

Topical corticosteroid inhibits interleukin-4, -5 and -13 in nasal secretions following allergen challenge

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Summary

Background Cytokines and chemokines produced by allergen-reactive T-helper type 2 (Th2) cells may be pivotal to the pathophysiology of allergic disorders.

Objective This study was performed to assess the effect of 7 days of topical corticosteroid on nasal allergen challenge (NAC) in terms of eosinophils, cytokines and chemokines obtained by nasal lavage and filter paper methods.

Methods Patients with grass pollen seasonal-allergic rhinitis ($n = 13$) out of season received nasal challenge following matched placebo (twice daily into each nostril for 7 days) and fluticasone propionate (100 µg twice daily into each nostril for 7 days). Chemokine and cytokine levels were analysed using a sensitive automated bead immunoassay system at intervals up to 8 h after NAC.

Results Levels of cytokines and chemokines from filter paper were generally higher than from nasal lavage. Fluticasone propionate caused a reduction in symptoms, total leukocyte counts and eosinophils, and abrogation of IL-4, IL-5, IL-6 and IL-13 responses in the filter paper taken in the late phase ($P < 0.05$ for IL-4 and IL-13, $P < 0.01$ for IL-5 and IL-6). Levels of chemokines (eotaxin, RANTES, MCP-1, MIP-1α, IL-8 and IP-10) were also reduced in the late phase ($P < 0.01$ at 8 h). However, levels of IL-2, IL-3, IL-7, IL-12 (p40 and p70), -15, TNF-α, IFN-γ and GM-CSF were not affected.

Conclusion Fluticasone propionate has selective inhibitory effects on Th2 cytokine synthesis following nasal challenge, while also decreasing release of chemokines, but not affecting levels of Th1 cytokines.

Keywords eosinophils, fluticasone propionate, nasal allergen challenge

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Introduction

There is strong evidence that allergen-reactive T-helper type 2 (Th2) cells play an important role in the induction and maintenance of the allergic inflammatory cascade [1]. Cytokines and chemokines produced by Th2 cells (IL-4, IL-5, and IL-13) may be pivotal to the pathophysiology of allergic disorders: involving production of IgE; recruitment and activation of mast cells and eosinophils; mucus hypersecretion; subepithelial fibrosis; and tissue remodelling [2,3]. Novel immunotherapeutic strategies target allergen-specific Th2 cells and their products in atopic individuals [4–7].

The nasal inflammatory exudates can be measured in nasal lavage fluid or fluid adsorbed on filter paper strips placed on the nasal mucosa. Following allergen challenge increases in

IL-5 and eotaxin have been detected in nasal lavage fluid [8, 9]. In addition, post-allergen challenge increases in GM-CSF, IL-5, IL-8, MIP-1α, and RANTES [10–12] have been detected using filter paper strips.

Topical corticosteroids are recognized as first line therapy for more severe and persistent forms of allergic rhinitis (AR) by the Allergic Rhinitis and its Impact on Asthma (ARIA) global guidelines [7]. A single dose of topical corticosteroid has been shown to reduce levels of GM-CSF and IL-5 detected by absorption with filter paper following nasal challenge with grass pollen in AR [11]. Maintenance treatment with topical steroids exerts a range of anti-inflammatory nasal effects, and following nasal allergen challenge (NAC) has been shown to inhibit levels of cytokines such as IL-1β, GM-CSF [12] and TNF-α [13], as well as chemokines such as eotaxin [9], RANTES, MIP-1α, and IL-8 [12]. One week of nasal fluticasone has been shown to decrease IL-6 levels detected by ELISA following NAC [14], and mRNA for IL-6 has also been shown to be decreased [15]. However, topical budesonide did not inhibit IL-6 levels in nasal lavage fluid following natural ragweed exposure [16].

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Monoclonal anti-IgE antibody has recently been shown to inhibit NAC responses [17].

NAC is a convenient and non-invasive challenge model to study the effects of anti-inflammatory therapy on allergic reactions. Following NAC it is possible to take repeated samples of nasal exudates by absorption on to filter paper or by nasal lavage. In the present study, we assessed the effects of 7 days of topical fluticasone propionate (FP) on nasal responses to grass pollen in patients with seasonal AR outside the hayfever season. In particular, we describe effects of corticosteroid therapy on a range of 22 chemokines and cytokines measured by a multiplexed bead array system.

Materials and methods

Patients

Thirteen patients with grass pollen AR were studied (Table 1). Specific allergy was confirmed by positive skin prick test (SPT) to Timothy grass pollen extract (Soluprick, *phleum pratense*; ALK, Horsholm, Denmark). The study was

Table 1. Demographic data of study subjects

Characteristic	
Age (years)	
Mean	26.9
Range	20–38
Sex (F/M)	6/7
Race	
White	11
Black	1
Asian	1
Total serum IgE (IU/mL)	
Geometric mean	163.6
Standard deviation	159.7
Baseline blood eosinophils ($\times 10^9$ cells/L)	
Mean	0.18
Standard deviation	0.09

approved by local ethics committee and all subjects gave informed written consent.

Study design

Patients received placebo for 7 days, and after washout (7 days), FP (100 μ g bd into each nostril) for 7 days in a single blind fashion.

Nasal allergen challenge

Nasal allergen challenge (NAC) with Timothy grass pollen (Aquagen, *phleum pratense*; ALK) was administered after each period (placebo/FP) using a nasal Bidose applicator (Valois S.A., Marly le Roi, France), which delivers 100 μ L into each nostril per actuation as a total nose dose of 1000 biological units [18]. Nasal lavage and contralateral filter paper sampling was taken at 0, 0.5, 1, 2, 4, 6, and 8 h after NAC.

Lavage procedure and processing

A modified nasal pool technique was adapted from previous studies [19]. The subjects were seated in a forward-flexed neck position (60° from the upright position) to prevent fluid from reaching the nasopharynx. To ensure adequate washing, the lavage fluid (6 mL) was passed slowly into the nasal cavity and then left to dwell for 30 s. The fluid was withdrawn into the syringe and flushed back into the nasal cavity. Care was taken to maintain a tight seal between the olive and nostril throughout.

The nasal lavage samples were initially centrifuged at 4 °C for 10 min at 400 *g* to generate a supernatant which was collected for later fluid phase analysis. The cell pellet was resuspended in 0.01%, dithiothreitol (DTT) in phosphate-buffered saline (PBS). The samples were gently agitated on a rolling mixer for 10 min and then centrifuged again to generate a supernatant. The supernatant was discarded and the remaining pellet resuspended in PBS. An initial estimate of total cell count was made using an improved Neubauer haemocytometer (Glaswarenfabrik, Sontheim, Germany). The cell suspension was diluted and cytospin slides were

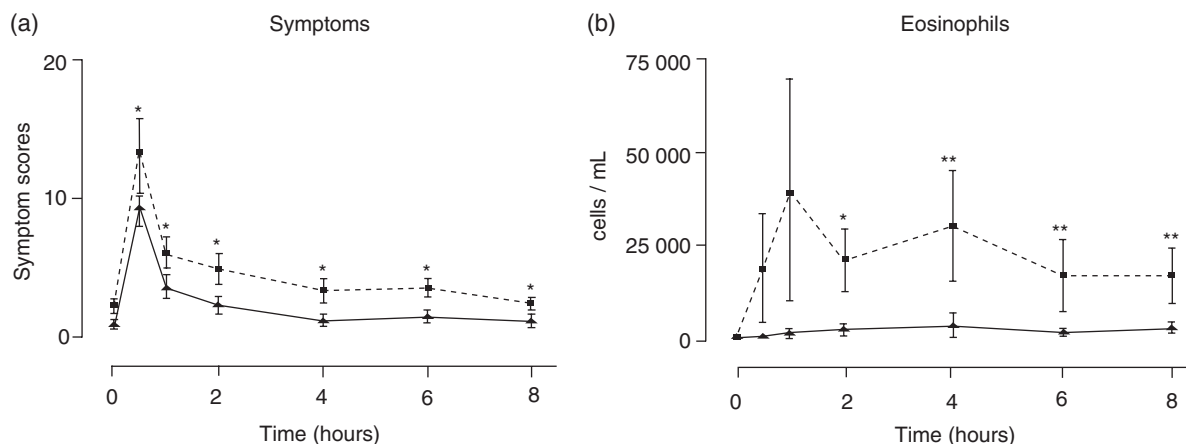


Fig. 1. Symptom scores (a) and eosinophils cell counts in nasal lavages (cells/mL) (b) before and at various time points following nasal allergen challenge in relation to either placebo (dotted line) or fluticasone (solid line) pre-treatment. Values presented are means $\pm$ SEM ($n = 13$). * $P < 0.05$ of post-corticosteroid allergen responses compared with post-placebo allergen responses at the same time interval. ** $P < 0.01$ of post corticosteroid allergen responses compared with post-placebo allergen responses at the same time interval.

produced. Slides were stained with Diff-Quik stain and differential cell counts were determined by assessment of 400 leucocytes on the cytospin.

Filter paper procedure and processing

A modified 'matrix method' was adapted from that used in previous studies [12, 20]. Two strips (7 × 30 mm/strip) of filter paper (Whatman No. 42; Whatman Paper Ltd, Maidstone, UK) were placed, one on the nasal septum and one on the inferior turbinate for 10 min. After removal the strips were placed into 500 µL of assay buffer and agitated on a rolling mixer. After centrifugation, the eluate was collected for later analysis.

Measurement of mediators

The lavage supernatants were centrifuged through 0.22 µm pore size cellulose acetate filters (Costar spin-X[®], Corning Inc., Corning, NY, USA) to remove residual mucus before analysis. The levels of human IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, eotaxin, RANTES, MCP-1, MIP-1α, IP-10, GM-CSF, IFN-γ and TNF-α in lavage and filter eluate were analysed using a multiplexed bead array system (Upstate, Milton Keynes, UK). Samples were read using a commercially available Luminex[®] 100 IS[™] instrument (Luminex Corporation, Austin, TX, USA). To increase sensitivity the gain of the RP1 reporter channel was increased from a value of 3885–9000 and a final late wash step was added to reduce the associated increase in background. The sensitivities for all cytokines and chemokines were 190 pM, except for RANTES which was 230 pM. Details on the methods are provided in the online data supplement.

Nasal symptoms

Nasal symptoms were recorded before and 0.5, 1, 2, 4, 6, and 8 h after allergen challenge. Nasal congestion, rhinorrhea, frontal headache, post-nasal drip, sneezing and nasal itch, were scored from 0 to 3 (0 = no, 1 = mild, 2 = moderate, 3 = severe symptoms).

Statistical analyses

The significance of the change from baseline and the difference between active/placebo was tested at each time point, with the non-parametric Wilcoxon's signed rank test and correlations were evaluated by the Spearman rank test. However, in order to avoid false-positive results arising from multiple testing of large numbers of samples, two-way analysis of variance (ANOVA) tests were used to exclude any spurious significance. Each two-way ANOVA test provided single *P*-values for the change in each cytokine and

chemokine in each treatment group following challenge, a single *P*-value for the comparison between placebo and fluticasone, and a third *P*-value for interaction between the

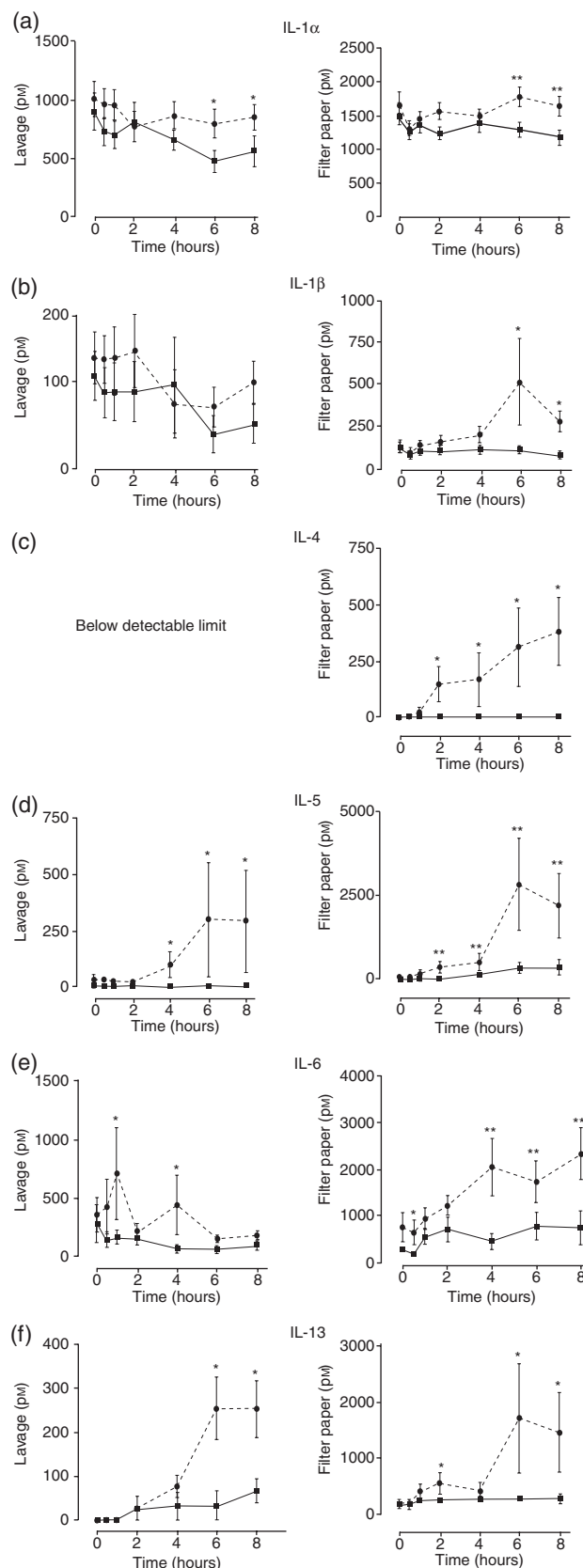


Fig. 2. Levels of cytokines (pm) significantly affected by topical corticosteroids in nasal lavage and filter paper. IL-1α (a), IL-1β (b), IL-4 (c), IL-5 (d), IL-6 (e), IL-13 (f) before and at various time-points following nasal allergen challenge either after placebo (dotted line) or fluticasone (solid line) pre-treatment. Values presented are means ± SEM (*n* = 13). **P* < 0.05 of post-corticosteroid allergen responses compared with post-placebo allergen responses at the same time interval. ***P* < 0.01 of post corticosteroid allergen responses compared with post-placebo allergen responses at the same time interval.

time and treatment factors. A P -value of <0.05 was considered significant.

Results

Effects of nasal allergen in placebo group compared with baseline

Following NAC symptoms were almost immediate, peaking at 0.5 and 2 h before gradually abating ($P < 0.01$ to 2 h compared with baseline). Data is shown in Fig. 1, but note that all significance values shown in Figs 1–4 are for comparison between responses after fluticasone compared with the same time interval after placebo, and are not for comparisons with baseline. The number of eosinophils in nasal lavage fluid increased to an early peak at 1 h, showed a second peak at 4 h after allergen challenge, and were still increased at 8 h ($P < 0.01$ at all three times compared with baseline).

Levels of cytokines and chemokines detected in filter paper were generally higher than those in nasal lavage. Compared with baseline levels there were significant increases compared with baseline in post-NAC lavage levels of IL-6 at 1 and 4 h and IL-13 at 6–8 h (Fig. 2). In contrast, in the filter paper the levels of IL-1 β , IL-4, IL-5, IL-6 and IL-13 levels were significantly increased compared with baseline at 6–8 h. No statistically significant increases in the levels of IL-2, IL-3, IL-12 (p40), TNF- α , IFN- γ or GM-CSF compared with baseline were seen following NAC in lavage or filter paper sampling methods (Fig. 3). In filter paper there were significant changes in the level of eotaxin in the early and late phases at 2 and 8 h, RANTES at 1–6 h, while MCP-1 and MIP-1 α were increased at 6–8 h.

Effects of nasal fluticasone compared with placebo

A 7-day treatment of nasal FP caused a statistically significant decrease in symptoms from immediately after NAC up to 8 h, and a significant decrease in eosinophils in nasal lavage at 2–8 h (Fig. 1).

In nasal lavage, IL-1 α was inhibited at 6–8 h, IL-1 β was not significantly inhibited, IL-4 was below the detection limit, IL-5 was inhibited at 4–8 h, IL-6 at 1 and 4 h and IL-13 at 6–8 h (Fig. 2). Using filter paper as a sampling method, IL-1 α , IL-1 β , IL-4, IL-5, IL-6 and IL-13 were all significantly inhibited at 6–8 h, with greater significance for some responses compared with nasal lavage (Fig. 2).

IL-2, IL-3, IL-12 (p40), TNF- α , IFN- γ and GM-CSF showed no significant inhibition in lavage or filter paper (Fig. 3). Measurement of IL-7, IL-12 (p70), and IL-15 in filter paper and lavage samples showed no significant changes and

data is not shown. IL-10 was below the detection limit and data is not shown.

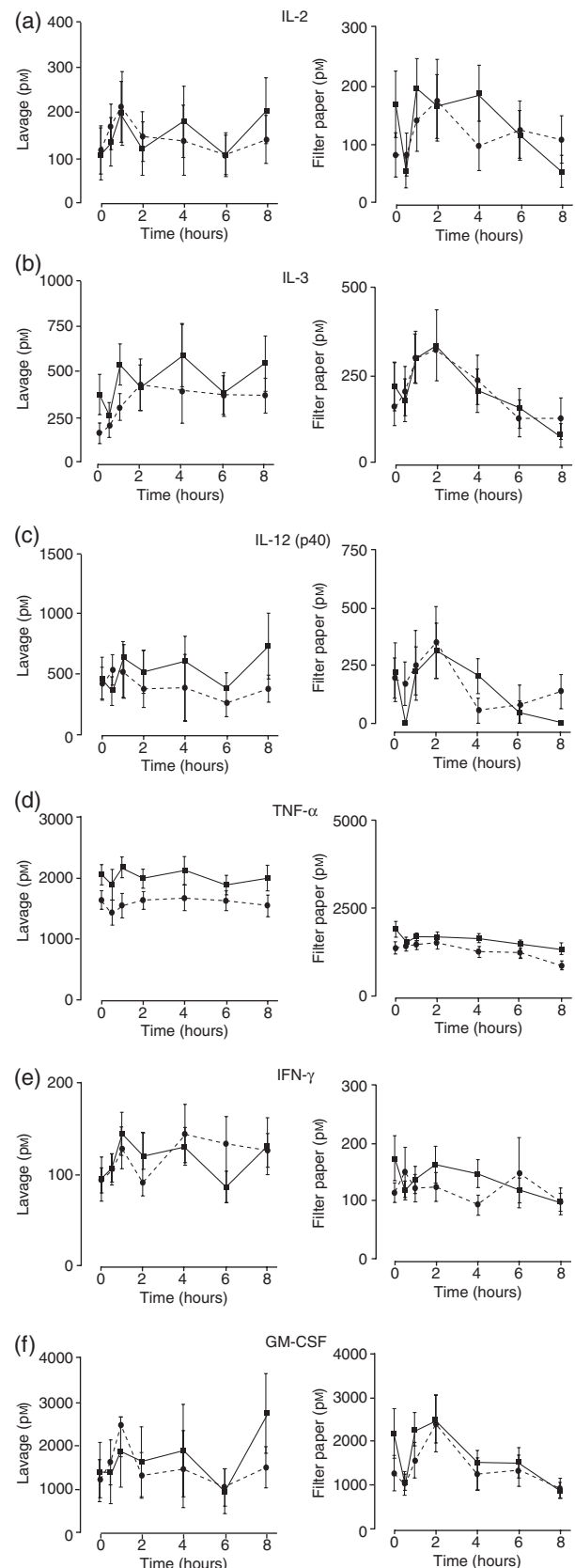


Fig. 3. Levels of cytokines (pm) unaffected by topical corticosteroids in nasal lavage and filter paper. IL-2 (a), IL-3 (b), IL-12 (p40) (c), TNF- α (d), IFN- γ (e), GM-CSF (f) before and at various time-points following nasal allergen challenge either after placebo (dotted line) or fluticasone (solid line) pre-treatment. Values presented are means $\pm$ SEM ($n = 13$). * $P < 0.05$ of post corticosteroid allergen responses compared with post-placebo allergen responses at the same time interval. ** $P < 0.01$ of post-corticosteroid allergen responses compared with post-placebo allergen responses at the same time interval.

In nasal lavage, FP caused significant decreases in certain chemokines at some time intervals after NAC; including RANTES, MCP-1, MIP-1 α , IL-8 and IP-10 (Fig. 4). In filter paper, FP caused generally more convincing decreases in levels of chemokines; including eotaxin, RANTES, MCP-1, MIP-1 α , IL-8 and IP-10.

Discussion

NAC is a convenient and non-invasive clinical study design to assess new therapies directed against allergic inflammation. Volunteers with hayfever outside the grass pollen season can readily be recruited, allergen can be delivered by a simple spray device, nasal exudates can be analysed following lavage or filter paper absorption, and a panel of cytokines and chemokines can be efficiently measured by a sensitive and semi-automated bead array system.

Nasal lavage and filter paper are non-invasive methods which can be used at frequent intervals before and after allergen challenge to describe nasal exudate responses. The nasal filter paper method has the advantage of directly sampling nasal secretions which are less diluted and can therefore pick up protein signals which are below the detection limits of nasal lavage.

However, it should be noted that nasal lavage probably represents an extracellular signal and nasal sampling by absorption into filter paper strips possibly represents both an intracellular and extracellular signal. This is because cells that adhere to the surface of the filter paper may lyse and release their intracellular contents which will be absorbed into the paper matrix along with the extracellular signals. This may be of particular importance as we have shown that the total cell counts are reduced by FP treatment and therefore fewer cells will adhere to the filter paper and release their contents. The contribution of decreased cell numbers may explain why the FP treatment group achieved a more statistically significant inhibition when sampled with filter paper. Changes in chemokine levels may be secondary to reductions in cell numbers, and may be a surrogate for the number of cells.

Nasal FP had selective inhibitory effects on 'Th2 cytokine' synthesis (IL-4, IL-5, IL-6, and IL-13) following NAC, while also decreasing release of chemokines (eotaxin, RANTES, MCP-1, MIP-1 α , IL-8 and IP-10), but not affecting levels of 'Th1 cytokines'. In addition, there was an ablation of the eosinophil response after challenge and an overall decrease in nasal symptom score. This suggests an important role for these cytokines in the pathogenesis of allergic disease and demonstrates a potential therapeutic mechanism of action of corticosteroids.

The effect on IL-4, IL-5, and IL-13 may be explained by the involvement of the transcription factor GATA-3 [21, 22]. It has been previously demonstrated that single doses of topical

steroid inhibit nasal GM-CSF and IL-5 levels detected on filter paper following NAC [23]. IL-5 has an established role as the major terminal differentiation factor during eosinopoi-

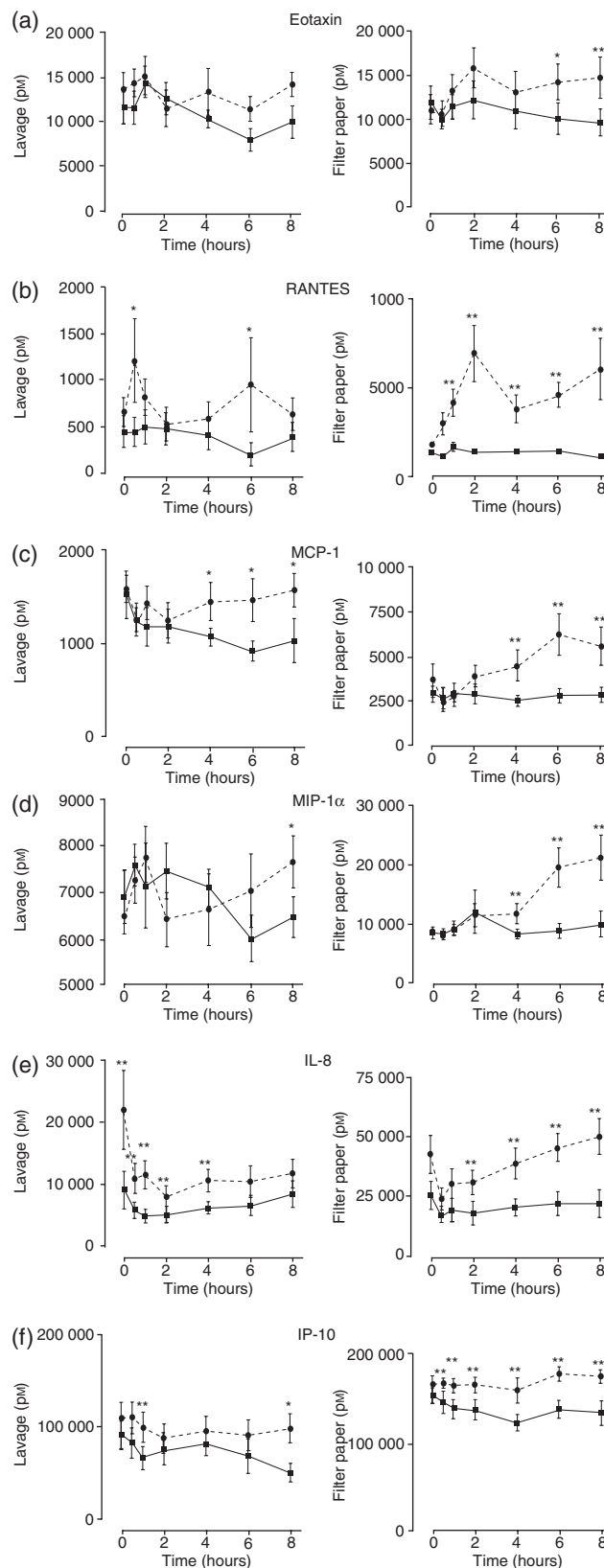


Fig. 4. Levels of chemokines (pm) in nasal lavage and filter paper. Eotaxin (a), RANTES (b), MCP-1 (c), MIP-1 α (d), IL-8 (e), IP-10 (f) before and at various time-points following allergen challenge either after placebo (dotted line) or fluticasone (solid line) pre-treatment. Values presented are means $\pm$ SEM ($n = 13$). * $P < 0.05$ of post-corticosteroid allergen responses compared with post-placebo allergen responses at the same time interval. ** $P < 0.01$ of post-corticosteroid allergen responses compared with post-placebo allergen responses at the same time interval.

esis, it is involved in eosinophil release from bone marrow [24], and is also thought to act as a survival factor for tissue eosinophils [25, 26]. In studies of human asthma, monoclonal antibodies against IL-5 fail to inhibit the late asthmatic reaction (LAR) [27] and do not appear to have symptomatic benefit [24, 28]. Anti-IL-5 treatment did not decrease bronchial mucosal eosinophils to the same extent as blood eosinophils [29], and eosinophils could play an important role in airways remodelling [30–34].

IL-13 shares several important functional properties with IL-4, including its ability to enhance MHC class II expression, CD23 expression, B cell switching to IgE, mast cell differentiation, and, via inhibition of monocyte-derived IL-12 synthesis, inhibition of Th1-associated T cell differentiation *in vitro* [35]. Furthermore, IL-13 appears to be more important than IL-4 in the development of airway hyper-reactivity and mucus production. IL-13 receptors are expressed on most inflammatory cells, however, functional IL-13 receptors do not appear to be expressed on human T cells. Thus, IL-13 appears to be important in the effector phase of allergic inflammation. This is further supported by many *in vivo* observations including that IL-13 administration resulted in allergic inflammation, and IL-13 blockade abolishes allergic inflammation independently of IL-4. Given the importance of IL-13 as an effector molecule its regulation might be key to the propagation of the allergic response.

The pattern of IL-4, IL-5 and IL-13 expression seen in this study along with the similarity in functional activity of IL-4 and IL-13 suggests that their expression may be co-regulated. IL-6 is an acute phase pro-inflammatory cytokine that is produced by a variety of cells and has the potential to promote Th2 differentiation and GATA-3 expression [36]. The reduction of levels of cytokines in nasal exudates may have resulted from steroid-induced inhibition of cytokine production, decreased cytokine secretion, reduced recruitment of cells and/or be secondary to local vasoconstrictor [37] and decreased vascular permeability.

NAC provides a relatively non-invasive model to identify effects of a topical corticosteroid. This study highlights the potential importance of IL-4, IL-5, IL-6 and IL-13 in the pathogenesis of allergic airways disease. We found that assay of proteins with a bead array system is a sensitive and semi-automated method to document these effects, and expect this model to be applicable to the assessment of novel anti-allergic drugs.

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