Phenotype and severity of asthma determines bronchial epithelial immune responses to a viral mimic

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The airway epithelial pro-inflammatory and anti-viral response to a viral stimulus differs with the phenotype and severity of asthma, indicating differences in immune drivers of exacerbations
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Abstract

Background Asthma is characterised by an aggravated immune response to respiratory viral infections. This phenomenon is a clinically well-recognised driver of acute exacerbations, but how different phenotypes of asthma respond immunologically to viruses is unclear.

Objectives To describe the association between different phenotypes and severity of asthma and bronchial epithelial immune responses to viral stimulation.

Methods In the Immunoreact study, healthy subjects (n=10) and 50 patients with asthma were included; 30 (60%) were atopic, and 34 (68%) were eosinophilic; 14 (28%) had severe asthma. All participants underwent bronchoscopy with collection of bronchial brushings. Bronchial epithelial cells (BECs) were expanded and stimulated with the viral replication mimic poly (I:C) (Toll-like receptor (TLR)3 agonist) *in vitro*. The expression of TLR3-induced pro-inflammatory and antiviral responses of BECs were analysed using reverse transcriptase quantitative PCR and multiplex ELISA and compared across asthma phenotypes and severity of disease.

Results Patients with atopic asthma had increased induction of interleukin (IL)-4, interferon (IFN)-β, IL-6, tumour necrosis factor-α, and IL-1β after poly (I:C) stimulation compared to non-atopic patients, whereas in patients with eosinophilic asthma only IL-6 and IL-8 induction was higher than in non-eosinophilic asthma. Patients with severe asthma displayed a decreased antiviral IFN-β, and increased expression of IL-8, most pronounced in atopic and eosinophilic asthmatics. Furthermore, induction of IL-33 in response to poly (I:C) was increased in severe atopic and in severe eosinophilic asthma, but thymic stromal lymphopoietin only in severe eosinophilic asthma.

Conclusions The bronchial epithelial immune response to a viral mimic stimulation differs between asthma phenotypes and severities, which may be important to consider when targeting novel asthma treatments.

Introduction

Acute exacerbations of asthma are associated with a high burden of morbidity, and reducing exacerbations is a key treatment management goal in asthma [1]. An important, but unresolved, question is why some asthma patients develop frequent exacerbations. A defining feature of exacerbation-prone asthma is an increased inflammatory response to triggers, with rhinovirus being the most common cause of exacerbations [2]. Understanding the mechanisms involved in this increased airway immune responsiveness is important for developing better asthma treatments.

The airway epithelium has become recognised as a central orchestrator of the inflammatory cascade induced during asthma exacerbations [3, 4], and a potential treatment target [5]. In asthma patients, viral infection induces the release of the epithelial cytokines thymic stromal lymphopoietin (TSLP), interleukin (IL)-33 and IL-25, all of which can generate the T2 inflammatory response involved in acute exacerbations, including release of IL-5, IL-4 and IL-13 [6–9]. Importantly, the epithelial cytokines may induce eosinophilic inflammation in non-atopic as well as atopic asthma, *via* the activation of type 2 innate lymphoid cells [10]. Furthermore, rhinovirus activates pattern recognition receptors, including Toll-like receptor (TLR)3, retinoic acid-inducible gene-I and melanoma differentiation-associated (MDA)-5, leading to release of cytokines, chemokines and antimicrobial peptides that attract and activate innate and adaptive immune cells [11].

In combination with the exacerbated inflammatory response to rhinovirus, asthma is associated with an impaired antiviral defence: in particular, the production of interferons (IFNs) in response to viruses has been shown to be reduced in asthma, resulting in increased viral replication and a protracted inflammatory response [12–14]. However, the evidence is slightly conflicting, with some studies not finding impaired IFN responses, which could reflect that it is only a feature in certain asthma phenotypes.

Overall, associations between airway bronchial epithelial responses to virus and different phenotypes of asthma are not well understood. Atopic asthma has been linked to an increased risk of virus-induced exacerbations, probably mediated *via* an aberrant epithelial inflammatory response [12, 15], which is further exacerbated by allergen exposure [16]. But for other phenotypes, such as the exacerbation-prone eosinophilic asthma, it is not clear to which extent exacerbations are triggered by virus-induced bronchial epithelial immune responses. Experimentally, T2 cytokines including IL-4 and IL-13 have been shown to reduce induction of IFN-β on infection [12], but no studies have so far compared the epithelial response in patients with eosinophilic *versus* non-eosinophilic asthma.

In this study, we hypothesised that the phenotype (allergic or eosinophilic asthma) as well as the severity of asthma are determinants of the epithelial immune response to a viral stimulus. To explore this hypothesis, we obtained bronchial epithelial cells (BECs) from adult healthy subjects and asthma patients and compared the *in vitro* response to a virus infection mimic and TLR-3 agonist, poly (I:C), across different phenotypes and asthma severities.

Methods

In the Immunoreact study, BECs obtained from healthy subjects and asthma patients were used to examine the impact of asthma phenotypes and severity on the airway epithelial response to virus. Samples from three studies were included, all approved by the local scientific ethics committee (H-16043663, H-16002008, H-17004938). All patients gave informed consent prior to participating in the studies.

Patient population

All participants with asthma had a confirmed diagnosis of asthma (either a current or historical positive bronchial provocation test with mannitol or methacholine, or significant reversibility to β_2-agonist). Healthy controls did not have any symptoms or signs of asthma or other lung diseases.

Patient examinations

All patients underwent clinical characterisation with spirometry and a bronchial challenge test with inhaled mannitol (patients with forced expiratory volume in 1 s (FEV_1) >70% predicted), assessment of fractional exhaled nitric oxide (F_{eNO}) and blood and induced sputum cell differential count, as well as bronchoscopy with mucosal brushings. All patients filled in a self-administered Danish version of the Asthma Control Questionnaire-5 [17]. For a full description of methods, refer to the supplementary material.

Subgroup comparisons were made based on phenotype (atopic *versus* non-atopic and eosinophilic *versus* non-eosinophilic) as well as severity, using the following definitions.

Atopy was defined as atopic sensitisation with elevated specific IgE or a positive skin-prick test, to at least one aeroallergen in a standard panel of ten aeroallergens (birch (*Betula*), grass (*Phleum pratense*), mugwort, horse, dog, cat (Fel d), house dust mite (Der p 1 and Der f 2) and fungi (*Alternaria* and *Cladosporium*).

Eosinophilia was defined as sputum eosinophilia ⩾3%.

Asthma severity: severe asthma was defined according to the European Respiratory Society/American Thoracic Society criteria; treatment with a daily inhaled corticoisteroid (ICS) dose of ⩾1600 µg budesonide equivalent and at least one second controller (long-acting β-agonist, leukotriene receptor antagonist or long-acting muscarinic antagonist) [18]. Patients were divided into mild, non-ICS treated asthma, and mild–moderate ICS treated asthma (<1600 µg budesonide equivalent).

Experimental assays

BECs were stimulated with 10 $\mu g \cdot mL^{-1}$ poly (I:C) (double-stranded (ds)RNA, TLR3 agonist; Invitrogen, Paisley, UK) for 3 or 24 h, as previously optimised and validated [19]. For further details, please refer to the supplementary material.

RNA isolation and reverse transcriptase quantitative PCR

RNA extraction kit (Nucleospin RNA II; Macherey-Nagel, Düren, Germany) was used for isolation of total RNA from BEC lysates, according to the manufacturer's protocol. RNA concentration was measured with Nanodrop and 1 µg RNA was reversely transcribed by using Precision Nanoscript Reverse Transcription kit (PrimerDesign, Southampton, UK). Finally, mRNA expression was measured by quantitative (q)PCR on an Mx3005P qPCR system (Stratagene, La Jolla, CA, USA). GAPDH and UBC were used as reference genes. All primers used were from PrimerDesign. A list of the primers used in qPCR analyses is shown in supplementary table S1. The $2^{-\Delta\Delta Ct}$ method was used for gene expression analyses [20].

ELISA analysis of released proteins

The analysis of protein release in cell culture supernatants from BECs both at baseline and after poly (I:C) stimulation was performed on the MAGPIX device (R&D Systems, Abingdon, UK), using a Luminex immunoassay (R&D Systems). The proteins measured were IFN-β (the most characteristic type-I IFN involved in the antiviral immune response), the alarmin IL-33, type 2 cytokines (IL-4, IL-5 and IL-13), pro-inflammatory cytokines implicated in virus-induced asthma exacerbations (IL-1β, IL-6 and tumour necrosis factor (TNF)-α) and the eosinophilic and neutrophilic chemo-attractants CCL-5 and IL-8, respectively. All protocols were performed according to manufacturer's instructions. Data was acquired on a calibrated and validated instrument.

Release of thymic stromal lymphopoietin (TSLP) was assessed using an individual ELISA kit (Duoset ELISA kit; R&D Systems) following the manufacturer's instructions. Optical densities were recorded in a Multiskan GO spectrophotometer (Thermo Scientific, Waltham, MA, USA) at 450 nm with a wavelength correction at 540 nm. Protein concentration was calculated from a standard curve.

Statistical analyses

Normality was assessed using Kolmogorov–Smirnov and Shapiro–Wilk tests. When normal, unpaired t-test analyses (two group comparisons) or one-way ANOVA with Holm-Šídák's multiple comparisons test (more than two group comparisons) were used. As non-parametric tests, the Mann–Whitney U-test for two group comparisons or Kruskal–Wallis with Dunn's *post hoc* test when more than two groups were compared, were used. Definitive outliers have been removed using the robust regression followed by outlier identification test with Q set at 0.1%. A p-value <0.05 was considered significant.

Results

Patient characteristics

The characteristics of the 50 patients enrolled in the study are summarised in table 1. Atopy was detected in 60% of patients, and eosinophilia in 59% of patients. 14 patients (28%) had severe asthma, 16 patients (32%) had mild–moderate asthma, defined as low–moderate doses of ICS, and the remaining 20 (40%) patients had mild asthma, without ICS use. Severe asthma was associated with more frequent exacerbations, higher symptom scores and lower lung function, as well as higher levels of sputum eosinophils and F_{eNO} (table 1).

When comparing characteristics between different asthma phenotypes, atopic asthma was associated with a higher exacerbation rate compared to non-atopic asthma, but the two groups were otherwise comparable (data not shown). Patients with eosinophilic asthma had more airflow obstruction (lower FEV_1/forced vital capacity ratio), were older and had a higher body mass index, but were otherwise comparable to patients with non-eosinophilic asthma.

TABLE 1 Immunoreact study population characteristics (n=50)

	Mild non-ICS treated asthma	Mild–moderate ICS-treated asthma	Severe asthma	p-value
Patients	20	16	14	
Age years	30.1±12.4	42±18.1	49.6±12.5	0.001
Female %	60	62.5	35.7	0.276
BMI kg·m^{-2}	24.0±4.16	28.4±5.2	28.12±4.92	0.012
Smoking status (never-smoker, ex-smoker, current smoker)	16/4/0	12/4/0	3/10/1	0.006
Smoking pack-years	0.73±2.05	1.07±2.11	12.5±14	0.000
Age at onset	14 (0–57)	23 (2–33)	16 (0–57)	0.942
ACQ-5 score	1.43±1.22	2.27±0.69	2.91±0.94	<0.05
Asthma exacerbation history (past 12 months)				
Emergency room visit or hospital admission	0.05±0.22	0.19±0.40	0.36±0.50	0.073
OCS courses	0.05±0.22	0.38±0.50	0.93±1.1	0.002
Medications				
ICS %	0	100	100	>0.001
ICS budesonide equivalent[#]	0±0	750±287.52	1661±221.88	0.000
LABA %	0	68.8	92.9	0.000
LTRA %	0	12.5	50	0.009
LAMA %	0	18.8	7.1	0.12
Lung function (pre-SABA)				
FEV_1 %	99.5±13.7	91.5±11.0	82.5±20.2	0.009
FVC %	108.7±11.3	105.2±17.3	96.6±20.9	0.115
FEV_1/FVC	0.78±0.08	0.73±0.06	0.70±0.09	0.017
AHR (mannitol) PD_{15}	180 (9–626)	127 (4–297)	125 (37–279)	0.27
T2 biomarkers				
F_{eNO} ppb	24.7 (5.8–208.9)	23 (4.55–140.1)	40 (9–67)	0.65
Atopy %	65	50	64.3	0.61
Total IgE kUA·L^{-1}	94.5 (3–1330)	40 (4–392)	182.5 (4–1420)	0.05
B-eosinophils ×10^9 cells·L^{-1}	0.17 (0.04–0.73)	0.3 (0.08–0.72)	0.36 (0.06–1.09)	0.27
Sputum cell counts				
Total cell count ×10^6 cells·mL^{-1}	313.1 (134.1)	286.1 (177.2)	348.4 (127.8)	0.58
Eosinophils %	3.3 (0.3–20.5)	1.4 (0–21.5)	18.1 (1–82.0)	0.01
Neutrophils %	11.9 (1.8–34.7)	54.8 (0–95)	27.0 (1.3–78.8)	0.001
Sputum phenotype %				
Eosinophilic	53	36	92	0.01
Neutrophilic	24	50	27	0.26
Allergy status % positive				
Birch	40	19	29	0.39
Grass	50	25	71	0.04
Ragweed	20	13	29	0.55
Horse	25	13	0	0.11
Dog	25	31	21	0.82
Cat	25	25	29	0.97
House dust mite	45	31	86	0.01
Alternaria	5	0	14	0.25
Cladosporium	0	0	7	0.30

Data are presented as n, mean±SD or median (range), unless otherwise stated. With increasing use of inhaled corticosteroids (ICS) ("severity"): older age, higher body mass index (BMI), more symptoms, increased risk of exacerbations, lower lung function, more smoking exposure, more eosinophilia, more neutrophilia and more house dust mite allergy. ACQ: Asthma Control Questionnaire; OCS: oral corticosteroids; LABA: long-acting β-agonist; LTRA: leukotriene receptor antagonist; LAMA: long-acting muscarinic antagonist; SABA: short-acting β-agonist; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; AHR: airway hyperresponsiveness; PD_{15}: provocative dose causing a 15% fall in FEV_1; F_{eNO}: fractional exhaled nitric oxide. [#]: ⩾1600 µg budesonide.

Bronchial epithelial response to the viral mimic poly (I:C) differs between healthy and patients with asthma

After stimulation with the TLR3 agonist poly (I:C), bronchial epithelial cells from patients with asthma showed a general release of both cytokines and chemokines, indicating that a broad immunological

response was raised towards this viral mimic (figure 1a). This included cytokines and chemokines closely related to asthma pathogenesis, such as the type-2 cytokines IL-4 and IL-13, the alarmins IL-33 and TSLP, as well as the eosinophilic chemoattractant CCL-5. Moreover, cytokines involved in a typical neutrophilic response (*i.e.* IL-1β, IL-6, IL-8 and TNF-α) and antiviral response (*i.e.* IFN-β) to rhinovirus infection was also clearly induced by poly (I:C) stimulation in BECs.

Additionally, TLR3 stimulation was conducted on BECs from 10 healthy subjects, demonstrating a similar broad immunological response (figure 1b–k), but with less release of IL-8 (figure 1e), IL-13 (figure 1g) and TSLP (figure 1i) compared to asthma patients ($p<0.05$).

Bronchial immune response to TLR3 stimulation is mainly associated to asthma atopic status, and to the eosinophilic phenotype

Next, we assessed the impact of sensitisation to aeroallergens (atopic, n=30 *versus* non-atopic, n=20); on the BECs response to TLR3 stimulation (figure 2a–e). Atopic patients responded with a higher induction of pro-inflammatory cytokine release after poly (I:C) stimulation compared with non-atopic patients, including IL-1β, IL-6 and TNF-α proteins (figure 2a). However, no difference was found between the two groups in poly (I:C)-induced release of the neutrophilic chemoattractant IL-8, and only a trend was found

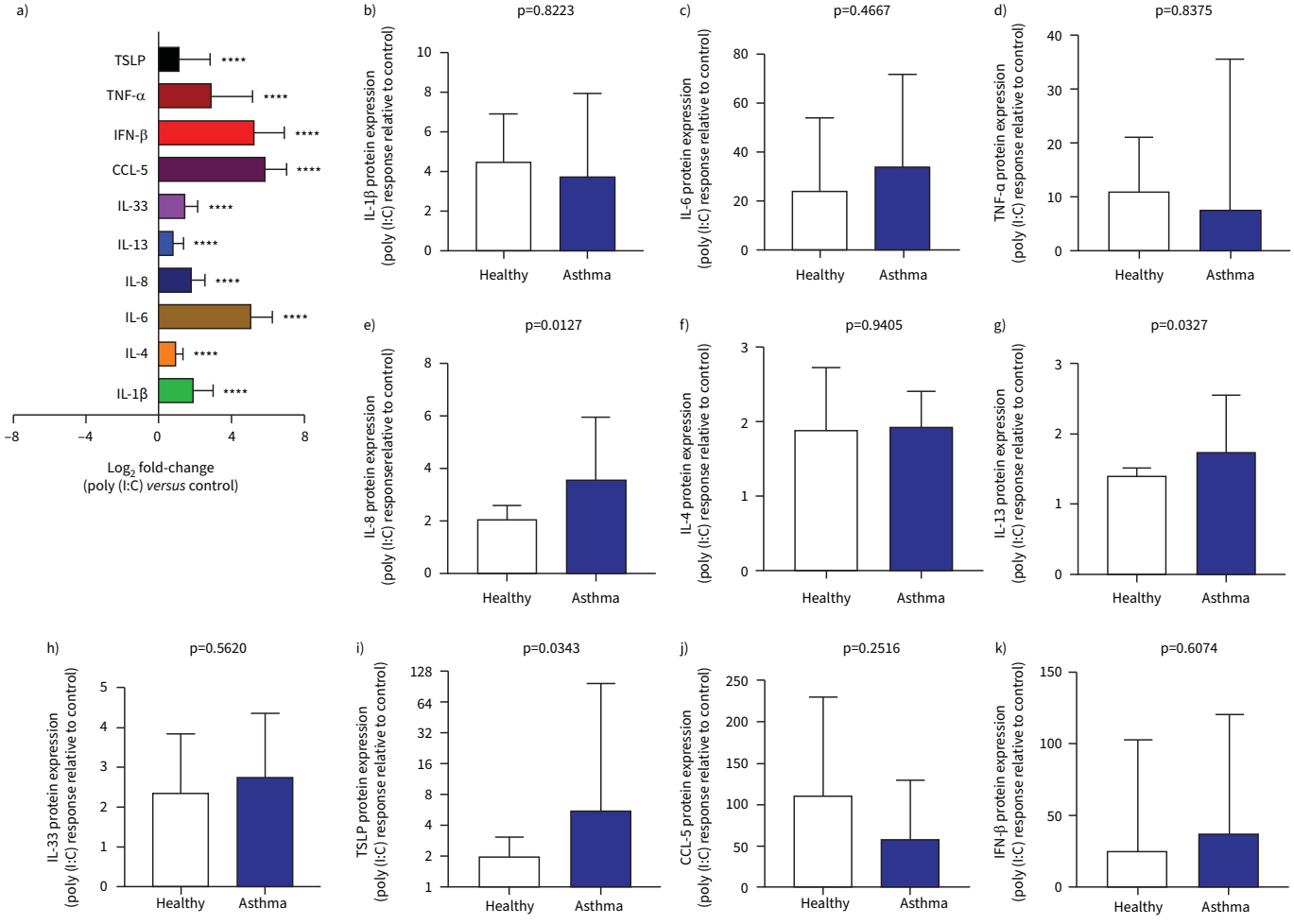

FIGURE 1 Immunoreactivity responses to poly (I:C) stimulation are different in human bronchial epithelial cells (HBECs) from asthmatic patients compared to healthy subjects. a) Release of multiple pro-inflammatory mediators from HBECs of patients with asthma (n=50) in response to 24 h poly (I:C) stimulation. Wilcoxon signed rank test. ****: $p<0.0001$. b–k) Upregulation of the neutrophilic mediators b) interleukin (IL)-1β, c) IL-6, d) tumour necrosis factor (TNF)-α and e) IL-8; the T2-cytokines f) IL-4 and g) IL-13; the epithelial alarmins h) IL-33 and i) thymic stromal lymphopoietin (TSLP); j) the eosinophilic chemokine CCL-5; and k) the antiviral mediator interferon (IFN)-β, in response to 24 h poly (I:C)-stimulation between healthy (n=10) and patients with asthma (n=50). Data are expressed as fold-change expression of poly (I:C)-stimulated HBECs relative to unstimulated cells. Mann–Whitney U-test.

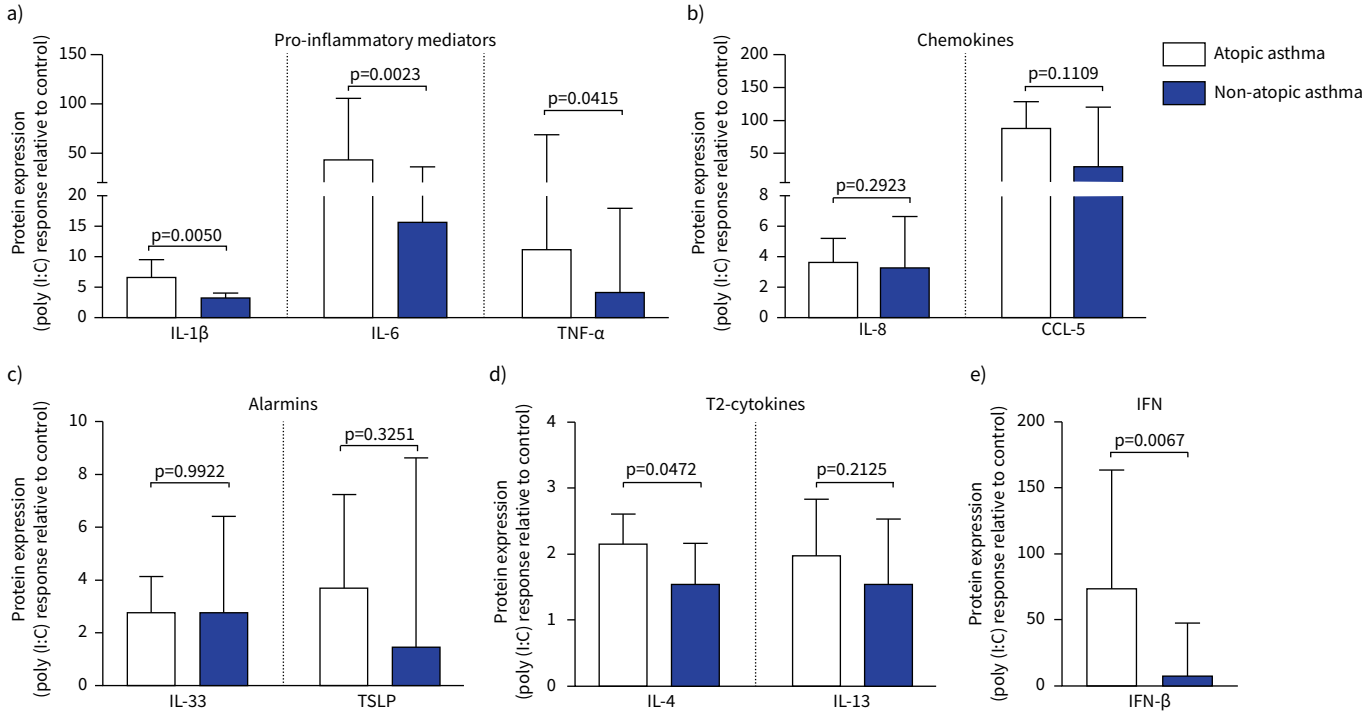

FIGURE 2 Immunoreactivity responses to poly (I:C) stimulation are higher in human bronchial epithelial cells (BECs) from atopic patients compared to non-atopic patients with asthma. a–j) Upregulation of a) the pro-inflammatory mediators interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α; b) the neutrophilic and eosinophilic chemokines IL-8 and CCL-5, respectively; c) the epithelial alarmins IL-33 and thymic stromal lymphopoietin (TSLP); d) the T2-cytokines IL-4 and IL-13; and e) the antiviral mediator interferon (IFN)-β, in response to 24 h poly (I:C)-stimulation between allergic (n=30) and non-allergic (n=20) patients with asthma. All data are expressed as fold-change expression of poly (I:C) stimulated BECs relative to unstimulated cells. Mann–Whitney U-test.

for CCL-5 release (p=0.1108) (figure 2b). The TLR3-dependent induction of the alarmins IL-33 or TSLP, was also not affected by atopic status of the patients (figure 2c), but IL-4 protein release was higher in atopic patients compared to non-atopic patients (figures 2d). Strikingly, IFN-β release after poly (I:C) stimulation was also greater in the atopic *versus* non-atopic groups (p<0.01) (figure 2e).

We did not find changes in the inducibility of most of the markers measured after stimulation of BECs with poly (I:C) when we subdivided asthmatic patients in eosinophilic (sputum eosinophils >3%; n=26) and non-eosinophilic (n=18) patients (figure 3a–e), except for an increased IL-6 and IL-8 protein release in eosinophilic patients compared to non-eosinophilic subjects (figure 3a,b). Indeed, when stratifying for both allergy and eosinophilia, atopic non-eosinophilic asthma was associated with an immune response to poly (I:C) similar to that of the group of atopic patients in general (supplementary figure S1), suggesting a predominant effect of atopy. However, IL-6 expression seemed to be affected by allergic and eosinophilic components (supplementary figure S1e), and IL-8 was more pronounced in non-atopic eosinophilic asthma compared to non-atopic non-eosinophilic asthma (supplementary figure S1f), although the number after stratification was too low to ensure statistical power.

Impact of asthma severity on bronchial epithelial immunoreactivity to TLR3 stimulation

Finally, we evaluated the overall impact of asthma severity, as well as the specific effect of asthma severity within the different asthma phenotypes studied.

We found no relevant changes associated with disease severity for the pro-inflammatory cytokines IL-1β, IL-6 or TNF-α (supplementary figure S2a–i). However, the release, as well as the gene expression of the main neutrophilic chemokine IL-8, after poly (I:C) stimulation, was induced in patients with severe disease compared with milder asthmatics (figure 4). The association of asthma severity with IL-8 protein release in response to poly (I:C) (24 h stimulation) was particularly evident in atopic and eosinophilic patients both at gene (figure 4e,f) and protein (figure 4b,c) level. BECs from severe asthmatics also displayed a higher release of the eosinophilic chemoattractant CCL-5 after poly (I:C) stimulation compared to BECs from

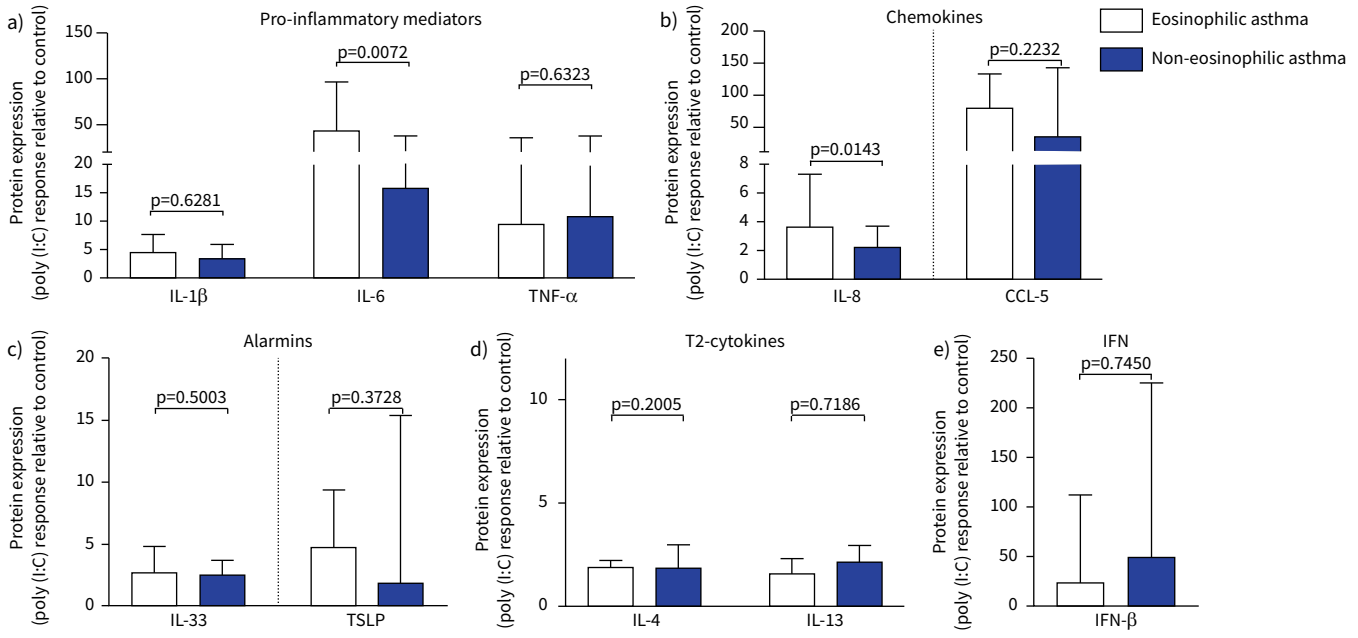

FIGURE 3 Eosinophilic phenotype in asthma has sparse effects on the immunoreactivity responses of the bronchial epithelium to poly (I:C) stimulation. a–e) Upregulation of a) the pro-inflammatory mediators interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α; b) the neutrophilic (IL-8) and eosinophilic (CCL-5) chemokines; c) the epithelial alarmins IL-33 and thymic stromal lymphopoietin (TSLP); d) the T2-cytokines IL-4 and IL-13; and e) the antiviral mediator interferon (IFN)-β, in response to 24 h poly (I:C)-stimulation between eosinophilic (n=26) and non-eosinophilic (n=18) patients with asthma. All data are expressed as fold-change expression of poly (I:C) stimulated bronchial epithelial cells relative to unstimulated cells. Mann–Whitney U-test.

mild asthmatics (supplementary figure 2j), although this was not significant after dividing patients into the different phenotypes (supplementary figure 2k,l).

Interestingly, IFN-β protein release after 24 h stimulation with poly (I:C) trended to decrease in severe asthmatics (figure 5a–c), and this reduction was evident at gene level after 3 h stimulation (figure 5d–f). Remarkably, although the IFN-β release in response to poly (I:C) was higher in mild atopic asthmatics compared with mild non-atopic patients (figure 5b), patients with severe atopic asthma displayed a significantly decreased level of IFN-β release compared with patients with less severity, both at gene and protein level (figure 5b,e). The same decrease in IFN-β in severe patients was observed within the eosinophilic phenotype (figure 5c,f).

Finally, the release of the main epithelial alarmins IL-33 and TSLP after poly (I:C) stimulation was modified with disease severity (figure 6). Particularly, IL-33 protein release after poly (I:C) stimulation was higher in severe patients with both atopic and eosinophilic asthma (figure 6a–c). TLR3-induced TSLP release was specifically increased in severe eosinophilic asthma (figure 6d–f).

In summary, cytokine responses to TLR3 stimulation in BECs were influenced by the phenotype and severity of asthma, with different impacts of asthma severity within atopic and eosinophilic asthma (figure 7). The present BEC data highlighted an impaired antiviral response in severe asthmatics (decreased IFN-β), in parallel to the exaggerated pro-inflammatory response (higher expression of IL-33, TSLP and IL-8), particularly evident in atopic and eosinophilic patients.

Discussion

We have shown that both the phenotype and severity of asthma impact the bronchial epithelial antiviral and pro-inflammatory responses to the viral infection mimic poly (I:C), suggesting that drivers of asthma exacerbations may depend on both of these dimensions of asthma. In general, we observed a broad release of pro-inflammatory cytokines, which was further increased in atopic asthma for IL-4, IL-6, IL1-β and TNF-α, and in eosinophilic asthma for IL-6 and IL-8. In patients with severe asthma, who were characterised by a higher exacerbation frequency, the pro-inflammatory responses observed in atopic

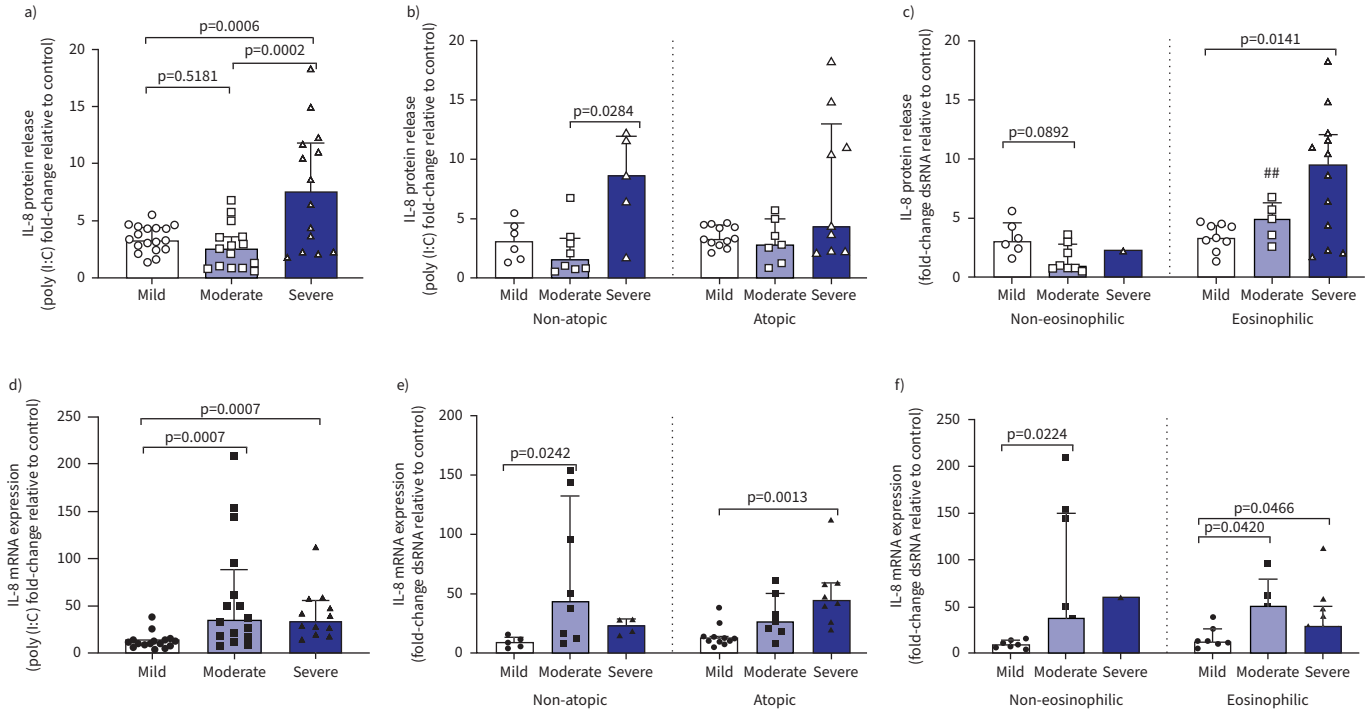

FIGURE 4 The neutrophilic response of the bronchial epithelium to the viral mimic poly (I:C) increases with the severity of asthma. a–c) Protein release and d–f) gene expression of the neutrophilic chemoattractant interleukin (IL)-8 in human bronchial epithelial cells (HBECs) from asthmatic patients a, d) with different degrees of severity (mild n=20; moderate n=16; severe n=14) in response to 24 h poly (I:C)-stimulation in the overall study population, or b, e) within the different asthma phenotypes: atopic (mild n=13; moderate n=8; severe n=9) *versus* non-atopic (mild n=7; moderate n=8; severe n=5) and c, f) eosinophilic (mild n=9; moderate n=5; severe n=12) *versus* non-eosinophilic (mild n=8; moderate n=9; severe n=1). All data are expressed as $\log_2$ fold-change expression of poly (I:C)-stimulated HBECs relative to unstimulated cells. One-way ANOVA with Holm–Šídák's multiple comparisons test (normality in the data) or Kruskal–Wallis with Dunn's multiple comparison test (absence of normality). Outliers have been removed using the robust regression followed by outlier identification test with Q set at 0.1%. ds: double-stranded.

asthma were further pronounced, with a higher release of IL-33 and IL-8. Patients with severe eosinophilic asthma displayed a similar response pattern, with increased release of IL-8 and IL-33, but also TSLP. Finally, the release of IFN-β was reduced in both severe atopic and severe eosinophilic asthma.

This is the first study to specifically compare bronchial epithelial inflammatory responses to a viral stimulus (TLR3 agonist poly (I:C)) across different clinically recognised phenotypes and levels of asthma severity. Mechanistically, the pro-inflammatory response to virus observed in atopic asthma, involving both type 2 and 1 T-helper cell related pathways, is in keeping with previous observations: rhinovirus infection has been demonstrated to induce a stronger pro-inflammatory response, involving IL-6 and IL-8, in the context of allergic inflammation mimicked by IL-4/IL-13 stimulation [21]. Furthermore, co-exposure with house dust mite and rhinovirus similarly induces higher release of IL-8 [12]. Experimentally, we have shown that rhinovirus infection induces airway neutrophilia specifically in mice with allergic sensitisation, as well as inducing release of TNF-α [22]. Together, these observations suggest that atopy and associated IL-4/IL-13 inflammation promotes a tendency to a stronger pro-inflammatory response to virus infection, potentially ultimately resulting in neutrophilic airway inflammation.

Several lines of evidence support the association between T2 inflammation and impaired IFN production in severe asthma [12]: experimentally, IL-4 and IL-13 exposure reduces IFN-β release, and we have previously shown that oxidative stress (a feature of active airway inflammation) may further impair IFN production [23]. The link between atopy and viral infection is further supported by the observation that anti-IgE specifically reduces viral induced exacerbations, arguably through an improvement in IFN-α production [24]. Our observations of reduced IFN release being specifically related to severe asthma are in line with inhaled interferon only being effective in severe asthma [25].

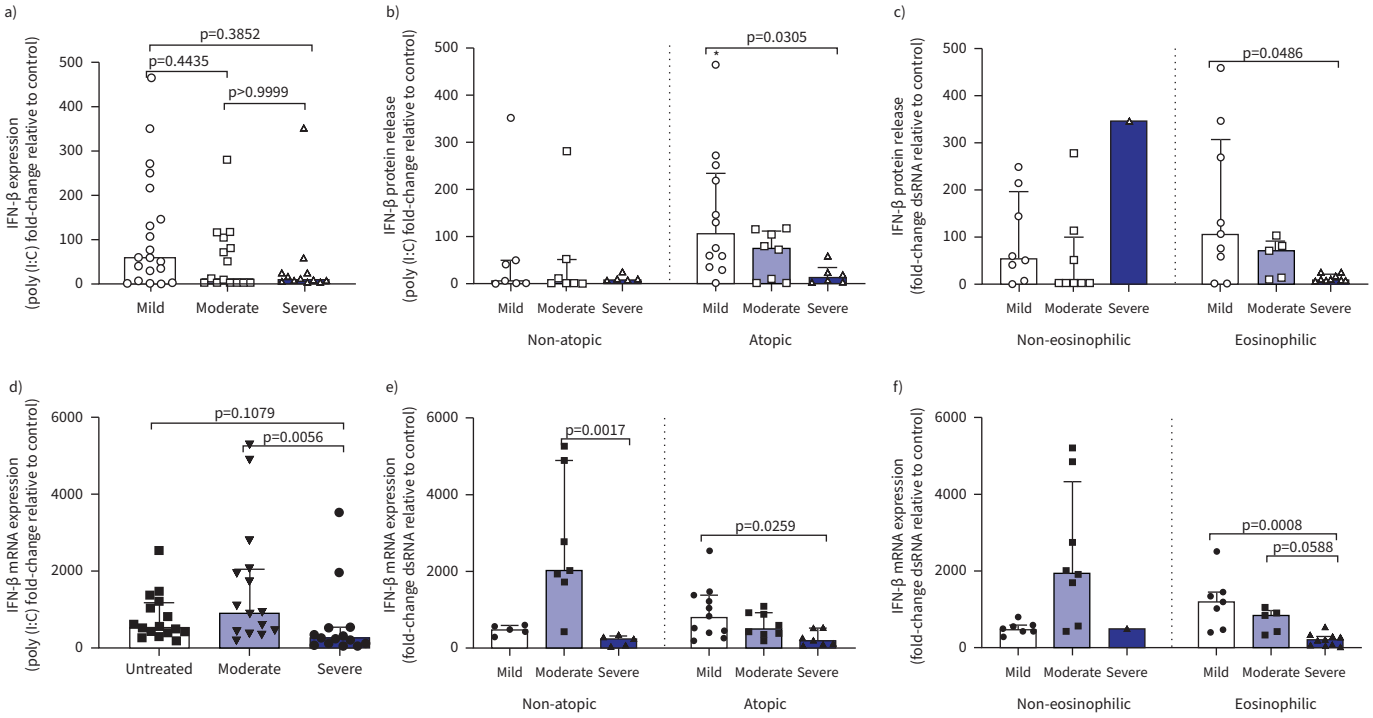

FIGURE 5 The interferon (IFN) response of the bronchial epithelium to the viral mimic poly (I:C) decreases with the severity of asthma, especially in atopic and eosinophilic patients. a–c) IFN-β release in cell culture supernatants of human bronchial epithelial cells (HBECs) from a) asthmatic patients with different degrees of severity (mild n=20; moderate n=16; severe n=14) in response to 24 h poly (I:C)-stimulation in the overall study population, or within the different asthma phenotypes: b) atopic (mild n=13; moderate n=8; severe n=9) *versus* non-atopic (mild n=7; moderate n=8; severe n=5) and c) eosinophilic (mild n=9; moderate n=5; severe n=12) *versus* non-eosinophilic (mild n=8; moderate n=9; severe n=1). IFN-β mRNA expression in HBECs from asthmatics after 3 h poly (I:C)-stimulation in d) the overall patient population, or within the different phenotypes: e) atopic *versus* non-atopic and f) eosinophilic *versus* non-eosinophilic. All data are expressed as $\log_2$ fold-change expression of poly (I:C)-stimulated HBECs relative to unstimulated cells. Kruskal–Wallis with Dunn's multiple comparison test. Outliers have been removed using the robust regression followed by outlier identification test with Q set at 0.1%. ds: double-stranded.

The impact of eosinophilic asthma on airway responses to virus has not been reported specifically outside the context of allergic asthma. Our observation of a moderate specific immune responses to poly (I:C) in eosinophilic asthma in general is novel and suggests that much of the aberrant immune response in asthma is connected to atopy. In patients with eosinophilic asthma, the release of the epithelial cytokines IL-33 and TSLP was increased in severe compared to mild asthma, suggesting that in patients with eosinophilic asthma, severity is a determinant of alarmin release.

A clear limitation of the present study is the relatively low numbers of patients per phenotype and severity strata, which effectively prohibited analysis of atopy in the absence of eosinophilia and conversely, eosinophilia in the absence of atopy. A subgroup analysis of patients with only atopy or eosinophilia did support our observations on different responses in atopic and eosinophilic asthma, but due to the low sample size in subgroups, it was not possible to assess clearly in the severe asthma group, whether the observed decrease in interferon induction and increase in IL-8 was related to atopy or to eosinophilia. This is to our knowledge the largest study to date examining epithelial bronchial responses across asthma phenotypes, further strengthened by a well-characterised patient population. However, the findings need to be validated in a separate cohort, which will need to include a sufficient number of patients with each phenotype (+/− atopy and +/− eosinophilia) within each severity strata. The cross-sectional study design represents a further limitation, and we cannot exclude the possibility that the reduced release of IFN in severe asthma is a result of high-dose ICS treatment; prospective studies are clearly warranted to assess the effect of specific interventions on the epithelial immune responses to a viral exacerbation trigger.

In order to make including a large cohort of asthma donors (n=50) with a range of clinical and immunological characteristics feasible, in the present study we used submerged BEC cultures instead of

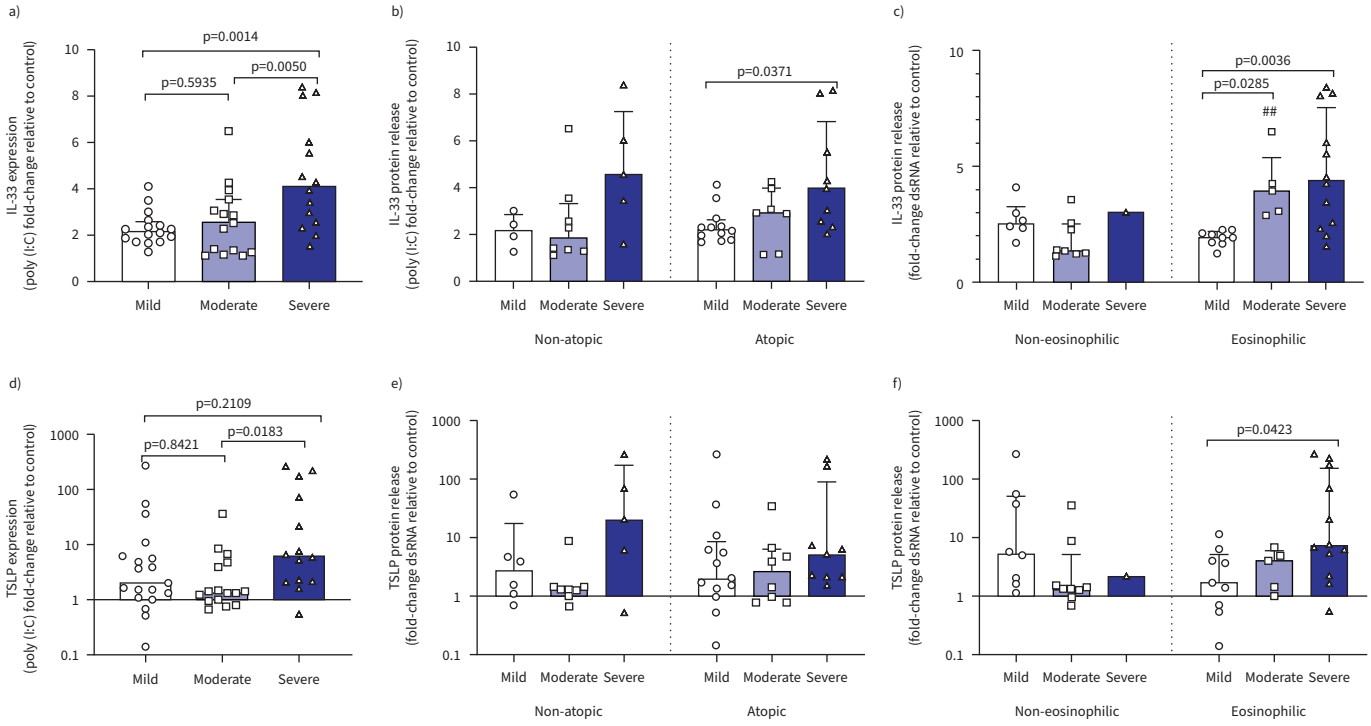

FIGURE 6 The alarmin response of the bronchial epithelium to the viral mimic poly (I:C) increases with the severity of asthma, especially in eosinophilic patients. a–c) Interleukin (IL)-33 and d–f) thymic stromal lymphopoietin (TSLP) release in cell culture supernatants of human bronchial epithelial cells (HBECs) from asthmatic patients with different degrees of severity (mild n=20; moderate n=16; severe n=14) in response to 24 h poly (I:C) stimulation in the a, d) overall study population or within the different asthma phenotypes: b, e) atopic (mild n=13; moderate n=8; severe n=9) *versus* non-atopic (mild n=7; moderate n=8; severe n=5) and c, f) eosinophilic (mild n=8; moderate n=9; severe n=12) *versus* non-eosinophilic (mild n=8; moderate n=9; severe n=1). All data are expressed as $\log_2$ fold-change expression of poly (I:C)-stimulated HBECs relative to unstimulated cells. Kruskal–Wallis with Dunn's multiple comparison test. Outliers have been removed using the robust regression followed by outlier identification test with Q set at 0.1%.

air–liquid interface cultures and poly (I:C) stimulations instead of live virus infections. Mucociliary differentiation of BECs is necessary for study of epithelial barrier function, remodelling or other features of asthma pathophysiology. However, several lines of evidence indicate that submerged BEC cultures are a good model for the study of cytokine response [23], and basal cells are main targets for the major group of human rhinovirus [26]. Poly (I:C) is a synthetic dsRNA TLR-3 agonist, that mimics rhinovirus infection, and has been demonstrated to induce a consistent and physiologically relevant induction of cytokines and interferons in the epithelium [6, 27]. In comparison, single-stranded RNA molecules or non-replicative rhinovirus only induce a very weak response of the epithelium. Nevertheless, it will be important to include rhinoviral infection in future studies to address consistency of the differences between asthma phenotypes suggested by the present data.

Clinically, our observations suggest that the immune responses involved in asthma exacerbations depend on the phenotype as well as the severity of asthma: the broad inflammatory response related to atopy tracks with the well-known clinical association between allergic asthma and viral-induced asthma exacerbations. Interestingly, TSLP and IL-33 were specifically induced in patients with severe eosinophilic asthma, suggesting a more central role of the epithelial cytokines in this group of patients. A better understanding the connection between distinct clinical traits, such as severity and phenotype, and the immunological drivers involved in asthma exacerbations may clearly improve our ability to develop better immune targeted treatments for asthma. Our findings suggest that it is the combination of the phenotype of asthma and the severity that determine the response to asthma exacerbation triggers, and which need to be taken into account when developing targeted treatments. Further studies are now warranted, to determine the relative contribution of eosinophilia *versus* atopy to the epithelial immune responses to asthma exacerbation triggers in severe asthma, by specifically assessing both eosinophilic asthma in the absence of atopy, and atopic asthma in the absence of eosinophilia.

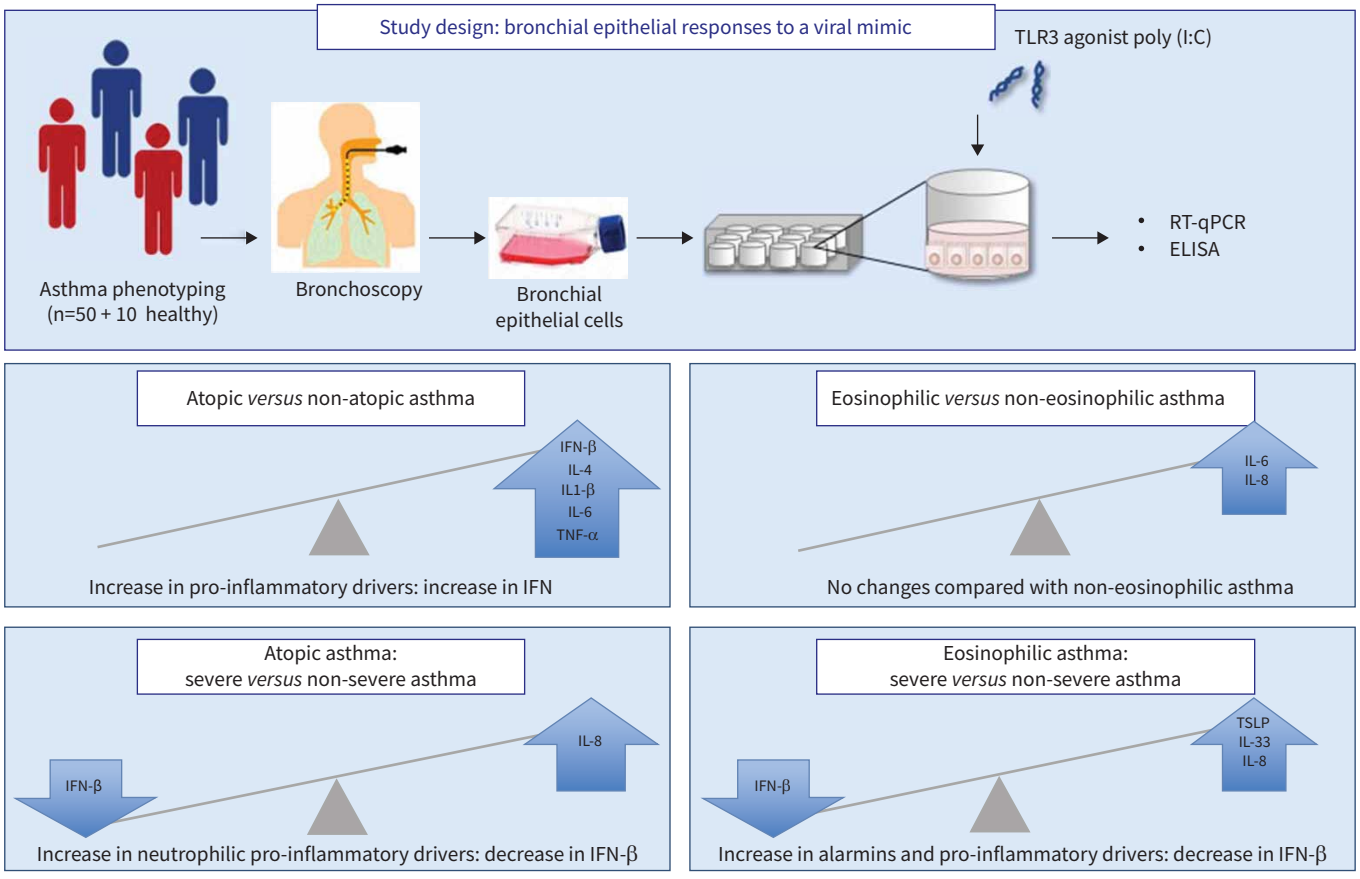

FIGURE 7 Summary of key findings. The release of pro-inflammatory and antiviral mediators is determined by the phenotype and severity of asthma: atopic asthma is associated with a broad pro-inflammatory response, whereas severe eosinophilic asthma is specifically associated with release of alarmins (thymic stromal lymphopoietin (TSLP) and interleukin (IL)-33). Furthermore, interferon (IFN)-β release is reduced in both severe atopic and severe eosinophilic asthma. TLR: Toll-like receptor; RT-qPCR: reverse transcriptase quantitative PCR; TNF: tumour necrosis factor.

In conclusion, we show that the bronchial epithelial immune response towards a viral mimic stimulation is determined by the phenotype of asthma, as well as the severity of asthma. Based on our findings, we propose the existence of "immunoreactivity phenotypes", and we believe that further studies are now needed to understand the potential role of epithelial immune response patterns in assessing response to specific targeted therapies, as well as predicting a response.

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