

PI3-Kinase Regulates Eosinophil and Neutrophil Degranulation in Patients with Allergic Rhinitis and Allergic Asthma Irrespective of Allergen Challenge Model

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Abstract—The PI3K pathway plays a major role in many vital cell processes. Our primary aim was to investigate signalling through PI3K for *in vitro* degranulation from allergen-primed eosinophils and neutrophils in allergic rhinitis and allergic asthma after seasonal and experimental allergen challenge. Nine patients with allergic rhinitis, eight with allergic asthma and four controls were studied during birch pollen season and after nasal and bronchial allergen challenge. Primed blood eosinophils and neutrophils were stimulated for *in vitro* degranulation with C3b-coated Sephadex particles, after prior incubation with Wortmannin, a PI3K inhibitor. The released amounts of eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and myeloperoxidase (MPO) were measured by radioimmunoassay. Wortmannin (10^{-6} to 10^{-9} M) inhibited ECP, EPO and MPO release in a dose-dependent manner in allergic rhinitis and allergic asthma in all three allergen challenge models. Inhibition of ECP release tended to be lower in the asthmatics in all allergen challenge models, statistically significant compared to the controls during season for 10^{-8} M Wortmannin ($p=0.01$). A clear propensity towards less inhibition in the rhinitic patients was seen after nasal and bronchial challenge compared to seasonal exposure, significant for ECP (10^{-8} M Wortmannin; $p=0.034$ and 0.002 , respectively). Signalling through PI3K is clearly involved in ECP, EPO and MPO release in allergic rhinitis and allergic asthma irrespective of allergen challenge model. Allergic asthma demonstrated less inhibition of ECP release via PI3K during pollen season, indicating that other pathways play a greater role in eosinophil degranulation in allergic asthma than allergic rhinitis.

KEY WORDS: eosinophil inflammation; allergic rhinitis; allergic asthma; eosinophil degranulation; PI3K; ECP.

INTRODUCTION

Allergic rhinitis and allergic asthma are chronic inflammatory diseases characterised by systemic and mucosal inflammation [1]. Eosinophils are considered to be key effector cells in mucosal allergic inflammation [2], but neutrophils are also known to participate in the

inflammatory response, especially in chronic severe asthma [3]. Epidemiological studies have demonstrated that rhinitis is a strong risk factor for the onset of asthma [4], but most patients with allergic rhinitis do not develop asthma [5].

The eosinophils are multifunctional leukocytes involved in host defence and inflammatory reactions, but also participating in immune modulation. Once activated, and recruited to the target tissue they secrete several tissue toxic granule proteins in addition to an array of inflammatory mediators [6]. The most studied eosinophil granule proteins are the eosinophil cationic protein (ECP), major basic protein and eosinophil peroxidase (EPO). On activation, the preformed granule products are released by exocytosis, cytolysis or by piecemeal degranulation. *In vitro* studies have demon-

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strated selective and differential release of granule proteins by piecemeal degranulation by transport vesicles [7, 8], and this route for degranulation has been suggested important in allergic reactions [9]. The recruitment and chemotaxis of the eosinophils to the site of action is fairly well studied [10], but the signal transduction for degranulation is less understood in allergic diseases. Complement factor 3 (C3b) is a potent mediator for eosinophil degranulation, and when attached to Sephadex particles, *in vitro* degranulation from granulocytes can be studied [11, 12].

The phosphoinositide-3-kinase family (PI3-kinases, PI3Ks) regulates cell membrane trafficking and controls many fundamental biological functions by downstream activation cascades [13]. PI3Ks are divided into three major classes based on structural features and lipid substrate preference. Class I PI3K is most studied and best understood [13]. PI3Ks generate 3-phosphorylated phosphoinositide lipids, and in the case of class I, the end-product is phosphatidylinositol(3,4,5)trisphosphate (PIP3) [13]. PIP3 acts as a critical second messenger and promotes downstream activation through a phosphorylation cascade of pleckstrin homology domain containing proteins, above all activation of the Akt pathway [13]. Further downstream, Akt phosphorylates numerous protein targets [13, 14]. Inhibitors have been useful for characterising the PI3Ks, and Wortmannin, a fungal metabolite, has widely been used for studying the PI3K pathway in leukocytes, though implications mainly have been drawn to class I PI3Ks [15]. It efficiently blocks class I PI3Ks, both class IA and class IB; class IA activated primarily through cell-surface receptor tyrosine kinases and class IB through G-protein-coupled receptors [16]. Wortmannin has been regarded as a selective PI3K inhibitor. Recent findings suggest that Wortmannin can compete with additional kinases and ATP, but only at much higher concentrations than in our assay [17, 18]. Class I PI3Ks can be activated by external stimuli [18] and are thought to be involved in mast cell and basophil degranulation in allergy [19, 20], but the role in eosinophil degranulation in answer to allergic reactions is so far not elucidated.

Patients with allergic asthma and allergic rhinitis demonstrate more or less the same degree of systemic eosinophilic inflammation both during pollen season and after nasal and bronchial challenge and yet they present with different clinical pictures. We hypothesise that the dissimilar clinical manifestations may be due to differently regulated signalling pathways. We previously found differences in the degranulation of ECP comparing

patients with rhinitis and asthma, with lower release in the asthmatics (submitted paper). After long-term allergen exposure, there might be alternative pathways for ECP degranulation in these patients; and in this study, we therefore compare the importance of PI3 kinase signalling for degranulation in the two patient groups.

MATERIALS AND METHODS

Patients

Seventeen birch pollen allergic patients were selected for the study, all diagnosed with seasonal allergic rhinitis or allergic asthma by a lung physician and allergologist at the Allergy out-patient clinic at Uppsala University Hospital. All patients were skin prick test-positive to birch pollen and none of the patients had symptoms or were on any regular treatment outside birch pollen season. Eight patients had a diagnosis of allergic seasonal asthma, having respiratory symptoms (wheeze and dyspnoea) and denying nasal symptoms during birch pollen season, and thus were categorised as having asthma as the predominant symptom. Nine patients were diagnosed with allergic rhinitis, having eye and nose symptoms and denying respiratory symptoms, and consequently, were categorised as having rhinitis as the predominant symptom. Topical steroids were not allowed during pollen season or outside season, and none of the patients were on any regular medication during season. None of the patients had smoked for the past 10 years. Forced expiratory volume in one second (FEV₁) out of season was more than 75% of predicted. FEV₁/forced vital capacity (FVC) was more than 70% in all patients (Table 1).

Control group

The control group consisted of four healthy, non-atopic, never-smoking subjects, having allergic symptoms neither outside nor during the birch pollen season. They were skin prick test-negative to all nine standard allergens, had no serum IgE antibodies, and had normal lung function with an FEV₁ >80% of predicted. The control group only completed investigations during the pollen season.

Study design

The study included altogether five visits to our out-patient clinic: inclusion, baseline, during birch pollen

Table 1. Demographic Data of Patients with Allergic Rhinitis and Allergic Asthma (Mean and Range)

	Allergic rhinitis (n=9)	Allergic asthma (n=8)	p Value
Gender (male/female)	8/1	3/8	0.36
Age	43 (24–66)	41 (19–56)	0.85
Ex-smokers (>10 yr)	2	1	0.79
SPT birch mm ²	47.1 (20–88)	43.6 (26–64)	1.0
IgE for birch, class 0–5	3.1 (2–4)	3.4 (2–5)	0.43
FEV ₁ (L)	4.0 (2.4–4.9)	3.5 (2.6–4.0)	0.12
FEV ₁ % of predicted	102 (75–139)	97 (83–108)	0.44
PEFR (L/min)	615 (415–826)	504 (347–652)	0.18
PEFR % of predicted	113 (82–140)	101 (73–133)	0.25
FEV ₁ -decrease in % ^a	0.42 (–5.6–5.8)	6.81 (1.55–16.4)	0.02
AllergenPD ₂₀ (SQE) ^b	34 500 (3,850–150,000)	3,700 (2,450–7,700)	0.04
Morning PEFR (L/min) diary during pollen season	575 (550–620)	475 (433–551)	0.02
Evening PEFR (L/min) diary during pollen season	610 (555–630)	478 (449–551)	0.005

SPT skin prick tests

^a After inhalation of hypertonic 4.5% saline solution

^b After bronchial allergen challenge (median and range)

season and after bronchial and nasal allergen challenge respectively. The season visit was made 2–3 weeks after the pollen counts had reached 4,000 grains/m³ of air, pollen grains counted by the Palynological Laboratory, Swedish Museum of Natural History, Stockholm, Sweden [21]. The study was performed during the birch pollen seasons in 2000 and 2002; the season of 2001 was excluded due to the low pollen counts. After inclusion, patients were investigated consecutively. Thus all patients were studied pre-season and during season the same year. Bronchial and nasal allergen challenges were performed during a 4-week period in January and February the following year. The subjects were told to avoid short-acting bronchodilators and anti-histamines for 24 h before the visit and nasal decongestants for 4 h before the visit. When pollen counts reached 4,000 grains/m³, the subjects were told to start recording their morning and evening peak expiratory flow rate (PEFR) in a diary until the season visit. The design of the present study has been described in detail in previous reports [21, 22].

Skin prick tests

Skin prick tests were performed with nine standard aeroallergen extracts (birch, timothy, moonwort, cat dander, dog dander, horse dander, *Dermatophagoides pteronyssinus*, *Cladosporium herbarum* and *Alternaria*) using Soluprick SQ ALK (Hørsholm, Denmark). The results were read after 15 min, measuring the largest diameter of the weal and its perpendicular diameter, and the product was expressed in square millimetre. Skin reactions were considered positive when larger than 9 mm².

Spirometry

Lung function tests were performed with a Vitalograph-Compact spirometer (Vitalograph Ltd., Buckingham, England). FEV₁, FVC, FEV₁/FVC%, and PEFR were recorded. The reference values were those from European Community for Coal and Steel [23]. Spirometry was performed before and after inhalation of hypertonic (4.5%) saline solution. The magnitude of the FEV₁ decrease after inhalation of hypertonic saline was used as a marker of bronchial responsiveness [21].

Nasal challenge test

The experimental nasal challenge test was performed by instillation in the same nostril of 0.3 mL diluent followed by birch pollen extract (Aquagen® SQ, ALK-Abelló, Hørsholm, Denmark) every 15 min in three steps: 1,000 SQ-U/mL, 10,000 SQ-U/mL and 100,000 SQ-U/mL. The symptom score was estimated; if pronounced local symptoms and sneezing occurred, the challenge test was stopped. The response to the allergen provocation was categorised into four groups: no response or response to one or more of the three allergen doses. Blood samples and nasal lavage were taken 18 h (±1 h) after the challenge test was completed.

Bronchial allergen challenge test

The experimental bronchial challenge test was performed using a DeVilbiss-40 nebuliser (particle size 0.5–5.5 µm, output 0.175±0.3 ml/min, mean±SD; Devillbiss Co, Somerset, PA, USA) [24]. Bronchial

challenge with birch pollen extract (Aquagen[®] SQ, ALK-Abelló, Hørsholm, Denmark) was performed in three steps with the doses 1,000 SQ-U, 10,000 SQ-U and 100,000 SQ-U, starting with inhalation of a diluent. The response to the allergen provocation was calculated as the cumulative dose that caused at least 20% decrease in FEV₁ (allergen provocation dose, PD₂₀). In cases when no significant fall in FEV₁ occurred, allergen PD₂₀ was arbitrarily given the value 150,000 SQ-U. Blood and sputum samples were taken 18 h ($\pm$ 1 h) after the challenge test was completed.

Inflammatory cell counts

Four milliliter of blood was collected in EDTA tubes for routine laboratory tests of eosinophil and neutrophil counts (Cell-Dyn 4000, Abbott Laboratories, Abbot Park, IL, USA) at the accredited laboratory at the Department of Clinical Chemistry, Uppsala University Hospital. Differential cell counts were obtained using a cytopsin preparation (Cytospin, Shandon, Southern Instruments, Sewickley, PA, USA), stained with May–Grünwald and Giemsa, and examined under light microscope.

Isolation of granulocytes

Isolation was performed on heparinised blood. The mononuclear leukocytes were removed by Percoll gradient centrifugation [25]. The granulocyte mixture obtained by this procedure had a purity of $99.8 \pm 0.2\%$ (SD).

Inhibitor

The PI3K pathway inhibitor Wortmannin (Calbiochem-Novabiochem Corp, La Jolla, CA, USA), was dissolved in dimethyl sulfoxide (DMSO; Sigma Chemical Company, St. Louise, MO, USA) and kept in dark at -18°C . For negative control, granulocytes were incubated with DMSO in dilutions corresponding to amount when inhibitor was present. On the day of use, dilutions from the stock material were made in assay buffer [Hanks' solution supplemented with 0.74 mM Ca^{2+} and 0.1% human serum albumin (HSA)] to the stated concentrations of 10^{-6} to 10^{-9} M of Wortmannin.

Inhibition of PI3K pathway

Granulocytes were preincubated with Wortmannin (10^{-6} to 10^{-9} M) for 10 min at 37°C , before induction of granule protein release. The concentration range was

tested in a previous study [26] and was found to inhibit eosinophil degranulation in a dose-dependent manner. The cell viability after this procedure was 99.0–99.5%, determined by staining with Tryptan blue. For negative control, the cells were pre-incubated for 10 min with assay buffer. All incubations were made in duplicates.

Measurement of eosinophil and neutrophil degranulation

The release assay for C3b-mediated degranulation by Sephadex particles was performed according to Winquist *et al.* [27], with some minor modifications as previously described [11]. The final concentration of granulocytes in the assay was $1.0 \times 10^9/\text{L}$. Incubation was then performed at 37°C for 0 and 20 min with either assay buffer for spontaneous granule release or with washed, serum-treated Sephadex G-15 particles (83.5 g/L; GE Healthcare (formerly Amersham Biosciences) NJ, USA) for stimulated release. Hanks' solution supplemented with 0.74 mM Ca^{2+} and 0.1% HSA was used as assay buffer. All incubations were made in duplicate. For measurement of total cell content of granule proteins; 300 mL of granulocytes ($3.0 \times 10^9/\text{L}$) was mixed with 1.5 mL of 0.5% N-acetyl-N,N,N-trimethylammonium bromide (cetyl-trimethyl-ammoniumbromide) in 0.15 mM NaCl and incubated for 1 h at RT followed by centrifugation at $600 \times g$ for 10 min at 4°C . A 1.5 mL volume of supernatant was removed and stored for later measurement of granule proteins.

Radioimmunoassays of granule proteins and RadioAllergoSorbent Test

The released amounts of ECP and MPO from the eosinophils and neutrophils, respectively, were assayed by means of specific radioimmunoassays (RIA; Pharmacia Diagnostics AB, Uppsala, Sweden) and EPO with ImmunoCAP FEIA (Pharmacia Diagnostics AB, Uppsala, Sweden). Specific IgE was determined by RadioAllergoSorbent Test (RAST; ImmunoCAP, Pharmacia Diagnostics AB, Uppsala, Sweden) at the Department of Clinical Immunology, Uppsala University Hospital (normal <0.35 kU/L).

Calculations of released amounts of ECP, EPO and MPO

The amounts of released ECP, EPO and MPO were expressed as percent of total cellular content, calculated from a standard curve of serial dilutions of respective

cell extracts. Results were calculated by regression analysis.

Ethical approval

The study was performed with the approval of the ethics committee at the Medical Faculty at Uppsala University and informed consent was obtained from each subject.

Statistical evaluation

The Kruskal–Wallis, ANOVA and Mann–Whitney *U* test were used to evaluate statistical differences between patient groups. For paired analyses, we used Friedman’s ANOVA and Wilcoxon’s matched pairs test. Correlations were investigated with Spearman’s test (ρ). A *p* value of <0.05 was considered significant. All the calculations were performed using the statistical software package Statistica (Statsoft Inc, Tulsa, OK, USA).

RESULTS

Clinical characteristics

No significant differences at baseline concerning gender, age, smoking, allergy variables and lung function were recorded between patients with allergic rhinitis and allergic asthma. However, patients with allergic asthma were more responsive as measured by FEV₁ decline to inhalation of hypertonic 4.5% saline solution at baseline, had a greater decrease in both morning and evening PEFR during pollen season and

also had a greater responsiveness expressed as allergen PD₂₀ for birch after bronchial challenge [21, 22] (Table 1).

PI3K inhibition of granule protein release during pollen season

Wortmannin (10^{-6} to 10^{-9} M) inhibited ECP and MPO release in a dose-dependent manner in allergic rhinitis and allergic asthma during the pollen season (Table 2) and this was also the case for EPO. However, there was a tendency towards less inhibition of ECP release in the asthmatic patients and for the suboptimal concentration of Wortmannin (10^{-8} M) this was significant compared to the control group ($p=0.01$; 37% inhibition in asthmatics, 70% in rhinitics and 73% in controls; Fig. 1). Inhibition of ECP release in allergic rhinitis and the control group was in the same range during season. The same pattern with less inhibition in the asthmatic group was also seen for MPO, being significant compared to the controls for 10^{-7} M Wortmannin (7.9% vs. 2.1%, $p=0.047$; Table 2). EPO release was low in assay buffer in all three patient groups and inhibition with Wortmannin displayed only a modest additional decrease in the release of EPO.

PI3K inhibition of granule protein release after experimental allergen challenge

Wortmannin (10^{-6} to 10^{-9} M) inhibited ECP, EPO and MPO release in a dose-dependent manner in allergic rhinitis and allergic asthma after nasal (Table 3) and bronchial allergen challenges (Table 4). Inhibition of granule protein release was in the same range in patients with allergic rhinitis and allergic asthma, though there

Table 2. Inhibition of C3b-induced Granule Protein Release (% of Total Cell Content after 20 min) in Allergic Rhinitis, Allergic Asthma, and the Control Group during Pollen Season (Median, IQR)

		Buffer	Wortmannin (10^{-9} M)	Wortmannin (10^{-8} M)	Wortmannin (10^{-7} M)	Wortmannin (10^{-6} M)
ECP	Controls	17.0 (14.1–18.0)	21.0 (19.4–22.9)	4.48 (3.65–7.39)	1.01 (0.92–2.28)	0.88 (0.56–2.40)
	Allergic rhinitis	17.9 (17.3–23.3)	17.1 (14.0–17.9)	5.41 (3.98–6.76)	2.72 (1.20–3.34)	2.07 (1.02–3.28)
	Allergic asthma	14.0 (7.72–18.1)	12.7 (8.84–19.2)	8.79* (3.14–10.6)	4.44 (1.62–6.32)	1.70 (1.23–6.32)
EPO	Controls	1.35 (1.17–1.72)	1.60 (1.36–1.82)	0.88 (0.53–1.17)	0.54 (0.36–0.71)	0.60 (0.38–0.64)
	Allergic rhinitis	2.01 (1.75–2.42)	1.83 (1.44–2.06)	1.03 (0.70–1.28)	0.62 (0.41–0.74)	0.47 (0.36–0.68)
	Allergic asthma	1.58 (1.52–3.46)	2.36 (1.79–3.20)	1.22 (0.62–2.16)	0.53 (0.26–0.93)	0.56 (0.20–1.1)
MPO	Controls	16.6 (14.9–18.0)	14.0 (12.4–16.9)	5.98 (4.37–8.00)	2.07 (1.62–4.54)	1.86 (1.75–4.53)
	Allergic rhinitis	15.9 (13.5–18.3)	12.6 (12.3–19.4)	6.13 (6.02–8.20)	4.97 (2.52–6.96)	5.24 (2.70–7.08)
	Allergic asthma	17.6 (12.4–18.6)	16.7 (13.7–18.9)	11.9 (5.36–16.3)	7.89** (3.10–8.61)	3.64 (3.16–4.00)

* $p=0.01$, significant difference in ECP release between allergic asthma and the control group

** $p=0.047$, significant difference in MPO release between allergic asthma and the control group

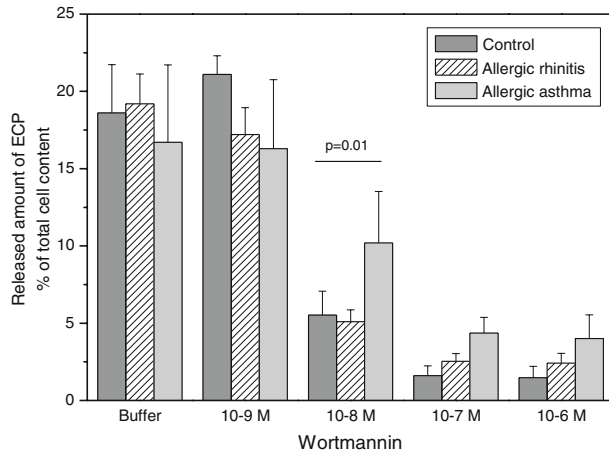


Fig. 1. PI3K inhibition with Wortmannin of C3b-induced release of ECP (after 20 min) in allergic rhinitis, allergic asthma and the control group during pollen season (mean+SEM).

was a tendency towards less inhibition in the asthmatic group (Figs. 2 and 3). There was also a clear propensity towards less inhibition in the rhinitic patients after allergen challenge compared to seasonal exposure, especially after bronchial challenge (Tables 3 and 4).

DISCUSSION

In this paper, we have studied signalling through PI3K for C3b-stimulated *in vitro* degranulation of blood eosinophils and neutrophils by using the PI3K inhibitor Wortmannin. The degranulation patterns from the granulocytes in allergic rhinitis and allergic asthma were compared, during pollen season and after nasal and bronchial allergen challenge, respectively. The main finding in our study was that the PI3K pathway clearly was involved in ECP, EPO and MPO release in both allergic rhinitis and allergic asthma after all three allergen challenge models. Inhibition of ECP and MPO

release followed a dose dependent pattern in all experiments. However, EPO release displayed only a modest decrease in release of granule proteins after inhibition with Wortmannin.

Eosinophil granulocytes are primarily tissue-residing cells in body mucosal linings and skin, involved in innate immune responses, tissue repair and immune modulating reactions [2]. After proper stimulation eosinophils express an array of surface receptors leading to granule protein release in target organs [28, 29], a strictly regulated process with a high threshold for degranulation. Previous studies indicate that the degree of priming is related to the degranulation status and activity of the disease [11, 29] and there is also a marked heterogeneity in degranulation levels in different eosinophilic disorders [30]. *In vitro* studies have also demonstrated selective release of the individual granule proteins, first demonstrated two decades ago for immunoglobulin-activated Fc receptors in both allergic and non-allergic subjects [31], but also for interaction with IgA locally in the mucosa [32].

The stimuli required for degranulation of the eosinophils both physiologically and in allergic disease is still much unexplored as well as the signal transduction from the cell membrane receptors. The binding of eosinophils to a surface by complement receptors induces a strong signal for degranulation involving the receptor for complement factor 3, which is also an important adhesion molecule involved in migration of leukocytes and is also implicated in secretory responses. Therefore, serum-opsonized sephadex particles can be used for attachment of eosinophils and investigation of degranulation [27, 33, 34]. It has been demonstrated that stimulation of the β_2 -integrin adhesion molecule (CD11b/CD18, Mac-1, CR1) plays a crucial role in triggering the intracellular signalling cascade leading to eosinophil degranulation [35]. Integrin (Mac-1) activation on the cell surface leads to activation of mainly cell surface tyrosine receptors on the eosinophils. This

Table 3. Inhibition of C3b-induced Granule Protein Release (% of Total Cell Content after 20 min) in Allergic Rhinitis and Allergic Asthma after Nasal Allergen Challenge (Median, IQR)

		Buffer	Wortmannin (10^{-9} M)	Wortmannin (10^{-8} M)	Wortmannin (10^{-7} M)	Wortmannin (10^{-6} M)
ECP	Allergic rhinitis	19.4 (17.7–23.4)	17.1 (12.1–21.9)	9.73 (6.58–10.7)	2.00 (1.53–2.09)	1.48 (0.85–2.08)
	Allergic asthma	14.9 (13.3–15.6)	14.1 (11.6–16.8)	11.0 (7.44–14.0)	3.61 (2.12–4.67)	2.26 (1.61–2.97)
EPO	Allergic rhinitis	1.4 (1.20–1.60)	1.58 (1.42–1.74)	0.86 (0.84–0.89)	0.56 (0.44–0.68)	0.53 (0.40–0.66)
	Allergic asthma	1.86 (1.65–2.60)	1.85 (1.36–3.19)	1.03 (0.67–1.44)	0.56 (0.55–0.57)	0.53 (0.52–0.60)
MPO	Allergic rhinitis	14.9 (14.8–18.2)	15.7 (13.4–18.4)	8.04 (7.19–8.32)	4.76 (4.47–4.76)	3.26 (2.41–4.28)
	Allergic asthma	18.4 (13.6–20.1)	15.8 (14.7–18.4)	10.8 (7.81–14.5)	6.81 (3.88–10.7)	4.76 (3.48–7.10)

Table 4. Inhibition of C3b-induced Granule Protein Release (% of Total Cell Content after 20 min) in Allergic Rhinitis and Allergic Asthma after Bronchial Allergen Challenge (Median, IQR)

		Buffer	Wortmannin (10^{-9} M)	Wortmannin (10^{-8} M)	Wortmannin (10^{-7} M)	Wortmannin (10^{-6} M)
ECP	Allergic rhinitis	19.8 (17.2–22.0)	18.1 (16.1–20.7)	15.7 (13.3–17.4)	3.84 (2.74–8.62)	2.12 (1.68–2.76)
	Allergic asthma	16.1 (10.1–17.1)	14.0 (11.4–16.2)	10.5 (8.45–11.4)	2.42 (1.99–4.34)	1.77 (1.66–1.86)
EPO	Allergic rhinitis	1.99 (1.96–2.00)	2.22 (1.84–2.34)	1.63 (1.59–1.75)	0.58 (0.54–0.86)	0.36 (0.35–0.42)
	Allergic asthma	1.84 (1.39–1.99)	1.60 (1.50–1.97)	1.04 (0.94–1.28)	0.47 (0.38–0.58)	0.44 (0.36–0.49)
MPO	Allergic rhinitis	21.6 (19.0–25.1)	19.8 (17.7–21.1)	17.6 (13.7–19.0)	5.62 (5.34–9.61)	4.55 (4.06–4.96)
	Allergic asthma	16.8 (13.5–21.8)	18.4 (12.4–21.6)	11.4 (9.28–13.8)	5.30 (3.08–6.27)	3.24 (1.87–4.82)

triggers the downstream signalling cascade leading to cellular degranulation in response to granulocyte-macrophage colony-stimulating factor (GM-CSF), platelet activating factor, IgG and C3b [33, 34, 36]. In the present study, we have investigated eosinophil and neutrophil degranulation by serum opsonized Sephadex particles, which mainly activate degranulation by binding to CD11b/CD18 [11, 26].

It is now accepted that activation of class I PI3K is one of the most important pathways for controlling downstream phosphorylation cascade events in neutrophils [37], and also in eosinophils *in vitro* in mice as well as in healthy blood donors [15, 26]. In this study, we investigated the involvement of the PI3K pathway in the degranulation of allergen primed granulocytes by using the PI3K inhibitor Wortmannin in patients with allergic rhinitis and allergic asthma in three allergen challenge models. In line with previous reports on healthy blood donors, we found that Wortmannin inhibited C3b-induced *in vitro* degranu-

lation of both eosinophils and neutrophils in allergic patients in a dose-related fashion, with slight differences between patients with allergic rhinitis and allergic asthma [26]. In healthy subjects Wortmannin (10^{-6} to 10^{-9} M) inhibited the degranulation of eosinophils (ECP, EPO, EPX/EDN) in a dose-dependent manner with 78% inhibition of ECP, 56% inhibition of EPO and 72% inhibition of EPX/EDN release at the suboptimal concentration of Wortmannin 10^{-8} [26]. The asthmatic patients demonstrated an overall tendency to less inhibition of ECP release by Wortmannin, indicating that other pathways apart from PI3K play a larger role in eosinophil degranulation in allergic asthma than in allergic rhinitis. There was also a clear propensity towards less inhibition in the rhinitic patients after allergen challenge compared to seasonal exposure, especially after bronchial challenge

EPO release, on the other hand, did not follow a strict dose-dependent mode of action and the absolute amount of EPO release compared to buffer control was

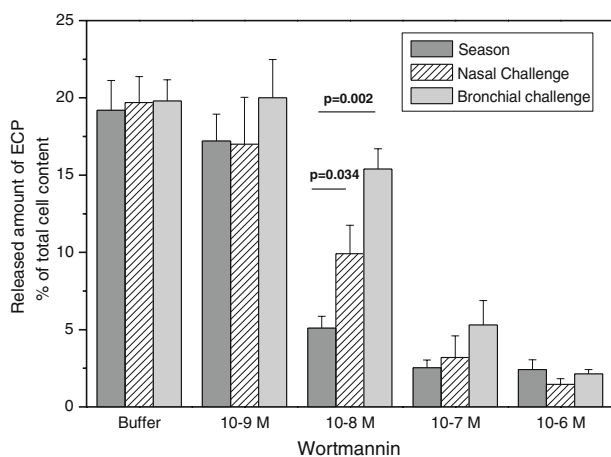


Fig. 2. PI3K inhibition with Wortmannin of C3b-induced release of ECP (after 20 min) in patients with allergic rhinitis during pollen season and after nasal and bronchial allergen challenge (mean+SEM).

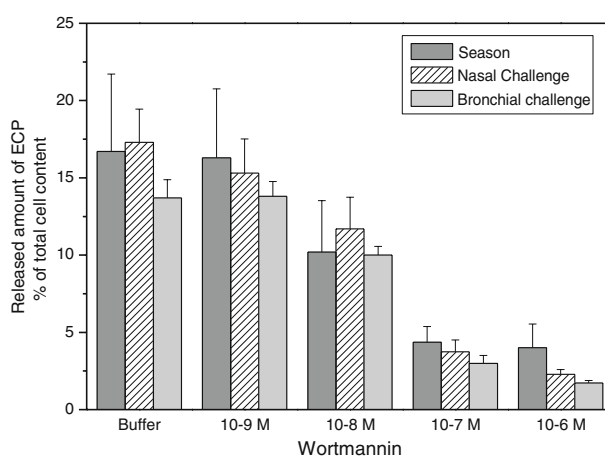


Fig. 3. PI3K inhibition with Wortmannin of C3b-induced release of ECP (after 20 min) in patients with allergic asthma during pollen season and after nasal and bronchial allergen challenge (mean+SEM).

very low in all groups, as has been reported in previously [11, 30, 38]. In the control group, a slight increase in EPO release (18%) could be recorded at the lowest concentration of Wortmannin (10^{-9} M) compared to buffer during pollen season. In the rhinitic patients, both nasal and bronchial challenge resulted in increased EPO release compared to buffer in 10^{-9} M Wortmannin after seasonal exposure (13% and 12%, respectively). The inhibition of released EPO by 10^{-6} M Wortmannin was significant in the rhinitic group compared to the controls during pollen season and after bronchial challenge. It has been postulated that the very low release values of EPO in degranulation assays depends on the sticky nature of the protein, due to a very high cationic charge with a isoelectric point of 10.8, and therefore adhering to the test tubes when investigated [39]. It has also been demonstrated that Wortmannin inhibits EPO release *in vitro*, possibly by blocking CD18, after incubation with a cytokine cocktail of IL-3, IL-5 and GM-CSF [39]. In addition, EPO has been shown to be the most potent eosinophil granule protein, on a molar basis, to kill helminths [2], and perhaps not playing an important role in allergic reactions.

The eosinophils are considered to be major effector cells in tissue damage in asthma. However, the role of the neutrophils is less understood in allergic airway inflammation except in severe chronic asthma [3]. Interestingly, it has recently been shown that neutrophils are prominently elevated even in mild asthma exacerbations [40] as well as in non-atopic asthmatic children [41]. Neutrophils are phagocytic cells participating in the first line of infectious defence and they are also a major source of pro-inflammatory cytokines, contributing to the onset and early orchestration of the inflammatory response in many diseases. Recently, it has been reported that neutrophils are the first cells recruited to the site of the allergic reaction [42] and there are indications that the neutrophil has tools to both limit the magnitude and length of the inflammatory response, i.e., may take part in the resolution process of both the allergic and other inflammatory responses [37, 42]. In another report it has been shown that the PI3K class IB isoform pathway in neutrophils is a key modulator in inflammation and allergy, but not needed in neutrophil phagocytic processes [37, 43]. In our study, MPO release was shown in both patients with allergic rhinitis and mild allergic asthma during pollen season and after experimental challenge. There was also a strict dose-dependent pattern for the MPO release,

except for a slight increase compared to buffer in the asthmatic patients (10%) after bronchial challenge. No significant differences in MPO release between allergic rhinitis and allergic asthma in either allergen challenge model could be seen. However, there was a clear tendency towards less inhibition in the allergic patients compared to the control group during pollen season, indicating a role for the neutrophils in the allergic inflammation.

The strength of the present study was the simultaneous evaluation of the signal transduction through PI3K in B-eosinophils and B-neutrophils in patients with allergic asthma and allergic rhinitis after both long-term natural allergen exposure and after experimental allergen challenge, i.e., different modes of allergen priming of the granulocytes. One drawback of this study is the relative small number of subjects in each allergic group which limited the opportunity to find differences between the two allergic groups. The results indicate that the two allergic groups, more or less, follow the same pattern for signalling through PI3-kinases. However, there are many questions to be resolved and further investigations are needed in order to study the more precise nature of signal transduction, both downstream of PI3K as well as the pathway for activating the individual PI3K isoforms, with specific repertoires of downstream signalling, to achieve tissue-selective inhibition of PI3K [44].

We conclude that the PI3-kinase pathway for degranulation of ECP, EPO and MPO clearly was involved in both patients with allergic rhinitis and allergic asthma in all three allergen challenge models. Patients with allergic asthma, though, demonstrated less inhibition of ECP release via the PI3K-mediated pathway during pollen season, indicating that other pathways play a larger role in eosinophil degranulation in allergic asthma than in allergic rhinitis.

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Competing interests. The authors declare that they have no competing interests.

Authors' contributions. Mary Kämpe, Marie Carlson and Gunnemar Stålenheim designed the study and were responsible, together with Christer Janson, for analysing and interpreting the results as well as critically revising the manuscript. Maria Lampinen was involved in drafting the manuscript and the figures. Ingrid Stolt carried out the assays and assisted in the statistical analyses.

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