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Characteristics associated with clinical severity and inflammatory phenotype of naturally occurring virus-induced exacerbations of asthma in adults

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ABSTRACT

Background: In experimental studies viral infections have been shown to induce type 2 inflammation in asthmatics, but whether this is a feature of naturally occurring virus-induced asthma exacerbations is unknown. Thymic stromal lymphopoietin (TSLP) released from the airway epithelium in response to damage, has been suggested as a link between viral infection and type 2 inflammation, but the role of TSLP in asthma exacerbations is unknown.

Objective: To assess whether type 2 inflammation, as measured by sputum eosinophils and fractional exhaled nitric oxide (FeNO), is a feature of naturally occurring virus-induced exacerbations of asthma and whether TSLP is associated with this type 2 inflammation.

Methods: Patients presenting to hospital with acute asthma were examined during the exacerbation, and after 4 weeks recovery. The assessments included spirometry, FeNO and induced sputum for differential counts and TSLP mRNA levels. Nasal swabs were collected for viral detection.

Results: Sputum eosinophils and FeNO were similar between virus-positive (n = 44) and negative patients (n = 44). In virus-positive patients, TSLP expression was lower at exacerbation than follow-up (p = 0.03). High TSLP at exacerbation was associated with lower sputum eosinophils (p = 0.01) and higher FEV1 (p = 0.03). In virus-positive patients, %-predicted FEV1 negatively correlated with both FeNO and sputum eosinophils (p = 0.02 and p = 0.05, respectively).

Conclusion: Our findings support that type 2 inflammation is present in patients during virus-induced asthma exacerbations, to the same degree as non-viral exacerbations, and correlate negatively with FEV1. However, in virus-positive patients, high TSLP expression during exacerbation was associated with low sputum eosinophils, suggesting that the effect of TSLP in vivo, in the setting of an asthma exacerbation, might be different than the type 2 inducing effects observed in experimental studies.

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1. Introduction

With an estimated prevalence of nearly 10%, asthma is one of the more frequent chronic diseases in Western countries, and exacerbations are the major cause of morbidity and health care
utilization [1]. Respiratory viruses are one of the most common triggers of asthma exacerbations [2], but the underlying mechanisms are not fully understood.

Overall, around half of asthma patients have prominent type 2 inflammation during stable disease [3], characterized by IL-5 driven eosinophilia in both sputum and blood and mucus hypersecretion, airway hyperresponsiveness and increased IgE production, driven by IL-4 and IL-13. This Th2-high phenotype has been associated with more severe disease [4,5] and an increased risk of exacerbations [6]. Whether aggravated type 2 inflammation is a feature of both virus-induced and non-viral exacerbations, is currently unknown and is likely to have implications for the efficacy of emerging biological treatment in reducing the rate and severity of these exacerbations.

Allergen sensitization and exposure is the established cause of increased type 2 inflammation. However, in vitro studies have suggested that respiratory viruses are capable of independently inducing type 2 inflammation, by causing a release of pro-inflammatory cytokines, including thymic stromal lymphopoietin (TSLP), from the airway epithelium [7,8]. In this innate immune pathway, TSLP causes release of type 2 cytokines IL-4, IL-5 and IL-13, from T-cells through activation of dendritic cells [9] and by direct action on innate lymphoid cells (ILC) [10]. TSLP is increased in patients with asthma, compared to controls [11] and anti-TSLP antibodies have been shown to reduce airway inflammation in response to an allergen provocation test [12]. Epithelial cells from asthmatics seem to be particular prone to TSLP release following response to an allergen provocation test [12]. Epithelial cells from asthmatics seem to be particular prone to TSLP release following exposure to viral infection in vitro [8] but whether TSLP is induced in asthmatics during viral infection in vivo and stimulates type 2 inflammation is unknown.

Asthma patients, particularly those with type 2 inflammation, experience more severe symptoms when experimentally infected with respiratory viruses [13,14] and, although controversial, the mechanism is proposed to involve a reduced production of interferons [15–18]. Whether patients with high type 2 inflammation during a real-life viral infection experience more severe exacerbations, is unclear.

The aim of the present study was to test the hypothesis that sputum eosinophilia and increased FeNO are features of naturally occurring virus-induced exacerbations. Secondly, that the level of sputum eosinophilia and FeNO are associated with the degree of TSLP expression in sputum. Finally we wished to test whether any type 2 inflammation during a virus-induced exacerbation would be associated with more severe exacerbations, measured by FEV1. Exploratory outcomes included rhinovirus subtype and systemic markers of inflammation.

2. Methods

2.1. Study design

The study was a prospective observational study conducted at Bispebjerg University Hospital between July 2013 and September 2015. Patients were recruited both from the hospital Emergency Department, and from the acute asthma clinic within the hospital research unit and were seen within 24 h of contact. Exacerbations were defined as worsening of symptoms surpassing the daily variation that required a change in treatment in line with ERS/ATS recommendations [19]. After 4 weeks, participants were re-examined in the research unit. All patients had a doctor’s diagnosis of asthma and had the diagnosis confirmed during the study in line with GINA recommendations [1]. Patients were between 16 and 45 years of age. Pregnant women and patients with pulmonary diseases other than asthma were excluded. Both current and past smokers were included.

The study was approved by the local scientific ethics committee (protocols nr: H-2-2013-046, H-3-2011-121, H-1500369).
covariates: gender, age, smoking, bacterial infection and ICS usage.

Among patients with a respiratory virus, six had a bacterial co-
detected were rhinovirus (77%), human coronavirus (16%), para-
(50%) had a detectable respiratory virus at exacerbation. Viruses

3. Results

3.1. Respiratory viruses and bacteria detected at exacerbation

A total of 88 patients were included in the study, of which 44 (50%) had a detectable respiratory virus at exacerbation. Viruses detected were rhinovirus (77%), human coronavirus (16%), parainfluenza virus (5%) and human metapneumovirus (2%) (Fig. 1). Among patients with a respiratory virus, six had a bacterial co-
fection (3 H. influenzae, 1 S. pneumoniae, 1 S. aureus and 1 M. catarrhalis). There were three patients with bacterial infections in the virus-negative group (2 S. pneumoniae and 1 Gemella hae-
molysans) (p = 0.29). In the comparisons of virus-positive and negative groups, the nine patients with bacterial infections were included. All conclusions remained unchanged when patients with bacterial infections were excluded from the analyses.

3.2. Clinical characteristics of virus-positive vs. virus-negative patients

Demographics of patients with, or without, viral infection were similar, as were the level of treatment required at exacerbation (Table 1). Likewise, lung function and inflammatory markers were largely similar, with the exception of a higher CRP in the virus-positive group (GM 8.4, 95% CI 3.5–11.5, p = 0.02) compared to the virus-negative group (GM 4.9, 95% CI 3.5–7.0). Sputum induction was successful in 67 patients (76%, 31 without virus and 36 with virus). The absolute number of both eosinophils and neutrophils were higher in virus-positive patients, compared to virus-negative patients, but the percentages of each cell type were not different between virus-positive and negative (Table 1). Consequently, the distribution of patients into established sputum phenotype [30] was similar between viral and non-viral exacerbations (Fig. 2).

In the virus-induced group FeNO was higher at exacerbation (GM 28, 95% CI 22–35, p = 0.001) compared to follow-up (GM 18, 95% CI 14–24), which was also the case in the non-viral group (GM 35, 95% CI 26–49 vs. 23, 95% CI 18–30, respectively, p = 0.001). Sputum eosinophils were not significantly different at follow-up compared to the percentages during exacerbation in either group.

In the period between the initial and the follow-up visit patients were treated following clinical guidelines (GINA). Following admission 75% received an oral course of prednisolone (37.5 mg once daily for 10 days), while the rest were managed by increasing their inhaled steroid. No other treatment was initiated by the study team, but existing treatment with LABA, leukotrienes or any other asthma medication was continued following exacerbation. There were no significant differences in clinical management between the virus-positive and virus-negative group.

3.3. TSLP mRNA expression

There was no difference in sputum TSLP mRNA expression between patients with, or without, a viral infection at exacerbation (GM 3.2, 95% CI 1.4–7.1 vs. 3.5, 95% CI 2.0–6.0, respectively, p = 0.84) (Fig. 3). In all, there were 36 paired measurements of TSLP (exacerbation and follow-up; 19 from virus-positive patients, 17 from virus-negative patients). In virus-positive patients TSLP expression was lower at exacerbation (GM 2.8, 95% CI 1.2–6.8, p = 0.03) compared to follow-up (GM 6.1, 95% CI 2.8–13.4), whereas there was no difference between exacerbation (GM 2.8, 95% CI 1.1–7.0, p = 0.74) and follow-up (GM 2.4, 95% CI 0.9–6.4) in the virus-negative group (Fig. 3).

In patients without a virus infection, there was no difference in inflammatory phenotype. FEV1 or ACQ between those with high or low TSLP expression (n = 12 and n = 9, respectively) (Fig. 4). However, in virus-positive patients, those with high TSLP expression levels (n = 15) had significantly lower sputum eosinophil percentage (GM 0.5, 95% CI 0.2–1.4, p = 0.01 vs. 3.8, 95% CI 1.1–13.8), higher FEV1 (86.2 ± 12.4, p = 0.03 vs. 75.1 ± 14.9), lower ACQ score (2.5 ± 1.2, p = 0.01 vs. 3.7 ± 1.4), higher blood neutrophils (median 4.3 × 10⁹ cells/L (range 2.3–10.2), p = 0.04 vs. 6.0 × 10⁹ cells/L (2.8–13.5)) and a trend for lower FeNO (GM 27 ppb, 95% CI 20–36, p = 0.16 vs. 38 ppb 95% CI 26–56) compared to those with low TSLP expression levels (n = 15) (Fig. 4). There was no difference in ICS usage between the TSLP high and low groups.

3.4. Type 2 inflammation and FEV1 at exacerbation

To examine the impact of type 2 inflammation on the clinical severity of exacerbation, the association with sputum eosinophils, FeNO and FEV1 at exacerbation was examined in both the virus-
positive and negative group.

The percentage of sputum eosinophils correlated negatively with FEV1 in patients with a virus detected at exacerbation.
In this study, type 2 inflammation and TSLP mRNA expression were present in virus-induced exacerbations to a similar extent as in non-viral exacerbations. Furthermore, both sputum eosinophils and FeNO were negatively correlated with FEV1 at exacerbation.

As exploratory predictors of FEV1, we also measured blood eosinophils, total IgE and sputum neutrophils. Using the same analysis, both blood eosinophils and total IgE were associated with FEV1 with no difference between virus-positive and negative patients, while sputum neutrophils were not associated with FEV1 in either group (data not shown).

### 3.5. Impact of the type of virus detected

Patients infected with a rhinovirus had lower %-predicted FEV1 (77.2 ± 16.0, p = 0.02) compared to patients with other respiratory viruses (90.8 ± 15.5), and a higher ACQ score (3.4 ± 1.4, p = 0.03 vs. 2.3 ± 1.4, respectively) (Table 2). There were no significant differences in demographics, spirometry or inflammatory markers between patients infected with RV-C (n = 10) compared to patients infected with RV-A (n = 23) (Table 3).

### 4. Discussion

In contrast to our hypothesis, we found that participants with high
Fig. 4. Inflammatory and clinical characteristics of virus positive patients with TSLP expression at exacerbation below the median (TSLP low) and above the median (TSLP high).

Table 2
Characteristics of patients at presentation with exacerbation with rhinovirus or other viruses at exacerbation.

|                      | Rhinovirus (n = 34) | Other viruses (n = 10) | p-value |
|----------------------|---------------------|------------------------|---------|
| Gender (% females)   | 68                  | 70                     | 0.89    |
| Age (years)          | 29 (9)              | 41 (17)                | 0.06    |
| Smokers (% never; former; current) | 65; 15; 20 | 60; 30; 10 | 0.47    |
| Packyears (among former and current smokers) | 6.0 (5.4) | 1.7 (1.6) | 0.15    |
| Atopic (%)           | 62                  | 40                     | 0.22    |
| ICS dose (daily budesonide eq.) | 200 (0–2400) | 800 (0–800) | 0.34    |
| FEV1%pred.           | 77.2 (16.0)         | 90.8 (15.5)            | 0.02    |
| FVC%pred.            | 87.1 (14.4)         | 94.9 (11.8)            | 0.13    |
| Beta-2-agonist within 8 h? (%) | 46          | 25                     | 0.29    |
| FeNO (ppb)*          | 29 (23–37)          | 24 (15–38)             | 0.41    |
| Blood eosinophils (× 10^6 cells/L) | 0.07 (0–1.32) | 0.13 (0–0.41) | 0.84    |
| Blood neutrophils (× 10^6 cells/L) | 6.2 (2.1–14.1) | 3.6 (2.9–10.2) | 0.13    |
| Total IgE (× 10^3 IU/L)* | 167 (85–328) | 96 (31–298) | 0.41    |
| CRP (mg/L)*          | 8.5 (6.0–12.0)      | 8.2 (3.5–19.1)         | 0.93    |

n = 27

|                      | n = 9               |
|----------------------|---------------------|
| Sputum total cell count (× 10^6/ml)* | 1.0 (0.6–1.7)   |
| Sputum eosinophils (× 10^6/ml)*    | 0.07 (0.04–0.13)  |
| Sputum %-eosinophils*             | 1.2 (0.5–3.0)     |
| Sputum neutrophils (× 10^6/ml)*   | 0.5 (0.2–0.9)     |
| Sputum %-neutrophils*             | 45.9 (32.0–66.0)  |

* Log-transformed and presented as geometric mean (95% CI).
sputum TSLP had lower sputum eosinophils, lower FeNO and higher %FEV1 at exacerbation than participants with low sputum TSLP suggesting that TSLP does not promote type 2 inflammation in the airways during virus-induced exacerbations of asthma. It is possible that the timing of sample collection and changes in post-translational modification of the TSLP mRNA could have impacted on the final amount of TSLP protein being produced, although, previous studies have found good concordance between TSLP gene expression and protein concentration [11,31]. An inhibitory effect of corticosteroids cannot be ruled out, but it would not explain the difference between the virus-positive and virus-negative group.

Although ex vivo studies have proposed that TSLP acts as a mediator of eosinophilia in response to viral infection [32], this has not been documented in vivo. Our findings suggest that this effect might be more complex in the setting of asthma exacerbations and suggests that other pathways (e.g. IL-33 or other modulators of ILC2s) in the innate immune system are contributing to initiating type 2 inflammation in response to viral infection.

We attempted to measure TSLP protein using a highly sensitive ELISA (Bio-Plex Pro, Bio-Rad on a Luminex platform, lower limit of detection 0.8 pg/ml) but did not detect TSLP in any of the samples. This may have been due to the addition of dithiothreitol (DTT) to detection 0.8 pg/ml) but did not detect TSLP in any of the samples.
seasonal differences in inflammatory markers at exacerbation were observed. Sensitization and exposure to allergens in conjunction with viral infection has been shown to increase the risk of admission [34] and biological treatment options targeting type 2 inflammation, have reported reductions in exacerbation rates of 50–80% [35–38]. Yet, whether predominately virus-induced exacerbations were being prevented, or non-viral exacerbations, is unknown.

It is possible that the inflammatory phenotype expressed during stable disease is representative of the response developed at exacerbation, regardless of trigger. A prospective study of asthma patients phenotyped during stable disease and followed until exacerbation is required to investigate this.

The mechanisms by which increased type 2 inflammation might negatively affect the course of a virus infection in asthmatics are not clear, but recent mechanistic studies have suggested type 2 inflammatory pathways may lead to reduced production of interferon [15–18]. The role of type 2 inflammation in relation to naturally occurring viral infections in asthma patients, has been much less examined. Denlinger and colleagues sampled asthma patients with symptoms of a cold, and found that patients who went on to develop a clinical exacerbation were characterized by higher sputum neutrophils, whereas an association with eosinophils or FeNO was not observed. However, it should be noted that the study was not aimed at describing the severity of the exacerbations, and the potential association with sputum inflammatory phenotype [39]. Wark and colleagues have found that degranulation of both neutrophils and eosinophils was associated with a longer time in hospital for patients admitted with virus-induced acute asthma [40]. In the present study, sputum neutrophil count, though elevated in virus-positive exacerbations compared to non-viral exacerbations, did not correlate with FEV1.

More severe wheezing illness has been reported in children infected with RV-C, compared to RV-A [41], however, this has not been established in adults [42]. In the present study, RV-C comprised 30% of all rhinovirus infections – a proportion similar to that reported by Wark and colleagues [43]. We found no difference in clinical measurements or sputum inflammatory phenotype between patients with RV-C and RV-A. We did note that patients infected with RV-C had higher blood eosinophils at exacerbation that were not mediated by a difference in ICS dose; however, this should be interpreted with caution as no other type 2 markers were increased.

In conclusion, we have found that type 2 inflammation was present during virus-induced exacerbations to the same extent as non-viral exacerbations, and was associated with a lower FEV1 at exacerbation. Unexpectedly, TSLP expression correlated negatively with sputum eosinophils in virus-induced exacerbations, suggesting that other pathways (including epithelial cytokines such as IL-33) might be responsible for driving innate pathways leading to type 2 inflammation in acute virus induced asthma.

Conflicts of interest

None.

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