Eur Respir J 2003; 22: 118–126
DOI: 10.1183/09031936.03.00031102
Printed in UK – all rights reserved

European Respiratory Journal
ISSN 0903-1936

Regular salbutamol use increases CXCL8 responses in asthma: relationship to the eosinophil response

J.R. Gordon*, V.A. Swystun#, F. Li*, X. Zhang*, B.E. Davis#, P. Hull¶, D.W. Cockcroft#

Regular salbutamol use increases CXCL8 responses in asthma: relationship to the eosinophil response. J.R. Gordon, V.A. Swystun, F. Li, X. Zhang, B.E. Davis, P. Hull, D.W. Cockcroft. ©ERS Journals Ltd 2003.

ABSTRACT: Regular salbutamol use can exacerbate allergen-induced airway eosinophilia in asthmatics, but its effect on airway eosinophil chemokine responses is unknown.

Asthmatic subjects (n=14) were treated for 10 days with placebo or salbutamol in a double-blind, cross-over study, then given same-dose allergen challenges. Their sputa were then analysed 1 and 7 h later for a panel of eosinophil-related cytokines. Eosinophils from five test and three control subjects were tested for expression of CXCL8/interleukin (IL)-8, and its receptors and responsiveness to CCL11/eotaxin and CXCL8/IL-8.

Sputum CXCL8/IL-8, but not IL-5, CCL5/regulated on activation, T-cell expressed and secreted, CCL7/monocyte chemotactic protein-3, CCL11/eotaxin, granulocyte-macrophage colony-stimulating factor or tumour necrosis factor levels, were increased (42%) by the salbutamol treatments. The CXCL8/IL-8 levels correlated with the proportions of sputum eosinophils and these cells, but not other sputum cells, stained strongly for CXCL8/IL-8. The circulating eosinophils of the tested subjects (n=5) expressed CXCL8/IL-8 receptors and secreted high levels of this chemokine. Neutralisation of sputum CXCL8/IL-8 reduced eosinophil chemotactic responses to these samples by 19±5%.

These data suggest that regular use of salbutamol can augment airway CXCL8/interleukin-8 responses to allergen challenge and that this CXCL8/interleukin-8 could contribute to the airway inflammatory response.

Eur Respir J 2003; 22: 118–126.

*Immunology Research Group, University of Saskatchewan, and the Divisions of #Respiratory Medicine and ¶Dermatology, Royal University Hospital, Saskatoon, SK, Canada.

Correspondence: J.R. Gordon, Dept of Veterinary Microbiology, 52 Campus Drive, University of Saskatchewan, Saskatoon, SK, Canada S7N 5B4.
Fax: 1 3069667244
E-mail: john.gordon@usask.ca

Keywords: Allergy
asthma
cytokines
eosinophils
salbutamol
sputum

Received: April 15 2002
Accepted after revision: February 28 2003

This work was supported by grants from the Saskatchewan Lung Association and the Canadian Institutes of Health Research.

Inhaled β_2-adrenergic receptor agonists (β_2-agonists) comprise a primary therapy for bronchoconstriction in allergic asthma patients. However, evidence suggests that regular use of β_2-agonists can reduce their effectiveness [1, 2] and lead to increased airway hyperresponsiveness [3–5] and eosinophil/mast cell responses [4, 5] to allergen challenge. Given the association of eosinophils with asthma severity and pathology [6], the cellular mechanisms that may regulate this eosinophil response were questioned.

Eosinophils express receptors for many molecules that are highly pertinent to asthma pathophysiology [6]. For example, the CCR3 receptor, which binds multiple CC chemokines (*e.g.* CCL11/eotaxin [6]), is broadly accepted as critical to the eosinophil response. However, CXCL8/interleukin (IL)-8 is also strongly expressed within allergic lesions [7–10] and eosinophils are responsive to this CXCR1/CXCR2 ligand [11, 12]. Indeed, the decreased eosinophilia after allergen immunotherapy may be related to reduced CXCL8/IL-8 responses to allergen challenge [13].

In a recent study in which mildly asthmatic subjects (n=14) regularly used salbutamol (or placebo) for 10 days, an exacerbation of asthmatic responses (forced expiratory volume in one second (FEV1), serum tryptase and sputum eosinophils) to allergen challenge was observed [5]. In order to gain insight into potential mechanisms behind this eosinophilia [4, 5], a panel of nine pertinent eosinophil-signalling cytokines/chemokines in sputum, previously obtained from these subjects [5], were examined and, in retrospect, the relative responsiveness of their eosinophils (n=5) to CXCL8/IL-8 and CCL11/eotaxin. The expression of CXCL8/IL-8 by their sputum cells and circulating eosinophils and the extent to which CXCL8/IL-8 and CCL11/eotaxin contributed to their sputum eosinophil chemotactic activities were also assessed.

Methods

Study subjects

As noted above, the authors have previously reported the baseline and 1- and 7-h FEV1 values, mast cell tryptase, and sputum eosinophil and metachromatic cell counts in the present subjects [5]. All subjects were aged 20–51 yrs, had baseline FEV1 values of $\geqslant$70% predicted, and all except one demonstrated airway responsiveness to methacholine with a provocation concentration leading to a 20% fall in FEV1 (PC20) of 2.8 mg·mL^{-1} (table 1). All showed atopy for one or more inhalant allergens by skin-prick test and had an early asthmatic response to allergen challenge with a $\geqslant$15% fall in FEV1. Five subjects regularly used inhaled corticosteroids and continued these in the same dose for the entire study. One used sodium cromoglycate during exercising 1–2 times per week, and all but two had used salbutamol in the past. All

Table 1. – Anthropometric and medical data for the study subjects

Subject	Age yrs	Sex	Height cm	MCh PC_{20} $mg \cdot mL^{-1}$	Baseline FEV_1 % pred	Medication $\mu g \cdot day^{-1}$	Allergen
1	20	F	160	0.50	103	FP 250, Salb.	Horse
2	51	M	170	0.45	72		Grass
3	22	F	160	0.56	100	Salb.	Horse
4	36	M	171		94	Salb.	Grass
5	21	F	161	0.71	85	SCG	Grass
6	29	F	154	0.31	86		Cat
7	23	F	177	0.80	97	BDP 50	Cat
8	21	M	188	0.39	85	BUD 800, Salb.	Weed
9	22	M	170	2.8	85	BDP 1000	Horse
10	33	F	178	0.72	75		Cat
11	23	F	154	1.2	89	BDP 200	Grass
12	25	M	182	0.35	86	Salb.	Weed
13	22	F	173	1.0	94	Salb.	Horse
14	28	F	160	0.81	82	Salb.	Cat

MCh: methacholine; PC_{20}: provocative concentration leading to a 20% fall in forced expiratory volume in one second (FEV_1); % pred: % predicted; F: female; M: male; FP: fluticasone propionate; Salb.: salbutamol (per required need); SCG: sodium cromoglycate; BDP: beclomethasone dipropionate; BUD: budesonide.

withheld β_2-agonist use for $\geqslant 2$ weeks prior to and during the study (ipratropium bromide was substituted for symptom relief, Atrovent®; Boehringer-Ingleheim Ltd, Burlington, ON, Canada). All subjects were free of respiratory tract infections or known allergen exposure for the duration of and $\geqslant 4$ weeks prior to this study, which was confined to the snow-cover season. The experimental protocol was approved by the institutional human research ethics committee and all subjects signed informed consent forms prior to participation in the study.

Study design

Fourteen, mild, stable, asthmatic subjects were enrolled in a double-blind, placebo-controlled, cross-over study to compare the effects of 10-day treatment periods of either salbutamol (Ventolin®; GlaxoWellcome Inc., Mississauga, ON, Canada) or placebo on allergen-induced airway eosinophil-related cytokine expression. The two treatments comprised randomly assigned salbutamol ($100\ \mu g \cdot puff^{-1}$) or identical placebo metered-dose inhalers, each two puffs, four times per day, and separated in time by a $\geqslant 7$-day washout period. Each subject received identical doses of allergen after both treatments, administered 12–15 h after the last dose of study inhaler and followed by sputum inductions 1 and 7 h later. Sputum analysis has been reported many times to well reflect airway inflammatory responses discernable by bronchoalveolar lavage [14], including upregulated chemokine responses [15]. Background sputum samples, generated without allergen challenge, were also taken from each subject 1–5 weeks prior to the study.

Methods

Allergen challenges and sputum induction and processing. Allergen (cat epithelium (10,000 biological activity units$\cdot mL^{-1}$), five-grass mix pollen (83,000 protein nitrogen units (PNU)$\cdot mL^{-1}$), western weed pollen (42,000 PNU$\cdot mL^{-1}$; Greer Laboratories Inc., Lenoir, NC, USA), or horse epithelium (32,000 PNU$\cdot mL^{-1}$; Western Allergy Services, Richmond, BC, Canada)), or diluent aerosols were generated using a Wright nebuliser (Aerosol Medical Ltd, Colchester, UK) calibrated to $0.13\ mL \cdot min^{-1}$ output. For sputum induction, sterile saline aerosols of 3, 4 and 5% (w/v; each 12 mL) sodium chloride were generated using an ultrasonic nebuliser (output $3\ mL \cdot min^{-1}$, Ultra-Neb 99; Devilbiss Co., Somerset, PA, USA) and administered in turn *via* a mouthpiece until 2–5 mL of sputum had been produced. Each subject thoroughly rinsed their mouth and pharynx prior to each round of sputum production. All sputum samples were held on ice, their volumes recorded and then processed by the addition of 2 mL of 1% (w/v) dithiothreitol (DTT; Sigma Chemical Co., Mississauga, ON, Canada) in saline, with gentle vortexing to ensure thorough dispersal of the specimens. The sputum fluids were separated from the cells by standard centrifugation, then aliquoted and stored at -85°C [5]. For the chemotaxis assays, aliquots were dialysed extensively against phosphate-buffered saline (PBS; pH 7.2). The sputum cells were washed in Dulbecco modified Eagle medium (DMEM) to remove any residual DTT, then fixed in acid-alcohol formalin [16] for 30 min on ice and subsequently stored at -20°C in 70% ethanol.

Assessment of cytokine expression. The levels of the target cytokines in the sputum fluids were assessed by enzyme-linked immunosorbent assay (ELISA), while the fixed cells were probed for CXCL8/IL-8 expression by immunohistochemistry. For the ELISA, matched capture and biotinylated detection antibody pairs and recombinant cytokine standards for CCL5/regulated on activation, T-cell expressed and secreted (RANTES), CCL7/monocyte chemotactic protein (MCP)-3, CCL11/eotaxin, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, IL-5, IL-6, CXCL8/IL-8, IL-12, IL-13, and tumour necrosis factor (TNF; all R&D Systems, Minneapolis, MN, USA) were titrated to optimal levels and employed using a standard protocol [17]. Each sample was assayed in duplicate. In preliminary experiments the impact of DTT on the sensitivity of each cytokine ELISA was assessed by adding exogenous DTT or buffer into the standard curves. These results were validated by also dialysing the DTT from a sputum aliquot and retesting its cytokine content. The presence of DTT consistently, but differentially, affected the standard curve for each mediator, thus, the standards were routinely diluted in DMEM supplemented with 0.33% DTT, and thereby "real" values for each mediator in the DTT-dispersed sputa were obtained.

For the immunohistochemical localisation of CXCL-8/IL-8 to the fixed sputum cells, a standard protocol was used with Fast Red RR as the chromogen [17]. The target cells'

immunoglobulin (Ig) Fc receptors were blocked with normal human IgG antibodies (Sigma Chemical Co.) and an irrelevant isotype control antibody (Sigma Chemical Co.) was employed to confirm the specificity of the staining.

Peripheral blood eosinophil purification and assessment of CXCR-1/-2 and CCR3 expression. Five of the 14 experimental subjects for these retrospective studies were chosen based on: 1) levels of CXCL8/IL-8 and CCL11/eotaxin in their sputa differentially spanning the range from low to high; 2) eosinophils comprising a substantial proportion of their sputum leukocytes (table 2); 3) their continued availability as blood donors; and 4) availability of archived sputa. Control subjects for these experiments included one mildly atopic nonasthmatic (a/NA), one nonatopic nonasthmatic (na/NA) and an individual with a hypereosinophilia related to an epitheliotropic T-cell lymphoma (TCL). "Untouched" eosinophils were purified by negative-selection magnetism-assisted cell sorting (MACS) using anti-CD16 paramagnetic beads (Miltenyii Scientific, Auburn, CA, USA). Briefly, the granulocyte fraction of ethylenediamine tetraacetic acid-anticoagulated peripheral blood was incubated for 30 min at 6–8°C with anti-CD16-conjugated paramagnetic beads, then sieved through MACS columns to remove the CD16-positive cells (*i.e.* neutrophils). The flow-through cells comprised 97–99.9% eosinophils. For fluorescence-activated cell sorter (FACS) analyses, the cells were incubated for 30 min on ice with normal human IgG (100 $\mu g \cdot mL^{-1}$) to block unoccupied Fcγ receptors, then stained for 30 min on ice with either buffer alone, a mixture of fluorescein isothiocyanate (FITC)-conjugated anti-CXCR1 and -CXCR2 (1:1) or phycoerythrin-conjugated anti-CCR3 antibodies (each at 1 $\mu g \cdot mL^{-1}$; Pharmingen Canada, Mississauga, ON, Canada) and analysed on a FACSort (Becton-Dickinson Canada, Oakville, ON, Canada).

Eosinophil microchemotaxis assays. The authors' basic protocol, utilising 5-μm polyvinylpyrrolidone-free filters, has been reported previously [18]. Test samples, diluted in Hank's balanced salt solution/1% foetal calf serum and assayed in duplicate, included human CXCL8/IL-8 and CCL11/eotaxin (R&D Systems; each 0–100 $ng \cdot mL^{-1}$) or serial dilutions (1:10–1:160) of sputum samples that had been dialysed extensively against PBS. To determine the contributions of individual mediators, neutralising or irrelevant specificity isotype (mouse IgG_1) control antibodies (5 $\mu g \cdot mL^{-1}$; R&D Systems) were added to the diluted sputum samples at the start of the assays. In preliminary tests, the authors confirmed that this level of specific antibodies was sufficient to neutralise the cytokine content of the samples, and also that the antibodies did not cross-react with one another (data not shown). Purified eosinophils (10^6 mL^{-1}) were run in the assays for 1 h at 37°C, then the membranes were stained and examined by light microscopy. For each sample, the numbers of eosinophils observed within at least nine 40× objective microscope fields were counted and the results expressed as the number of eosinophils per 40× field.

Analyses

Comparisons of the response for each mediator were performed using nonparametric Wilcoxon signed-rank tests [5]. Correlational analyses included *post hoc* Fisher's r-to-z transformations to determine the probabilities of statistical significance. Comparisons in the chemotaxis assays utilised analysis of variance assays with *post hoc* Fisher-protected least significant difference testing. All group data are reported as mean±SEM.

Results

CXCL8/IL-8, but not CCR3 ligand, expression is upregulated following regular use of salbutamol

The authors have previously reported increased eosinophilia in sputum after regular salbutamol use (~28% and ~39% eosinophils in the placebo and salbutamol samples, respectively, p=0.045) [5]. The present study shows that while the levels of CCL5/RANTES, CCL7/MCP-3 and CCL11/eotaxin in these sputa were high (fig. 1), they were not significantly affected by the salbutamol treatments (n=14). The levels of each in the 1-h placebo or salbutamol samples were not significantly different from those in the background samples. In addition, there were no correlations between the proportions of eosinophils and the levels of CCL5/RANTES (r=-0.241, p=0.09), CCL7/MCP-3 (r=-0.164, p=0.26) or CCL11/eotaxin (r=0.065, p=0.65). This suggested that alternate mediators may be more integral to this heightened eosinophil response,

Table 2. – Eosinophil counts and chemokine values for the test and control subjects

Subject	Eosinophils % of total wbc	Chemokine level·pg·mL^{-1}			
		CXCL8/IL-8	CCL11/eotaxin	CCL7/MCP-3	CCL5/RANTES
Test					
1	32.5 Sputum	1220	6142	1296	2202
2	69 Sputum	1760	1205	157	1784
3	11 Sputum	5583	1290	246	<10
4	41 Sputum	172	1274	106	<10
5	90 Sputum	6177	12255	1170	570
Control					
a/NA	0.2 Sputum	23	<10	12	<10
TCL[#]	46 PB	-	++++	+	

Hypertonic saline-induced sputum samples were obtained from five allergen-challenged subjects regularly using salbutamol for 10 days, then the levels of sputum CXLC8 and CCL5/regulated on activation, T-cell expressed and secreted (RANTES), CCL7/monocyte chemotactic protein (MCP)-3, and CCL11/eotaxin were determined by enzyme-linked immunosorbent assay (ELISA) and the proportions of sputum eosinophils were determined by differential counting of Giemsa-stained cytocentrifuge preparations. Control samples included hypertonic saline-induced sputum from one atopic, nonasthmatic (a/NA) subject that was not challenged with allergen, and extracts of a lesional skin biopsy from a hypereosinophilic patient with an epitheliotropic T-cell lymphoma (TCL). wbc: white blood cells; IL: interleukin. [#]: the eosinophil data of the TCL patient refer to their peripheral blood (PB) differential counts, while the cytokine data relates to the mediators extracted from lesional biopsies and tested using *in vitro* chemotaxis assays with specific antibodies. Since the chemotaxis assay results are not directly comparable with the ELISA results, they are indicated as relative levels (-: negative; +: low-level positive; ++++: very high-level positive).

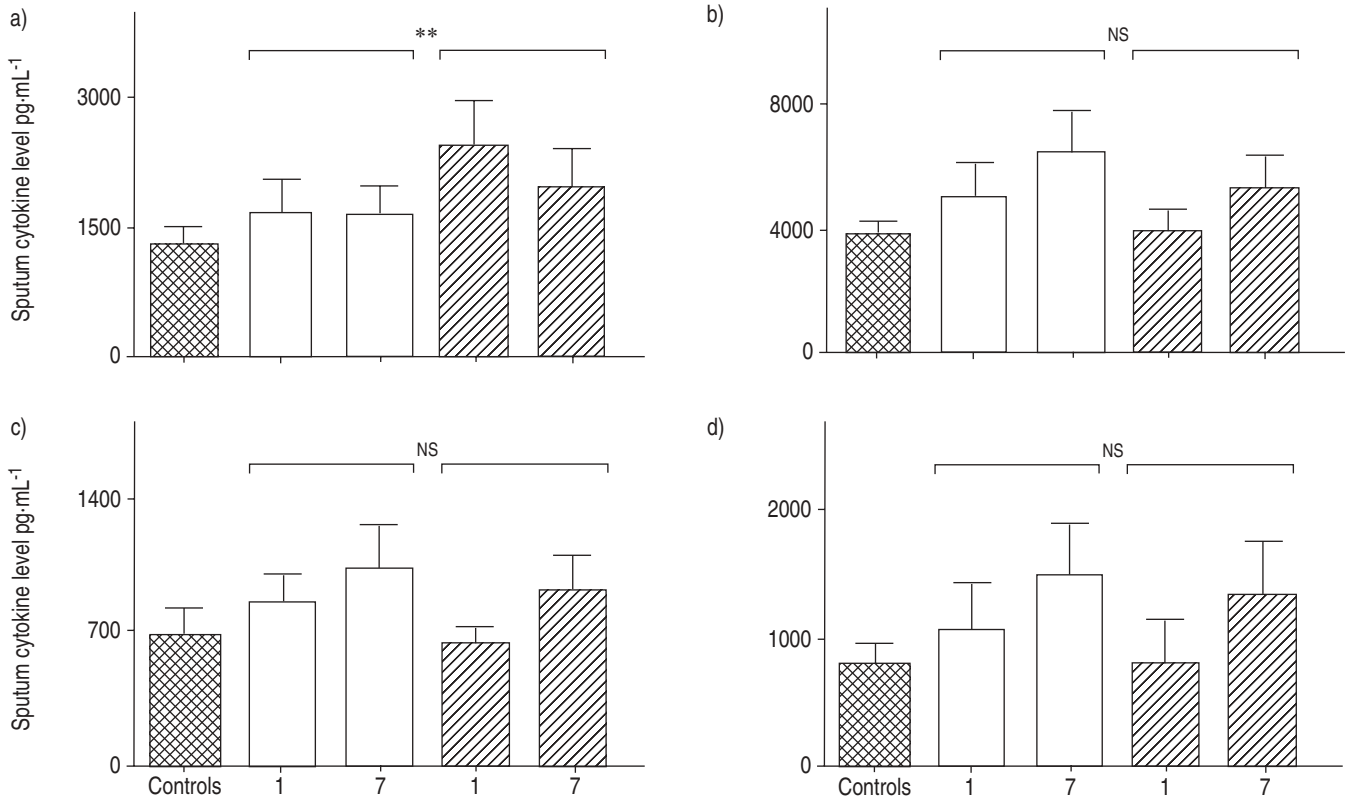

Fig. 1. – Effect of regular use of salbutamol on the levels of a) CXCL8/interleukin (IL)-8, and the eosinophil chemokines b) CCL11/eotaxin, c) CCL5/monocyte chemotactic protein-3 and d) CCL7/regulated on activation, T-cell expressed and secreted (RANTES) in the sputa of mild, stable, asthmatic subjects. Fourteen subjects with mild stable asthma were used in a double-blind, cross-over study of the effects on airway eosinophil chemokine responses of regular 10-day treatments with salbutamol (▨) or placebo (□). During the preliminary screening of the subjects, sputum samples were obtained as background controls (■). At the end of each treatment period, each subject was challenged with the same doses of allergen; then 1 and 7 h later, sputum samples were obtained and analysed by enzyme-linked immunosorbent assay for their mediator content. Regular salbutamol treatments significantly augmented the airway CXCL8/IL-8, but not CCL5/RANTES, CCL7/RANTES or CCL11/eotaxin, responses to allergen challenge relative to the placebo treatments. NS: nonsignificant, for placebo *versus* salbutamol groups. **: $p \leqslant 0.01$.

so the analyses were extended to include CXCL8/IL-8. The levels of CXCL8/IL-8 in the 1- and 7-h salbutamol treatment sputum samples were elevated by 43% (p=0.045) and 23% (p>0.05), respectively, relative to the paired placebo treatment samples (overall increase 38%, p=0.05; fig. 1), and the levels of CXCL8/IL-8 correlated significantly with the proportions of eosinophils (r=0.372, p=0.045) in their paired sputum samples (fig. 2). The salbutamol treatments did not affect the sputum neutrophil counts (1- and 7-h placebo values, 10.3±1.7 and 10.3±1.4%, respectively; 1- and 7-h salbutamol values, 9.5±1.1% and 10.2±1.3%, respectively) and the sputum CXCL8/IL-8 levels did not correlate with the proportions of sputum neutrophils at the times assessed herein (r=0.92, p>0.05). Several other cytokines important to eosinophils or allergic disease were also assessed, including GM-CSF, IL-4, -5, -6 and -13, and TNF [6, 12], but none of these were significantly affected by the salbutamol treatments (table 3). Taken together, these data indicate that despite the substantial levels of each of the nine chemokines/cytokines assessed, CXCL8/IL-8 was the only one that correlated significantly across all 14 subjects with the salbutamol-associated eosinophilia.

In order to determine whether cells recoverable within sputum could have contributed feasibly to the observed CXCL8/IL-8 response, cytocentrifuge slides of the sputum cells were immunostained for CXCL8/IL-8. No CXCL8/IL-8 staining was observed within the sputum lymphocytes, monocytes or macrophages, and there was essentially imperceptible staining within the neutrophils, but the eosinophils stained strongly for this chemokine (data not shown). No staining was observed in any cells probed with the isotype control antibody. As an alternative approach to confirming whether eosinophils of the subjects could express CXCL8/IL-8, the peripheral blood

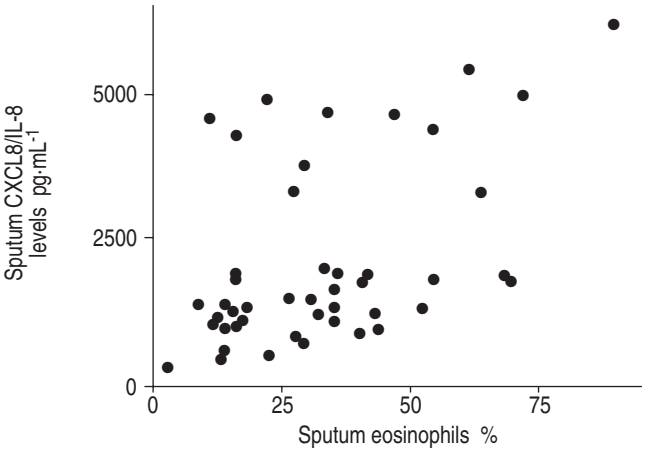

Fig. 2. – Scatterplot depicting the correlation between the proportions of sputum eosinophils and the levels of CXCL8/interleukin (IL)-8 in the matched sputum samples. The subjects and samples assessed were as noted in figure 1, as were the sputum levels of CXCL8/IL-8. The proportions of sputum eosinophils were determined previously [5] by direct counting of Wright's solution-stained cytocentrifuge preparations. There was a significant correlation between these two parameters (r=0.372; p=0.045).

leukocytes were retrospectively fractionated from five of them, as well as an a/NA control subject, and their release of CXCL8/IL-8 during overnight culture without additional stimuli was assessed. The cells employed comprised 97–99.9% pure eosinophils, as determined by FACS laser side- and forward-scatter (fig. 3a) and by morphological criteria on stained slides. The eosinophils of each subject released more CXCL8/IL-8 than their other blood leukocytes. Taken together, the mononuclear cells secreted 1,090±175 pg·mL^{-1} CXCL8/IL-8, the neutrophil fractions (~9% contaminating eosinophils) released 1,814±252 pg·mL^{-1} CXCL8/IL-8 and the eosinophils (⩾98% purity) 9,960±498 pg·mL^{-1} of this chemokine, as determined by ELISA. While this experiment provides no insight into the contributions of intact lung structural cells (*e.g.* epithelium) to the CXCL8/IL-8 response, it does suggest that the infiltrating eosinophils could contribute to a sputum CXCL8/IL-8 pool.

The peripheral blood eosinophils of allergic asthma subjects express CXCL8/IL-8 and CCL11/eotaxin receptors and respond chemotactically to both cytokines

The expression of CXCL8/IL-8 receptors (*i.e.* CXCR1 and CXCR2) on the freshly isolated peripheral blood eosinophils were also retrospectively assessed from these same five subjects, as well as three control subjects, simultaneously probing the cells for CCR3 (*i.e.* the receptor for CCL5/RANTES, CCL7/MCP-3, and CCL11/eotaxin) expression. FITC anti-mouse major histocompatibility complex II was used as the negative control antibody. The eosinophils of each asthmatic subject, as well as the a/NA and TCL control patients, expressed CXCR1/CXCR2 and CCR3, with the proportions of positive cells being variable (fig. 3 and table 4). The eosinophils of the na/NA donor were largely CXCR1/CXCR2-negative but expressed the CCR3 (table 4), while their neutrophils strongly expressed both CXCL8/IL-8 receptors but not CCR3 (data not shown).

That these receptors were functional was confirmed using chemotaxis assays with CXCL8/IL-8 and CCL11/eotaxin (figs 3e–f). The eosinophils of each test subject responded in a dose-dependent manner to both chemokines, with CCL11/eotaxin appearing to be a stronger chemoattractant at the higher doses. There was minimal subject-to-subject variability in the responses of the cells to either chemokine, as attested to by the low variance in the data (fig. 3e). The eosinophils of the a/NA (fig. 3f) and TCL (data not shown) control subjects also responded strongly to both CXCL8/IL-8 and CCL11/eotaxin, while the cells of the na/NA control subject did not respond to CXCL8/IL-8 but were responsive to CCL11/eotaxin (fig. 3f).

CXCL8/IL-8 can contribute significantly to the overall eosinophil chemotactic activities expressed in airways of allergen-challenged, allergic, asthma patients

It was demonstrated that immunologically detectable CXCL8/IL-8 was present at high levels in the sputum samples and that each subject′s eosinophils expressed the CXCR1/CXCR2. However, the sputum of the subjects also contained high levels of the more "traditional" eosinophil chemoattractants, CCL5/RANTES, CCL7/MCP-3 and CCL11/eotaxin (fig. 1), so that a critical issue was the relative importance of CXCL8/IL-8 to the eosinophil responses. In order to specifically target the issue of the levels of eosinophil chemokines in the sputum samples, in the assays of the eosinophil chemotactic activities of archived salbutamol-treatment group sputum samples, purified CXCR1/CXCR2- and CCR3-positive eosinophils of a common donor, the a/NA control (fig. 4), were used, and the samples from four of the subjects tested (fig. 3; sputa was no longer available from subject 2). The authors had already demonstrated that the cells of each subject responded more or less equally well to CXCL8/IL-8 (fig. 3e). The positive control for this assay comprised aqueous extracts from a lesional skin biopsy of an epitheliotropic TCL patient, which contained no discernable CXCL8/IL-8 but high levels of CCL11/eotaxin (unpublished observations). For a negative control sample, hypertonic saline-induced sputum from the a/NA control subject was used, which contained negligible CXCL8/IL-8 or CCL11/eotaxin (table 2). The CXCL8/IL-8 or CCL11/eotaxin within all the samples was neutralised using specific antibodies and then their residual activities assessed, relative to samples treated with the isotype control antibodies.

The level of each cytokine in the sputum samples (table 2)

Table 3. – Concentrations of allergy-related cytokines in sputa obtained from mild, stable, asthmatic subjects regularly treated with salbutamol or placebo medications

Cytokine	Cytokine concentration pg·mL^{-1} sputum						p-value[¶]
	Placebo			Salbutamol			
	1 h	7 h	p-value[#]	1 h	7 h	p-value[#]	
GM-CSF	1060±256	1220±244	NS	673±242	941±286	NS	1 h=0.20 7 h=0.06
IL-4	229±59	605±246	0.01	294±74	655±277	0.02	1 h=0.22 7 h=0.18
IL-5	202±66	387±117	NS	127±63	225±61	NS	1 h=0.38 7 h=0.31
IL-6	232±47	250±59	NS	505±138	380±141	NS	1 h=0.08 7 h=0.65
IL-13	88±48	180±79	NS	54±36	101±51	NS	1 h=0.22 7 h=0.62
TNF-α	426±110	2382±860	0.01	1095±494	1457±528	0.05	1 h=0.24 7 h=0.86

Data are presented as mean±SEM. Sputum samples were obtained as in table 2 and were assayed for the indicated cytokines by enzyme-linked immunosorbent assay. GM-CSF: granulocyte-macrophage colony-stimulating factor; IL: interleukin; TNF: tumour necrosis factor; NS: nonsignificant. [#]: between the levels of each cytokine in the 1-h *versus* 7-h samples; [¶]: salbutamol *versus* placebo treatment samples at both 1 and 7 h were determined using a (nonparametric) Wilcoxon signed-rank test.

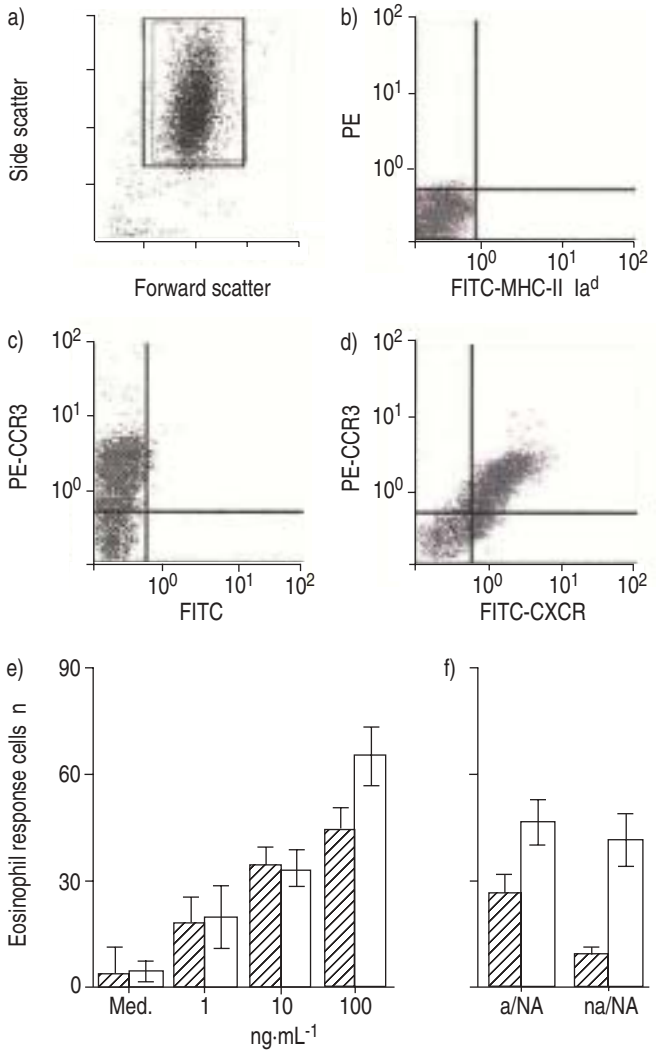

Fig. 3. – Fluorescence-activated cell sorter profiles of the cells (>99% eosinophils) from one test subject (number 4), including a) laser forward- and side-scatter characteristics, b) negative control staining with fluorescein isothiocyanate-conjugated goat antimouse major histocompatibility complex (MHC) II alone, c) phycoerythrin (PE)-conjugated anti-CCR3 alone or d) a combination of PE-conjugated anti-CCR3 and fluorescein isothiocyanate (FITC)-conjugated anti-CXCR-1/-2. CXCL8/interleukin-8 (▨) and CCL11/eotaxin (□) chemotaxis assay dose/response curves for the eosinophils (mean±SEM number of cells per 40× field) of e) the test subjects and f) the responses of the control subjects′ cells to 10 ng·mL^{-1} of either ligand. Med.: median; a/NA: atopic nonasthmatic; na/Na: non asthmatic.

correlated very well with the extent to which the antibodies downregulated their eosinophil chemotactic activities (r=0.921 for CXCL8/IL-8 and r=0.825 for CCL11/eotaxin). Thus, for subject 1, with much higher sputum levels of CCR3 ligands than CXCL8/IL-8, the anti-CXCL8/IL-8 antibodies had only moderate effects, while the anti-CCL11/eotaxin antibody was more, though still not fully, effective (fig. 4). The assay background in these experiments was 7±2 eosinophils per 40× field. For subject 3, with abundant sputum CXCL8/IL-8 and lower levels of CCL11/eotaxin, the reverse effects were observed. Subject 4 had negligible sputum CXCL8/IL-8 and lower levels of CCL11/eotaxin, while subject 5 had very high levels of both chemokines and, for both subjects, the chemotaxis data reflected these trends. Overall, CXCL8/IL-8 contributed 19±5% ($p<0.01$ *versus* control antibody treatment) of the eosinophil chemotactic activity of these samples,

Table 4. – The peripheral blood eosinophils of allergic asthma subjects express both CXCL8/interleukin (IL)-8 and CCL11/eotaxin receptors

Subject	Receptor-positive cells %		
	IL-8R	CCR3	IL-8R/CCR3 double-positive
Test			
1	97.7	69.0	68.6
2	98.6	13.5	12.2
3	88.4	86.3	80.4
4	74.7	72.5	68
5	24.6	28	21.8
Control			
a/NA	68.5	75.0	67.0
TCL	59.1	91.8	62.3
na/NA	3.1	75.1	2.2

Eosinophils were purified (>97%) from the peripheral blood of five allergic asthma test subjects, as well as from an atopic, nonasthmatic patient (a/NA), an eosinophilic T-cell lymphoma patient (TCL), and a nonatopic, nonasthmatic (na/NA) control subject, by negative selection magnetism-assisted cell-sorting with anti-CD16 paramagnetic beads. The cells were immunostained with fluorochrome-conjugated antibodies against CXCR1 and CXCR2 (IL-8R), anti-CCR3 (CCR3), anti-CXCR1/2 + anti-CCR3 (IL-8R/CCR3 double-positive), or antimouse major histocompatibility complex II (negative control, data not shown). The proportions of the population staining for each marker set are indicated.

while CCL11/eotaxin contributed 46±11% of this activity. The sputum of the a/NA control subject induced no significant responses and the antibody treatments did not affect this, while anti-CCL11/eotaxin, but not anti-CXCL8/IL-8, treatment of the lesional biopsy extracts from the TCL patient neutralised the eosinophil responses to the samples (fig. 4). These data suggest that unlike eosinophil responsiveness to CXCL8/IL-8, there is substantial subject-to-subject variability in the proportion of the sputum eosinophil chemotactic activities attributable to this chemokine. Nevertheless, the

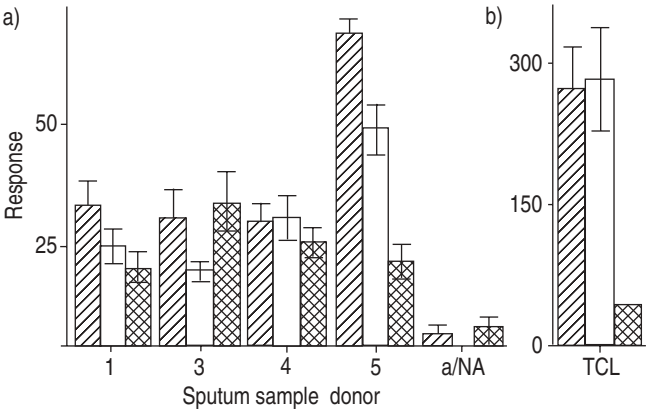

Fig. 4. – The eosinophil chemotactic activities in the sputum of allergic asthma subjects include CXCL8/interleukin (IL)-8 and CCL11/eotaxin. Eosinophils were purified as in figure 2 and used to assess the chemotactic activities present in the sputum (diluted 1:160) of a) several allergic asthma subjects and one atopic, nonasthmatic subject (a/NA), using a medium control (▨) and monospecific-neutralising antibodies (5 μg·mL^{-1}) against CXCL8/IL-8 (□) or CCL11/eotaxin (■). b) Extracts from a lesional biopsy of a T-cell lymphoma patient (TCL) were used as a control in this assay, as indicated in table 2. The residual eosinophil chemotactic activities of the samples were then assessed as in figure 2. Overall, the anti-CXCL8/IL-8 antibodies blocked ~20% of the eosinophil chemotactic activities of the sputum samples, while neutralisation of CCL11/eotaxin in the samples reduced their eosinophil chemotactic activities by ~45%.

impact of CXCL8/IL-8 on the sputum eosinophil chemotactic activities (~<50% of eotaxin) was significant.

As a final check on the relevance of these data to the CXCL8/IL-8 ELISA results, chemotaxis assays with sputum harvested at the time of these subjects' background assessments were run against those taken after the placebo and salbutamol treatments. By this time, however, the stocks of some of the archived sputum were extremely low, almost to the point of vanishing altogether, so that the subject's sputa was necessarily pooled (in equal proportions). Even so, the authors had sufficient sputa only to assess the contributions of CXCL8/IL-8 to the chemotactic activities of the samples. The data confirmed that after 10 days of placebo treatments, allergen challenge had induced an upregulation of eosinophil chemotactic activities in the airways (background sputum activity 25±2 *versus* placebo sputum 34±7 cells per 40× field; p=0.01) and that the salbutamol treatments had led to further augmentation of this response (43±4 cells per 40× field; p<0.001 *versus* the background samples and p=0.02 *versus* the placebo samples). Anti-CXCL8/IL-8 treatments reduced the chemotactic activities of the 7-h background, placebo and salbutamol samples by 12.0±10.6 (p>0.05), 17±7.1 (p<0.01), and 23.1±3.4% (p=0.012), respectively, relative to their paired isotype control antibody-treated samples.

Discussion

The authors have demonstrated that regular use of salbutamol can augment airway CXCL8/IL-8 responses to same-dose allergen challenges in mild, asthmatic subjects. While IL-5, GM-CSF, TNF and several CCR3 ligands were each strongly expressed in the samples, the salbutamol treatments had no discernable impact on these mediators. The CXCL8/IL-8 response was the only parameter that significantly correlated with the increased eosinophil responses to allergen challenge that occur under these conditions [4, 5]. It was also documented that the eosinophils of the subjects coexpress CXCL8/IL-8 receptors and CCR3, and can essentially respond equally well to their respective ligands. Finally, the authors confirmed that both CXCL8/IL-8 and CCL11/eotaxin can contribute significantly to sputum eosinophil chemotactic activities.

Historically, a role for CXCL8/IL-8 in allergic disease has not been very broadly contemplated, perhaps because this chemokine was considered to be largely neutrophil-specific and therefore little more than a marker of an underlying inflammatory response. However, allergen-induced CXCL8/IL-8 expression within the airways has been reported by multiple laboratories [7, 19], as has a significant correlation between nasal eosinophilia and CXCL8/IL-8 levels in allergic rhinitis subjects after, but not prior to, budesonide treatment [9]. Eosinophils have been reported by others as being CXCL8/IL-8-responsive [11, 12, 20], although this has been questioned as an artefact attributable to neutrophil contamination [21]. However, "untouched" eosinophils were employed, isolated by negative selection MACS to population purities of ⩾97%, which, by two independent means, confirmed that the CXCR1/CXCR2^{+} and chemotaxis-responder cells in the assays were unequivocally eosinophils. Thus, these data again confirm that the eosinophils of atopic subjects do express fully functional CXCL8/IL-8 receptors.

As noted, CXCL8/IL-8 is strongly expressed during allergic responses [7, 12, 19], but neutrophils are not widely reported as being integral to allergic disease. These cells do infiltrate lung tissues in allergen-challenged asthmatics [22]; within 4 h of segmental allergen challenge the airways of human subjects contain neutrophils [7], but it is not until ~18 h post-challenge that their levels are markedly elevated [19]. Significant numbers of neutrophils were detected in the sputa of the asthmatic subjects, but no increases (above background levels) associated with allergen challenge were observed. At first glance, the observed CXCL8/IL-8 upregulation without a concomitant increase in neutrophil levels could seem incongruent. While it is likely that the epithelium and other structural cells would express some CXCL8/IL-8 [23], it has been shown that eosinophils or their secretory products (*e.g.* major basic protein and IL-17) also strongly induce expression of CXCL8/IL-8 by endothelial cells [24] or fibroblasts [25, 26]. Thus, if airway-infiltrating eosinophil products contribute importantly to the total airway CXCL8/IL-8 response, the bulk of a CXCL8/IL-8-dependent neutrophil response would probably trail the eosinophil response by several hours. Neutrophils are the primary cells to infiltrate passive anaphylaxis lesions in the skin of mice; this response takes 4–6 h to develop [27, 28]. It is likely that mast cells release CXCL8/IL-8 [29] during these responses or that mast cell products, such as TNF [30] or tryptase [31], induce local secretion of chemokines containing the neutrophil-specific motifs glutamic acid-lysine-arginine, cysteine-alternate amino acid-cysteine (ELR-CXC; *e.g.* CXCL8/IL-8) by endothelial or epithelial cells. Indeed, allergen-induced mast cell tryptase levels in the sera of the subjects were significantly elevated following the salbutamol treatments [5]. During active allergic responses in mice and humans, a circulating eosinophilia often exists, such that the effects of CXCL8/IL-8 could readily be masked by high-level expression of alternate chemoattractants, such as the CCR3 ligands. The fact that CXCL8/IL-8 responses surface as significant to allergic rhinitis eosinophilia only after budesonide treatment [9] tends to substantiate this hypothesis.

CXCL8/IL-8 contributed less than one-half of the total eosinophil chemotactic activities represented by eotaxin in the sputa. However, it must be kept in mind that the ELR-CXC chemokines are most often redundantly expressed [32], and that each is chemotactically active on cells that express the CXCR1 or CXCR2 (*e.g.* eosinophils in this report). In fact, evidence from the present authors' studies with a combined CXCR1/CXCR2 antagonist, recently engineered by this group [33, 34], shows that CXCR1/CXCR2 ligands contribute ~30% of the total eosinophil chemotactic activities present in the sputum samples used in this study (unpublished data). Of course this still leaves chemoattractants such as the CCR3 ligands as the likely dominant influence in the response, but when comparing situations wherein the levels of these CCR3 ligands are essentially equivalent, which occurred with the 7-h placebo and salbutamol sputum samples, the impact of additional, alternate, chemokines such as CXCL8/IL-8 would become much more relevant. It seems logical to suggest that the correlation between the CXCL8/IL-8 levels and eosinophil numbers would be related to some extent to the fact that the eosinophils comprised a source of this chemokine, but whether the CXCL8/IL-8 that was detected free in the sputum was derived from these cells or from other sources (*e.g.* the epithelium) is open to speculation. Since eosinophil levels were increased in the sputa following the salbutamol treatments, it is reasonable to suggest that the total pool of CXCL8/IL-8 potentially available (*i.e.* eosinophil-associated and free) would be substantially greater than that detected in the 7-h sputum samples tested.

Multiple other studies have addressed the effects of exposure to β_2-agonists on cytokine production by isolated cells or cell lines *in vitro*. For example, addition of salbutamol to cultures of stimulated monocytes [35] or neutrophils [36] reduces their secretion of CXCL8/IL-8, although it increases or does not affect expression of this chemokine by transformed epithelial cells [23] or airway smooth muscle cells [37], respectively. Some β_2-agonists can downregulate the expression by human mast cells [38] or eosinophils [39] of TNF or

superoxide, respectively, and a single dose of salmeterol can inhibit allergen-induced changes in nasal mucosa vascular permeability, but does not reduce mast cell activation or cellular influx *in vivo* [40]. Conversely, prolonged *in vitro* exposure of eosinophils to β_2-agonists increases cellular superoxide release [39]. Thus, the impact of salbutamol on cellular responses can vary substantially with the system employed. The authors assessed its impact *in vivo* under clinical experimental conditions and found that the sputum eosinophils, and most likely some lung structural cell populations [23, 37, 41], strongly expressed CXCL8/IL-8. Unlike structural cells, the eosinophils would have infiltrated the lungs in response to the allergen challenge [5] 16–20 h following the last dose of salbutamol, so they may not have been directly exposed to substantial concentrations of this agonist.

As this study design did not examine CXCL8/interleukin-8 expression by resident nonairway cells, the authors cannot draw any definitive conclusions about their potential contributions to the CXCL8/interleukin-8 pool present in the sputa. However, the demonstration that regular salbutamol treatments augment pulmonary eosinophil and metachromatic cell influx, mast cell tryptase release [5] and CXCL8/interleukin-8 production suggests that acute *in vitro* challenge experiments may not adequately model the complexities of regular use of this agent *in vivo*.

References

1. O′Connor BJ, Aikman SL, Barnes PJ. Tolerance to the nonbronchodilator effects of inhaled beta 2-agonists in asthma. *N Engl J Med* 1992; 327: 1204–1208.
2. Cheung D, Timmers MC, Zwinderman AH, Bel EH, Dijkman JH, Sterk PJ. Long-term effects of a long-acting beta 2-adrenoceptor agonist, salmeterol, on airway hyperresponsiveness in patients with mild asthma. *N Engl J Med* 1992; 327: 1198–1203.
3. Cockcroft DW, McParland CP, Britto SA, Swystun VA, Rutherford BC. Regular inhaled salbutamol and airway responsiveness to allergen. *Lancet* 1993; 342: 833–837.
4. Gauvreau GM, Watson RM, O′Byrne PM. Kinetics of allergen-induced airway eosinophilic cytokine production and airway inflammation. *Am J Respir Crit Care Med* 1999; 160: 640–647.
5. Swystun VA, Gordon JR, Davis EB, Zhang X, Cockcroft DW. Mast cell tryptase release and asthmatic responses to allergen increase with regular use of salbutamol. *J Allergy Clin Immunol* 2000; 106: 57–64.
6. Kay AB, Barata L, Meng Q, Durham SR, Ying S. Eosinophils and eosinophil-associated cytokines in allergic inflammation. *Int Arch Allergy Immunol* 1997; 113: 196–199.
7. Nocker RE, Out TA, Weller FR, Mul EP, Jansen HM, van der Zee JS. Influx of neutrophils into the airway lumen at 4 h after segmental allergen challenge in asthma. *Int Arch Allergy Immunol* 1999; 119: 45–53.
8. Turner MO, Hussack P, Sears MR, Dolovich J, Hargreave FE. Exacerbations of asthma without sputum eosinophilia. *Thorax* 1995; 50: 1057–1061.
9. Benson M, Strannegard IL, Wennergren G, Strannegard O. Interleukin-5 and interleukin-8 in relation to eosinophils and neutrophils in nasal fluids from school children with seasonal allergic rhinitis. *Pediatr Allergy Immunol* 1999; 10: 178–185.
10. KleinJan A, Dijkstra MD, Boks SS, Severijnen LA, Mulder PG, Fokkens WJ. Increase in IL-8, IL-10, IL-13, and RANTES mRNA levels (*in situ* hybridization) in the nasal mucosa after nasal allergen provocation. *J Allergy Clin Immunol* 1999; 103: 441–450.
11. Sehmi R, Cromwell O, Wardlaw AJ, Moqbel R, Kay AB. Interleukin-8 is a chemo-attractant for eosinophils purified from subjects with a blood eosinophilia but not from normal healthy subjects. *Clin Exp Allergy* 1993; 23: 1027–1036.
12. Lampinen M, Rak S, Venge P. The role of interleukin-5, interleukin-8 and RANTES in the chemotactic attraction of eosinophils to the allergic lung. *Clin Exp Allergy* 1999; 29: 314–322.
13. Hauser U, Wagenmann M, Rudack C, Bachert C. Specific immunotherapy suppresses IL-8 levels in nasal secretions: A possible explanation for the inhibition of eosinophil migration. *Allergology* 1997; 20: 184–191.
14. Keatings VM, Evans DJ, O′Connor BJ, Barnes PJ. Cellular profiles in asthmatic airways: a comparison of induced sputum, bronchial washings, and bronchoalveolar lavage fluid. *Thorax* 1997; 52: 372–374.
15. Taha RA, Laberge S, Hamid Q, Olivenstein R. Increased expression of the chemoattractant cytokines eotaxin, monocyte chemotactic protein-4, and interleukin-16 in induced sputum in asthmatic patients. *Chest* 2001; 120: 595–601.
16. Gordon JR, Galli SJ. Promotion of mouse fibroblast collagen gene expression by mast cells stimulated *via* the Fc epsilon RI. Role for mast cell-derived transforming growth factor beta and tumor necrosis factor alpha. *J Exp Med* 1994; 180: 2027–2037.
17. Gordon JR. TGFbeta1 and TNFalpha secreted by mast cells stimulated *via* the FcepsilonRI activate fibroblasts for high-level production of monocyte chemoattractant protein-1 (MCP-1). *Cell Immunol* 2000; 201: 42–49.
18. Cairns CM, Gordon JR, Li F, Baca-Estrada ME, Moyana T, Xiang J. Lymphotactin expression by engineered myeloma cells drives tumor regression: mediation by CD4+ and CD8+ T cells and neutrophils expressing XCR1 receptor. *J Immunol* 2001; 167: 57–65.
19. Virchow JC Jr, Walker C, Hafner D, *et al.* T cells and cytokines in bronchoalveolar lavage fluid after segmental allergen provocation in atopic asthma. *Am J Respir Crit Care Med* 1995; 151: 960–968.
20. Ulfman LH, Joosten DP, van der Linden JA, Lammers JW, Zwaginga JJ, Koenderman L. IL-8 induces a transient arrest of rolling eosinophils on human endothelial cells. *J Immunol* 2001; 166: 588–595.
21. Petering H, Gotze O, Kimmig D, Smolarski R, Kapp A, Elsner J. The biologic role of interleukin-8: functional analysis and expression of CXCR1 and CXCR2 on human eosinophils. *Blood* 1999; 93: 694–702.
22. Ordonez CL, Shaughnessy TE, Matthay MA, Fahy JV. Increased neutrophil numbers and IL-8 levels in airway secretions in acute severe asthma: Clinical and biologic significance. *Am J Respir Crit Care Med* 2000; 161: 1185–1190.
23. Linden A. Increased interleukin-8 release by beta-adrenoceptor activation in human transformed bronchial epithelial cells. *Br J Pharmacol* 1996; 119: 402–406.
24. Tonnel AB, Gosset P, Molet S, Tillie-Leblond I, Jeannin P, Joseph M. Interactions between endothelial cells and effector cells in allergic inflammation. *Ann N Y Acad Sci* 1996; 796: 9–20.
25. Furuta GT, Ackerman SJ, Varga J, Spiess AM, Wang MY, Wershil BK. Eosinophil granule-derived major basic protein induces IL-8 expression in human intestinal myofibroblasts. *Clin Exp Immunol* 2000; 122: 35–40.
26. Molet S, Hamid Q, Davoine F, *et al.* IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol* 2001; 108: 430–438.
27. Wershil BK, Wang ZS, Gordon JR, Galli SJ. Recruitment of neutrophils during IgE-dependent cutaneous late phase reactions in the mouse is mast cell-dependent. Partial inhibition of the reaction with antiserum against tumor necrosis factor-alpha. *J Clin Invest* 1991; 87: 446–453.
28. Gordon JR. Monocyte chemoattractant peptide-1 expression during cutaneous allergic reactions in mice is mast cell dependent and largely mediates the monocyte recruitment response. *J Allergy Clin Immunol* 2000; 106: 110–116.

29. Moller A, Lippert U, Lessmann D, *et al.* Human mast cells produce IL-8. *J Immunol* 1993; 151: 3261–3266.
30. Gordon JR, Galli SJ. Mast cells as a source of both preformed and immunologically inducible TNF-alpha/cachectin. *Nature* 1990; 346: 274–276.
31. Compton SJ, Cairns JA, Holgate ST, Walls AF. The role of mast cell tryptase in regulating endothelial cell proliferation, cytokine release, and adhesion molecule expression: tryptase induces expression of mRNA for IL-1 beta and IL-8 and stimulates the selective release of IL-8 from human umbilical vein endothelial cells. *J Immunol* 1998; 161: 1939–1946.
32. Van Damme J, Wuyts A, Froyen G, *et al.* Granulocyte chemotactic protein-2 and related CXC chemokines: from gene regulation to receptor usage. *J Leukoc Biol* 1997; 62: 563–569.
33. Li F, Zhang X, Mizzi C, Gordon JR. $CXCL8_{(3–73)}$K11R/G31P antagonizes the neutrophil chemoattractants present in pasteurellosis and mastitis lesions and abrogates neutrophil influx into intradermal endotoxin challenge sites *in vivo*. *Vet Immunol Immunopathol* 2002; 90: 65–77.
34. Li F, Zhang X, Gordon JR. $CXCL8_{(3-73)}$K11R/G31P antagonizes ligand binding to the neutrophil CXCR1 and CXCR2 receptors and cellular responses to CXCL8/IL-8. *Biochem Biophys Res Commun* 2002; 293: 939–944.
35. Farmer P, Pugin J. β-adrenergic agonists exert their "anti-inflammatory" effects in monocytic cells through the IkappaB/NF-kappaB pathway. *Am J Physiol Lung Cell Mol Physiol* 2000; 279: L675–L682.
36. Au BT, Teixeira MM, Collins PD, Williams TJ. Effect of PDE4 inhibitors on zymosan-induced IL-8 release from human neutrophils: synergism with prostanoids and salbutamol. *Br J Pharmacol* 1998; 123: 1260–1266.
37. Hallsworth MP, Twort CH, Lee TH, Hirst SJ. Beta(2)-adrenoceptor agonists inhibit release of eosinophil-activating cytokines from human airway smooth muscle cells. *Br J Pharmacol* 2001; 132: 729–741.
38. Bissonnette EY, Befus AD. Anti-inflammatory effect of beta 2-agonists: inhibition of TNF-alpha release from human mast cells. *J Allergy Clin Immunol* 1997; 100: 825–831.
39. Nielson CP, Hadjokas NE. Beta-adrenoceptor agonists block corticosteroid inhibition in eosinophils. *Am J Respir Crit Care Med* 1998; 157: 184–191.
40. Proud D, Reynolds CJ, Lichtenstein LM, Kagey-Sobotka A, Togias A. Intranasal salmeterol inhibits allergen-induced vascular permeability but not mast cell activation or cellular infiltration. *Clin Exp Allergy* 1998; 28: 868–875.
41. Denburg JA, Gauldie J, Dolovich J, Ohtoshi T, Cox G, Jordana M. Structural cell-derived cytokines in allergic inflammation. *Int Arch Allergy Appl Immunol* 1991; 94: 127–132.