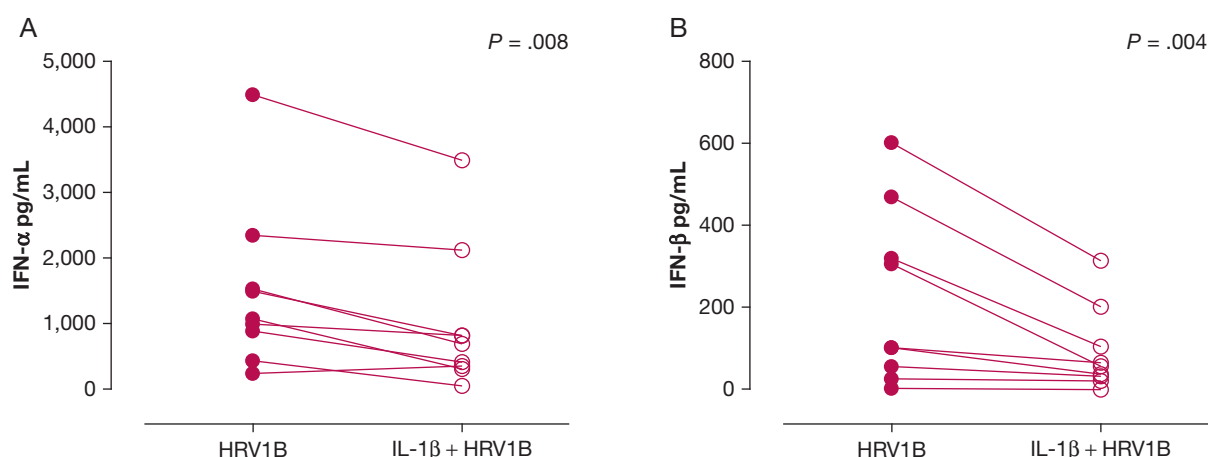


Figure 3 – Effects of IL-1β on rhinovirus-stimulated IFN-α (A) and IFN-β (B) production from healthy control peripheral blood mononuclear cells. HRV = human rhinovirus. See Figure 1 legend for expansion of other abbreviation.

dendritic cells and to a lesser extent from monocytes.^{6,30} In vivo, acute respiratory viral infections induce specific gene expression signatures in peripheral blood,³¹ and emerging data indicate that HRV-stimulated PBMCs exhibit gene expression signatures highly correlated with results obtained during in vivo HRV infections.³² Reduced IFN- α release from PBMCs has been reported in children with asthma,³³ in adults with asthma,⁶ and in women with asthma both during pregnancy and postpartum.³⁴ Wright et al³⁵ recently reported an asthma-specific impairment in toll-like receptor-9-dependent IFN- α production by PBMCs that was not observed in those with COPD or healthy control subjects. This finding is particularly interesting given that the asthma and COPD groups that were studied were both taking high doses of ICS and the participants with asthma were of similar severity to those studied in the present study. Airway epithelial cells from children with severe asthma also show impaired type I IFN production,¹¹ whereas some studies report that airway epithelial cells from adults with well-controlled asthma exhibit type I IFN production that is similar to that of healthy subjects.⁹ One recent study indicated that impaired type I IFN synthesis in asthma was confined to BAL cells, whereas IFN synthesis by rhinovirus-activated blood mononuclear cells was similar in patients with asthma and in healthy subjects.³⁶ Most of the participants in that study had relatively well-preserved lung function and mild eosinophilia in BAL fluid; no participants appeared to have had neutrophilic airway inflammation.³⁶ Our findings extend these observations, to show that patients with asthma who are most likely to have impaired type I IFN production are those with a neutrophilic inflammatory subtype. Our findings contrast with a smaller study of 25 patients with severe asthma that did not find a relationship between sputum differential leukocyte counts and IFN- α production³⁵; sample size and statistical power may explain the discrepancy between the two studies.

The finding that ICS dose was inversely associated with IFN synthesis use (Table 3) warrants discussion. Bratke et al³⁷ showed that toll-like receptor-9 mediated IFN- α secretion from plasmacytoid dendritic cells in asthma was lower in those taking ICS than in those not taking ICS. It is conceivable that systemic absorption of high doses of ICS might inhibit the capacity of HRV-stimulated PBMCs to synthesize IFN- α . We have shown that such inhibition can occur in vitro, but only at steroid concentrations of 10^{-8} M or more³⁸; such concentrations are unlikely to have been present systemically in the present study. Moreover, as discussed

earlier, others have noted that ICS use in asthma, but not in COPD, is associated with deficient IFN- α production by PBMCs.³⁵ We propose that the association between high-dose ICS and reduced IFN- α production is indirect, in that patients with noneosinophilic airway inflammation are often refractory to standard ICS doses, but are treated with high ICS doses nonetheless. High-dose ICS use is probably a marker of asthma severity, and direct pharmacological effects of ICS on IFN production are probably minor. A future study to examine the effects of ICS dose reduction on airway inflammation and capacity for virus-induced IFN synthesis would shed light on these uncertainties.

The mechanisms leading to reduced IFN- α and IFN- β synthesis by circulating leukocytes in neutrophilic asthma remain uncertain. One possibility is that patients with neutrophilic asthma carry an intrinsic (possibly germ line) defect in IFN- α or IFN- β synthesis, affecting all cell types, which contributes to the pathogenesis of neutrophilic airway inflammation. Although an interesting concept, currently there is little supporting evidence. Alternatively, neutrophilic airway inflammation may alter the function of circulating cells. Systemic inflammation appears to be prominent in neutrophilic asthma, relative to other asthma phenotypes,¹⁹ and perhaps this may alter the function of immune cell progenitors in bone marrow. In the present study we observed that IFN- α synthesis was inversely proportional to sputum IL-1 β and serum IL-6 by univariate analysis (Table 3), although this association was no longer significant by stepwise multiple linear regression. Initial experiments with cells from healthy donors showed that IL-1 β , but not IL-6, was able to inhibit HRV-stimulated IFN production in vitro (Fig 3). Although this provides proof of concept that such an effect may occur in vivo, further studies are required.

There are a number of limitations of the current study that should be acknowledged. The cross-sectional design means we are limited in our ability to assign cause and effect. At this stage we do not know the clinical relevance of our findings, and it will be important for future studies to determine whether the subset of asthma patients with the lowest capacity for IFN- α and IFN- β synthesis are at higher risk of asthma exacerbations. Our cohort are a group of older adults with poorly controlled asthma, and further studies are required to understand whether these differences apply to younger adults and children, although the nature of neutrophilic asthma is that it is associated with older age and therefore is less prevalent in younger adults and children with asthma.

Although a recent study found no relationship between impaired IFN- α release in severe asthma and exacerbation frequency, that study may have been underpowered to assess exacerbations as an end point.³⁵ It is also possible that the mechanism by which sputum eosinophilia is linked to asthma exacerbations may be independent of type I IFN. Neutrophilic airway inflammation has been linked to poor asthma outcomes, in particular more severe airflow obstruction, and our results suggest that this may be partly related to low type I IFN production. A prospective study is needed to examine the link between type I IFN and virus-associated asthma exacerbations and secondly, to explore the underlying mechanisms. Although we have studied HRV 1b, which is an HRV type A, evidence suggests responses to other serotypes of rhinovirus may be different,^{39,40} and future studies will also need to

examine IFN production in response to a wider range of HRVs. Finally, we recognize the absence of a group of age-matched healthy subjects in this study, but justify this on the basis that the primary aim of the study was to examine variations in IFN- α and IFN- β production between different asthma phenotypes, rather than comparing asthma with healthy subjects. An important strength of our study is the inclusion of a large number of carefully characterized participants with poorly controlled asthma.

In conclusion, antiviral IFN- α and IFN- β production was impaired in patients with neutrophilic airway inflammation and in those prescribed high doses of ICS. Our study is therefore an important step toward identifying those with poorly controlled asthma who might respond best to inhaled IFN during exacerbations.

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Additional information: The e-Appendix, e-Figure, and e-Tables can be found in the Supplemental Materials section of the online article.

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Reduced Antiviral Interferon Production in Poorly Controlled Asthma Is Associated With Neutrophilic Inflammation and High-Dose Inhaled Corticosteroids

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e-Appendix 1.***Supplementary material****Study design and participants*

The diagnosis of asthma was established using the American Thoracic Society guidelines based upon the presence of current episodic respiratory symptoms in the preceding 12 months, a doctor's diagnosis and evidence of variable airflow obstruction ¹⁴ (airway hyperresponsiveness, bronchodilator response or diurnal variation of peak expiratory flow).

Committee names and approval numbers

The samples were obtained from a number of centres across Australia. The research was approved by the following ethics committees (approval number is given in brackets)

Sir Charles Gairdner Group Human Research ethics committee (2008-147)

Royal Adelaide Hospital Human Research Ethics Committee (081108f)

Hunter New England Research Ethics Committee (08/11/19/3.03)

The Prince Charles Hospital Metro North Hospital and Health Services (HREC/08/QPCH/4)

Metro South Health Service District Human Research Ethics Committee (HREC/09/QPAH/015)

Assessments

Participants underwent a clinical assessment, allergy skin test (described below), spirometry with bronchodilator response, sputum induction and blood sampling in turn. All tests were performed on the same day. Measurements were carried out by observers blinded to other results.

Exclusion criteria

Participants with an FEV₁ <40% predicted, current smokers, ex-smokers who had ceased smoking in the previous year and those with a recent (past four weeks) exacerbation or respiratory infection were excluded. Those with significant smoking related air-space disease (ex-smokers >10 pack year history and DLCO/VA <70% predicted OR smoking history >10 pack years and exhaled carbon monoxide >10ppm) were also excluded.

Allergy skin test

Atopy was determined by the presence of at least one immediate reaction to skin-prick testing (wheal > 3mm) to common allergens Grass Mix #7, D. Pteronyssinus, Cat and Dog Hair, Mould Mix #10, Aspergillus fumigatus (EBOS Healthcare, Kingsgrove NSW Australia). Participants were asked to withhold any antihistamine medication for the five days prior to the test.

Smoking assessment

A smoking history was taken and smoking pack-years determined. Participants underwent exhaled carbon monoxide (eCO) measurements, determined by electrochemical detection with a Smokerlyzer® (Bedfont Scientific Ltd, Kent, UK; detection limit of 1ppb). All included participants had an eCO of less than 10ppm confirming their non-smoking status ³³.

Sputum induction and analysis

Selected sputum portions were dispersed by dithiothreitol (DTT) (Merck Millipore, Darmstadt, Germany) and a total cell count performed. Cytospins were prepared and stained with May-Grünwald Giemsa. Cell counts were performed on 400 non-squamous cells. Cell viability and differential cell counts were recorded.

Blood sampling, PBMC isolation and culture

To provide consistency, PBMC separation, cryopreservation and culturing were performed at a single site [Princess Alexandra Hospital (PAH) in Brisbane]. After collection, blood samples were transferred by courier to the PAH laboratory, arriving within 24 hours of collection. PBMC were prepared by density gradient centrifugation and cryopreserved in liquid nitrogen for later batch analysis. Techniques for cryopreservation and thawing have been optimised and used extensively in the laboratory of JWU over many years. Such techniques are commonly used in large-scale multi-centre studies of immunity to vaccines.

Statistical analysis

Categorical data are reported using frequencies and percentages. A Kruskal-Wallis test was performed in the different subgroups of subjects with asthma, followed by (post-hoc rank sum test). Spearman's rank correlation coefficient was used to assess the associations between non-parametric data. Multiple linear regression was conducted using a stepwise regression with the significance level for removal from the model was $p=0.100$ and the significance level for addition to the model was $p=0.050$. Age and sex were included in the multiple linear regression model and other variables were included when $p<0.200$ in univariate regression. A Mann-Whitney test was performed to compare the IFN α production in atopy vs non-atopy. Results were reported as significant when $p<0.05$.

Results

e-Table 1 is shown below

e-Table 1: Clinical characteristics of the asthmatic subjects classified based in their inflammatory phenotype.

	Eosinophilic	Paucigranulocytic	Neutrophilic	Mixed Granulocytic	p value
N	35	35	12	4	
Age, (years) mean (range)	57 (20 – 76)	59 (25 – 80)	63 (39- 79)	66 (46 – 78)	0.930
Sex, female (%)	25 (71)	22 (63)	5 (42)	2 (50)	0.270
Atopy, n (%)	26 (77)	28 (80)	9 (75.0)	2 (50)	0.562
Ex-smoker, n (%)	19 (58)	17 (50)	6 (50)	1 (25)	0.661
Pack years, median (q1,q3)	15 (6, 30)	15 (4, 57)	41 (25, 65)	26	0.488
FEV ₁ (% predicted), mean (SD)	84.5 (17.0)	78.7 (16.6)	67.5 (15.8)*	85.9 (24.8)	0.027
FEV ₁ /FVC (%), mean (SD)	0.70 (0.09)	0.71 (0.10)	0.69 (0.12)	0.74 (0.12)	0.818
ACQ6, mean (SD)	1.57 (0.99)	1.77 (0.68)	1.82 (0.46)	1.53 (0.89)	0.670
ICS dose (micrograms/day), median (q1,q3)	1000 (800, 2000)	1000 (800, 2000)	2000 (1480, 2000)	1250 (820, 2750)	0.217
Courses of oral corticosteroids for asthma exacerbation in the past year, median (q1, q3)	1 (0, 3)	0 (0, 2)	0 (0, 1.5)	1 (0.5, 2.5)	0.275
Peripheral blood lymphocytes x 10 ⁹ /L, median (q1, q3)	2.20 (1.81, 2.56)	2.21 (1.79, 2.8)	1.51 (1.25, 1.82)#	2.06 (1.46, 2.51)	0.003
Peripheral blood monocytes x 10 ⁹ /L, median (q1, q3)	0.60 (0.50, 0.70)	0.56 (0.40, 0.70)	0.50 (0.40, 0.67)	0.445 (0.305, 0.555)	0.265

FEV₁ percent predicted refers to the forced expiratory volume in 1 sec expressed as a percentage of the predicted value; ICS dose expressed as median refers to the total micrograms per day of beclomethasone equivalents.

*p < 0.05 compared with eosinophilic asthma (Bonferroni), # p<0.008 rank sum compared with eosinophilic asthma

e-Table 2: Cytokine production (pg/ml) by unstimulated PBMC (Median, q1, q3)

	IFN α	IFN β	CXCL-10	CXCL-8	IL-1 β	IL-6
Neut	3.2 (0.75, 16.9)	2.2 (1.0, 5.7)	61.5 (29.1 , 118.6)	14783 (7202, 40331)	99.0 (18.2, 577.4)	2916 (152.5 , 66257)
Eos	1.6 (0.0, 6.0)	2.6 (1.0 , 8.0)	57.1 (15.6, 217.4)	15554 (7732, 41216)	47.0 (10.3 , 179.4)	983.3 (83.2, 11062)
Pauci	1.05 (0.0 , 3.05)	3.6 (1.0, 10.23)	84.3 (26.6 , 254.7)	13346 (6736, 44667)	20.8 (8.1, 73.9)	871.4 (132.5, 5828)

e-Figure 1: Inflammatory cytokine levels in supernatant from PBMC cultured with HRV1b. Bars represent median levels for for A) IFN- α B) IFN- β C) CXCL10 D) CXCL8 E) IL-1 β F) IL-6. The inflammatory subtypes have been abbreviated from neutrophilic to neut, eosinophilic to eos and paucigranulocytic to pauci. The p value on each panel shows the Kruskal-Wallis results in which results for all 3 groups were compared. Significant differences between any two groups are indicated by * compared with eosinophilic asthma and # p<0.05 compared with paucigranulocytic asthma (post-hoc ranksum test). Dotted lines show the median values for PBMC incubated with media alone.

