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Clinical Immunology

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Tumor necrosis factor alpha (TNF- α) autoregulates its expression and induces adhesion molecule expression in asthma[☆]

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Received 6 April 2010; accepted with revision 8 March 2011

KEYWORDS

Asthma;
Airway inflammation;
Tumor necrosis factor;
Adhesion molecule;
Allergen

Abstract Subjects with mild asthma underwent repeated low-dose allergen exposure and bronchial biopsies were examined for the expression of TNF- α and adhesion molecules. Bronchial biopsies from moderately severe asthmatics were then tested in an explant culture system to assess the effect of *Der p* and CDP-870, a TNF- α blocking pegylated-antibody Fab, on expression of TNF- α and adhesion molecules. Low-dose allergen challenge significantly upregulated sub-mucosal mast cells, TNF- α ⁺ cells, and VCAM. When bronchial explants were exposed to *Der p* and CDP 870 for 24 h, CDP 870 caused a significant reduction in TNF- α release both at baseline and following stimulation with *Der p* allergen. The bronchial biopsies showed significant upregulation of TNF- α positive cells and ICAM-1 following exposure to *Der p* ($p=0.03$) and this was reduced in the presence of CDP-870. So, allergen exposure up-regulates TNF- α expression in asthma and down-stream targets, including adhesion molecules that contribute to airway inflammation.
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[☆] Research funding: this study was supported by a grant from UCB Celltech which also kindly provided the CDP-870 (TNF-alpha blocking monoclonal antibody).

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1. Introduction

Airway inflammation in asthma is orchestrated by various cytokines. One of these is TNF- α , a multifunctional cytokine involved in a range of inflammatory diseases including rheumatoid arthritis, inflammatory bowel disease (IBD), and psoriasis [1]. Although these disorders are all

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doi:[10.1016/j.clim.2011.03.005](https://doi.org/10.1016/j.clim.2011.03.005)

Please cite this article as: S.K. Babu, et al., Tumor necrosis factor alpha (TNF- α) autoregulates its expression and induces adhesion molecule expression in asthma, Clin. Immunol. (2011), doi:[10.1016/j.clim.2011.03.005](https://doi.org/10.1016/j.clim.2011.03.005)

characterized by a Th1-type immune response, asthma is usually considered as an eosinophilic disorder involving Th2 type cytokines. Nonetheless, several studies have shown a role for TNF- α in asthma [2]. Acute allergen exposure in asthmatic subjects is associated with increased TNF- α expression in bronchoalveolar lavage fluid (BALF) and bronchial biopsies [3]. Inhalation of TNF- α in both normal or asthmatic humans [4,5] leads to the development of bronchial hyperresponsiveness (BHR) accompanied by airway neutrophilia.

In our recent studies, we found that TNF- α mRNA levels were significantly higher in biopsies from the severe asthmatic compared to mild asthmatic subjects and there were significantly greater numbers of TNF- α positive immunoreactive mast cells in severe asthma compared with mild asthma. In a small proof of concept, open labeled study, symptomatic severe asthmatics treated for 12 weeks with the recombinant soluble TNF- α binding protein etanercept resulted in improved asthma control, reduced BHR, and lowered the need for rescue inhaled β_2 -agonist [6]. Similar findings have been reported by Berry et al. [7] in a 10 week randomized placebo controlled cross over trial involving 10 patients with refractory asthma treated with etanercept [8].

However, these positive studies have not been validated by other studies. Erin et al. investigated the role of infliximab—a TNF- α blocking monoclonal antibody in patients with moderate asthma. Infliximab did not change the morning PEF, their primary endpoint, but decreased the number of asthma exacerbations [9]. Furthermore, in a randomized, double-blind, placebo-controlled, parallel group trial of etanercept, we have reported only a small, but significant, improvement in asthma control and systemic inflammation, with no significant improvement in AQLQ scores, lung function, or BHR [10]. More recently, 309 patients with severe persistent asthma treated with golimumab, a human monoclonal antibody against TNF- α , did not show significant improvements in their FEV₁ and asthma exacerbations. Furthermore, the treatment group had significant risk of serious infections and an increased incidence of malignancies [11].

In view of the mixed outcomes from the clinical trials of TNF- α blockade in asthma, we have investigated a likely mechanistic role for TNF- α in asthma. We employed two alternative approaches: examining the expression of TNF- α following low dose allergen exposure in vivo and then investigating effects of blocking TNF- α in bronchial biopsy explants obtained from moderately severe asthmatics.

2. Materials and methods

2.1. Subjects

Adults aged between 18–55 years with a history of physician diagnosed mild-moderate allergic asthma for at least 1 year were enrolled into this study. The study was approved by the Local Research Ethics Committee and informed written consent was obtained from all the participating subjects. Baseline lung function measurements including FEV₁, FVC, and PEF were performed. Two groups of subjects were studied; their characteristics are given in the next sections.

2.2. Repeated low dose allergen exposure

Nine subjects were recruited into this study. The inclusion criteria were physician diagnosed asthma (mild asthmatics) of more than one year duration, asthma symptoms less than once per day on average, FEV₁ of >80% predicted as needed (prn), bronchodilator as the only treatment required, and a PC₂₀ of <8mg/mL on methacholine bronchial provocation test. Subjects underwent standard allergen challenge to determine the PD₂₀ (provocation dose to cause a 20% fall in FEV₁) and then underwent repeated low dose allergen exposure with 10% of the PD₂₀ 3 times a week for 4 weeks. Bronchoscopy was performed before and after the low dose allergen exposure (within 5 days after the last low dose allergen exposure) and bronchial biopsies obtained for immunohistochemistry. The study plan is shown in Fig. 1. Further details of the methodology can be found in the online supplement section.

2.3. Bronchial explant culture

Twelve moderately severe allergic asthmatic subjects (nine males and three females) were recruited into this study (clinical trial number NCT01161303). All subjects had a history compatible with asthma and bronchodilator reversibility of at least 12%. The subjects were atopic as determined by positive skin prick testing to a panel of common aero-allergens (ALK; Abello, Horsholm, Denmark) and were sensitive to the house dust mite allergen, *Dermatophagoides pteronissinus* (*Der p*). Subjects were receiving regular inhaled corticosteroids (mean $\pm$ SEM daily dose of 658 $\pm$ 97 μ g/d of beclomethasone dipropionate or equivalent) and short acting β_2 -agonists as required for symptom relief. Subjects withheld their inhaled corticosteroids for a minimum of one week prior to bronchoscopy and

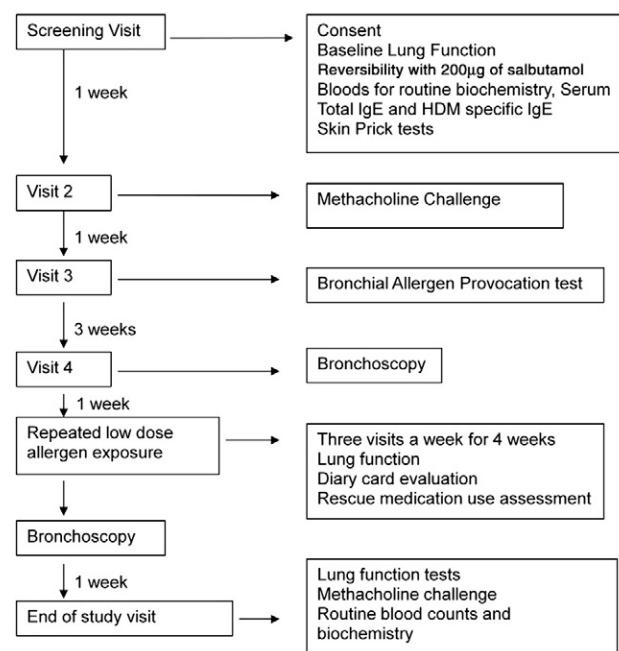


Figure 1 Study plan.

Table 1 Baseline characteristics of mild asthmatics recruited for repeated low dose allergen challenge.

Subject no.	Age	Sex	FEV ₁ , L/min	FEV ₁ , % predicted	FEV ₁ /FVC	SPT mean diameter	PC ₂₀ mg/mL
1	21	M	4.79	102.4	82.9	13	2.49
2	30	M	3.13	96.3	82.8	8.5	1.81
3	29	M	4.00	91.7	76.3	10.5	0.22
4	37	M	3.95	99.7	79.3	13	0.9
5	47	M	3.93	104.1	81.0	12.5	3.22
6	20	M	6.17	103.7	100.0	8.5	7.32
7	32	M	3.68	88.7	75.1	12	1.97
8	22	M	4.34	104.3	86.1	15	5.38
9	23	F	3.32	106.8	109.2	6.5	2.74

Table 2 Changes in lung function before and after low dose allergen exposure.

Lung function	Pre-allergen	Post-allergen	P value
FEV ₁ (L/min) (mean $\pm$ SEM)	4.17 $\pm$ 0.30	3.87 $\pm$ 0.13	NS
FVC (L/min) (mean $\pm$ SEM)	4.86 $\pm$ 0.32	4.59 $\pm$ 0.18	NS
PEF (L/s) (mean $\pm$ SEM)	577 $\pm$ 18	527 $\pm$ 20	NS
PC ₂₀ (mg/mL) (geo mean)	2.49	2.63	NS

NS indicates not significant; SEM, standard error of mean; geo mean, geometric mean.

bronchial biopsy to optimize inflammatory responses in the bronchial tissue culture. Subjects were provided with a peak flow diary to monitor PEF for any deterioration of their asthma symptoms and rescue medication use. A physician was available by telephone at all times to deal with any worsening of their asthma symptoms.

Bronchoscopy was performed according to the NIH guidelines and 6 bronchial biopsies were obtained from each subject [12]. The biopsies were cultured for 24 h in a serum free medium alone (500 μ L, AIMV; Life Technologies, Paisley, UK) or serum free medium plus *Der p* allergen (5000 SQ-U/mL; ALK, Horsholm, Denmark) or serum free medium plus *Der p* plus anti TNF- α (CDP-870, 10 μ g/mL [certolizumab pegol]). CDP-870 was kindly supplied by UCB Celltech Laboratories, Berkshire, UK, and has been evaluated in patients with rheumatoid arthritis and IBD [13,14]. It comprises a

pegylated humanized antibody Fab' fragment which potently neutralizes TNF- α . At the end of the incubation, the biopsies were then processed for immunohistochemistry and the supernatants were analyzed for IL-8, IL-5, IL-1 β , and TNF- α by ELISA. Control experiments showed that detection of TNF- α by ELISA in the presence of CDP-870 was reduced by 33%; therefore, a correction factor was used to calculate the levels of TNF- α in the supernatants containing CDP-870. Although the explant cultures were performed using samples from all 12 subjects, there was only 450 μ L of supernatant available for cytokine analysis. Since it was necessary to repeat some assays (when the readings lay off the standard curve), this affected the number of analyses that could be performed for each cytokine. Hence, the numbers of samples are different for the analysis of TNF- α , IL-8, and IL-1 β . Further details of the methodology are available from the online supplement.

2.4. Statistical analyses

Data were not normally distributed and were analyzed using non-parametric tests. The data were analyzed using SPSS 11.0 for Windows. The Wilcoxon's signed rank test was used for paired data comparisons. Results are expressed as median (interquartile ranges). Values of $p < 0.05$ were accepted as statistically significant.

3. Results

3.1. Repeated low dose allergen exposure increases TNF- α and adhesion molecule expression

Nine subjects were recruited for the low dose allergen challenge study and comprised eight males and one female. The mean age was 29.6 (± 2.8) years and none of the subjects were on inhaled corticosteroids for their asthma control. The baseline characteristics of the subjects are shown in Table 1. There was no significant difference in the FEV₁, FVC, PEF, or PC₂₀ before and after low dose allergen exposure (Table 2). Paired bronchial biopsies for immunohistochemical analysis were available from seven subjects, as two subjects were unable to undergo bronchoscopy due to their airway sensitivity following low dose allergen exposure. Immunostaining of the paired bronchial biopsies with validated monoclonal antibodies confirmed the presence of CD3⁺ T lymphocytes, neutrophil elastase (NE⁺) neutrophils, EG2⁺ eosinophils, and AA1⁺ mast cells in the mucosa and

Table 3 Changes in inflammatory cells before and after low dose allergen exposure.

Inflammatory cells	Submucosa [cells/mm ²] Median (range)		Mucosa [cells/mm ²] Median (range)	
	Pre-allergen	Post-allergen	Pre-allergen	Post-allergen
Lymphocytes (CD3 ⁺)	38.98 (18.77)	63.97 (47.64)	0.00 (0–0.12)	0.00
Eosinophils (EG2 ⁺)	10.53 (2.39)	3.31 (1.13)	0.00 (0–1.17)	0.00 (0–1.57)
Neutrophils (NE ⁺)	22.62 (16.69)	20.07 (10.37)	0.00	0.00 (0–0.25)
Mast cells	19.75 (17.27)	33.28* (31.00)	0.6 (0–4.43)	0.00

Results are expressed as medians (interquartile ranges); $n = 7$.

* $P = 0.018$.

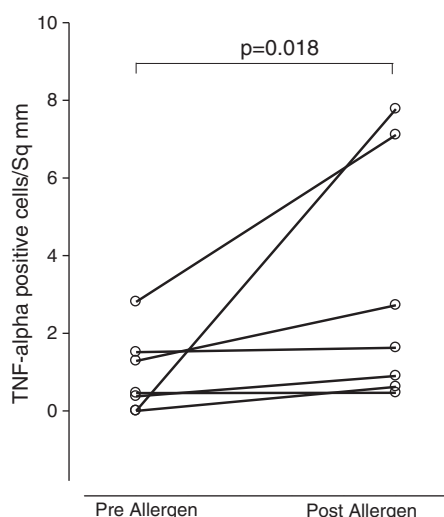


Figure 2 TNF- α positive cells in bronchial biopsies before and after low dose allergen exposure.

submucosa of mild asthmatics. Following low dose allergen inhalation, sub-mucosal mast cell numbers were significantly increased from 19.8 mm^2 (13.0–30.3) to 33.3 mm^2 (21.8–51.1); $p=0.01$ (Table 3). Meanwhile, there was no difference in other inflammatory cells including eosinophils, neutrophils, and CD3 lymphocytes. Numbers of submucosal cells that stained positively for TNF- α also increased following allergen exposure, from 0.46 mm^2 (0.19–1.40) to 1.63 mm^2 (0.76–4.91); $p=0.018$ (Fig. 2). TNF- α^+ cell numbers showed a positive correlation with mast cell numbers in the bronchial biopsies ($r=0.53$, $p=0.05$), suggesting that, in response to allergen exposure, mast cells were the major source of TNF- α expression. In contrast, there was no significant change in the TNF- α^+ cells in the bronchial epithelium. To examine expression of endothelial adhesion molecules, the expression of VCAM and ICAM was analyzed as percentage staining of the

endothelial cell marker EN4. VCAM was upregulated from 0% to 9.72% (5.33–71.28), $p=0.046$ (Fig. 3), while ICAM levels did not show a significant change (48.49% (32.77–59.02) to 85.63% (67.31–100.00) ($p=0.07$)).

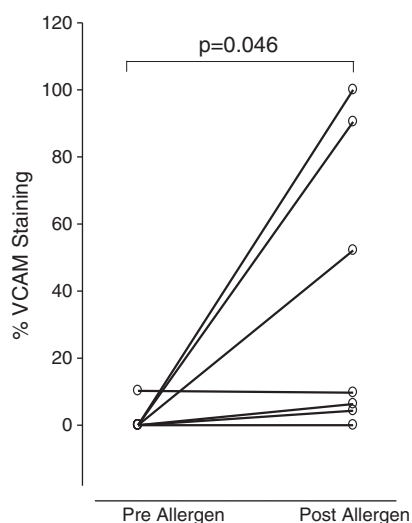
3.2. Anti TNF- α antibodies prevent allergen-induced increases in TNF- α and adhesion molecule expression in bronchial explant cultures of moderately severe asthmatic subjects

As TNF- α expression increases according to disease severity [15], we investigated TNF- α responses to allergen in more severe disease. Since it was not ethically acceptable to perform allergen challenges in severe asthma in vivo, allergen exposures were performed in vitro using a bronchial explant model, that we have previously characterized [16,17]. Twelve moderately severe allergic asthmatic subjects (9 males and 3 females) with a mean age of $33.6 (\pm 3.7)$ years were recruited for bronchoscopy to obtain bronchial biopsies. The baseline characteristics of the subjects are shown in Table 4.

Firstly, experiments were conducted to optimize the dose of CDP-870. Due to the small number of bronchial biopsies available for explant culture, airway epithelial cell cultures obtained from the same study subjects were used to optimize the dose of CDP-870 to ensure complete neutralization of any TNF- α produced in the explants. Based on these studies, a dose of $10 \mu\text{g/mL}$ was chosen to block the effects of TNF- α in the explant culture system. Further details are available in the online supplement.

When bronchial explant cultures were incubated for 24 h, the baseline levels of TNF- α were 11.3 (5.9–19.5) pg/mg and exposure to *Der p* produced no significant change in the levels of TNF- α (8.8 (5.1–30.2) pg/mg; $p=\text{NS}$). However, in the presence of CDP-870, the levels of TNF- α were significantly decreased (1.6 (0.2–6.9) pg/mg; Fig. 4) when compared both to the baseline levels ($p=0.005$) and

(A) Change in VCAM Staining



(B) Change in ICAM Staining

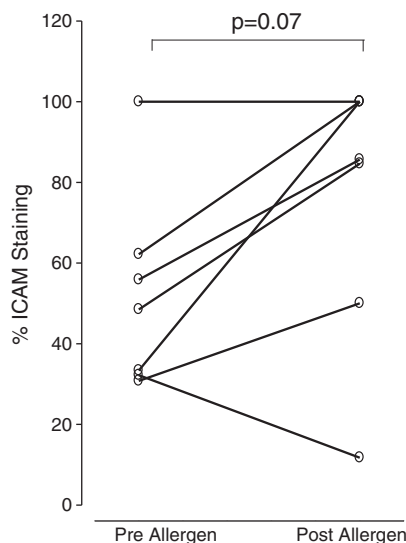


Figure 3 Staining for adhesion molecules VCAM and ICAM before and after low dose allergen exposure. (A) Percentage of staining for VCAM; (B) Percentage of staining for ICAM.

Table 4 Baseline characteristics of the subjects.

Subject no.	Age/sex (y)	SPT to HDM (mm)	Dose of inhaled steroids (mcg)	Baseline FEV ₁ (L)	Predicted FEV ₁ (%)	Baseline FVC (L)	PC ₂₀ (mg/mL)
01	33/M	7	600	2.46	69.9	3.22	9.13
02	42/M	10.5	500	4.36	108.2	4.93	4.94
03	58/M	5.5	400	2.6	77.6	5.41	2.51
04	57/M	5.5	1000	3.21	89.2	3.8	5.62
05	20/F	6.5	800	3.05	89.2	3.55	11.28
06	29/F	6	400	3.22	97.3	3.62	7.97
07	32/M	6	700	3.22	83.6	3.62	2.26
08	22/F	4.5	1500	2.25	74.8	2.81	0.42
09	26/M	5.5	400	4.64	97.7	6.02	3.97
10	21/M	8	400	4.23	91.2	5.02	7.97
11	28/M	7.5	800	2.91	91.5	3.5	0.29
12	35/M	7	400	2.92	78.2	4.08	11.14

SPT indicates skin prick test (wheal diameter in millimeters); PC₂₀, provocative concentration for 20% fall in FEV₁ with methacholine.

following stimulation with *Der p* ($p=0.01$). The inhibitory effects of CDP-870 were even apparent after correcting for the masking of TNF- α by CDP-870.

The spontaneous release of interleukin (IL)-8 from the bronchial explants of moderately severe asthmatics was 28.1 (13.0–39.0) pg/mL. Upon exposure to *Der p* extracts, there was considerable variability in IL-8 production ranging from 18.1–44.1 pg/mL with a mean of 38.1 pg/mL; $p=0.7$. In the presence of CDP-870, the levels of IL-8 decreased to 26.4 (14.0–34.7) pg/mL, $p=0.05$ (*Der p* vs. CDP-870) (Fig. 5). There was no significant difference between the levels of IL-8 following stimulation with *Der p*, but in the presence of CDP-870, the levels of IL-8 were significantly reduced ($p=0.05$). The cytokines IL-5 and IL-1 β were also assayed in the supernatants from the bronchial explants by ELISA; however, the levels of these cytokines did not change significantly following stimulation with *Der p* or in the presence of CDP-870.

3.3. Expression of inflammatory cells and cytokines in bronchial biopsies

Immunohistochemistry confirmed the presence of CD3⁺, CD4⁺, CD8⁺ T lymphocytes, neutrophil elastase (NE⁺) neutrophils, EG2⁺ eosinophils, AA1⁺ mast cells, and CD68⁺ macrophages in the submucosa of the explanted tissue. The inflammatory cell population in the epithelium was not assessed as the epithelial layer of the bronchial biopsies tended to become denuded during culture. Following exposure to *Der p*, there was no significant change in numbers of any of the cell types, as the explant model is an isolated system that does not allow influx of new inflammatory cells. In contrast, exposure to *Der p* caused the number of TNF- α positive cells to increase from 1.1 cells per square millimeters (0.2–1.6) to 2.4 cells per square millimeters

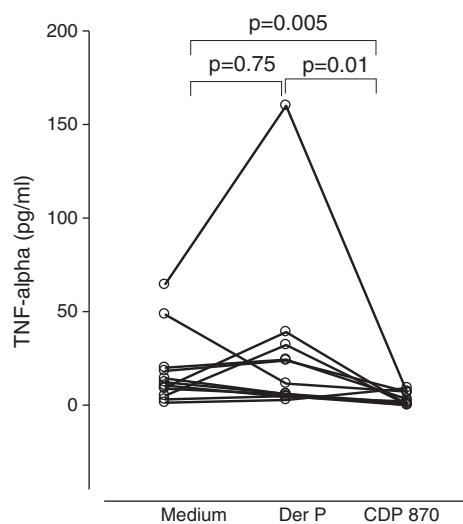


Figure 4 TNF- α protein production by bronchial explant cultures of moderately severe asthmatics in medium alone, after ex vivo stimulation with house dust mite (*Der p*) allergen (5000 SQ-IU/mL) for 24 h and in the presence of CDP-870 (10 ng/mL).

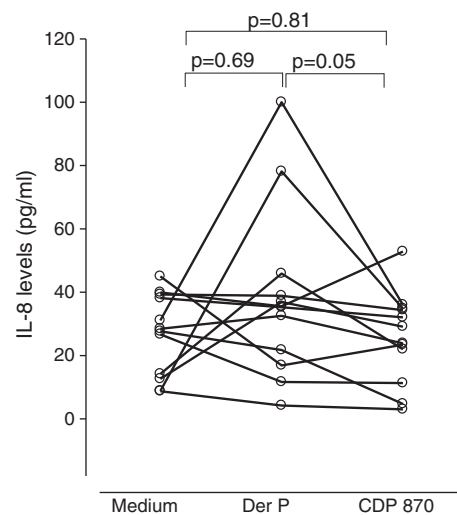


Figure 5 IL-8 production by bronchial explant cultures from moderately severe asthmatics after culture asthmatics in medium alone, after ex vivo stimulation with house dust mite (*Der p*) allergen (5000 SQ-IU/mL) for 24 h and in the presence of CDP-870 (10 ng/mL).

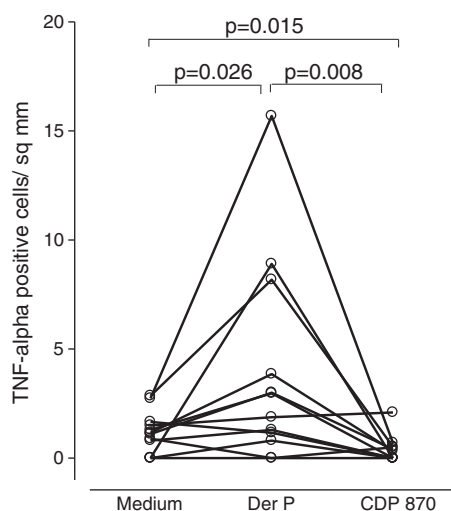


Figure 6 TNF- α expression in explant bronchial biopsies when cultured in medium; medium+*Der p* and medium+*Der p*+CDP-870.

(0.9–7.1) ($p=0.02$). This effect of *Der p* was suppressed by CDP-870 with only 0.2 cells per square millimeters (0.0–0.5), $p<0.008$, which stained positively for TNF- α (Fig. 6). In the presence of *Der p*, the expression of ICAM-1 in the submucosa increased from 79.03% (62.05–86.72) to 85.65% (75.49–99.63) ($p=0.15$) and this was reduced to 59.89% (36.52–80.12) ($p=0.01$) in the presence of CDP-870 (Fig. 7).

4. Discussion

Bolus allergen challenge models have been used to evaluate cells and mediators involved in acute allergen induced airway inflammation [18,19]; however, repeated small doses of allergen resemble more closely the natural asthmatic state and may be more relevant to the pathogen-

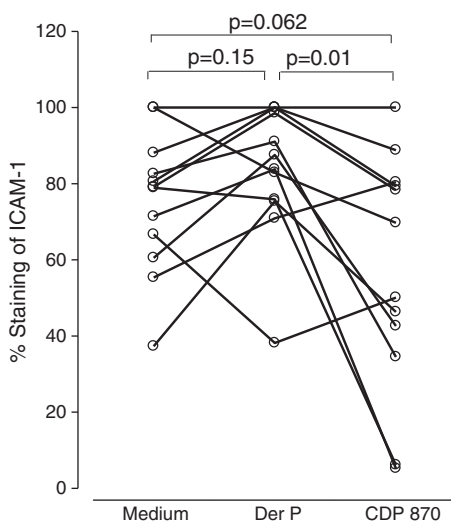


Figure 7 ICAM-1 expression in explant bronchial biopsies after culture in medium; medium+*Der p* and medium+*Der p*+CDP-870 for 24 h.

esis of chronic allergic asthma [20,21]. Low dose allergen challenge studies were designed to produce sub-clinical changes in the airway inflammation while not affecting lung function or airway responsiveness. Therefore, during this stage, the inflammatory response is low as compared to the repair processes occurring after the asthmatic bronchoconstriction [22,23].

An increase in the number of mast cells is reported in the airways of asthmatic patients even at an early stage of the disease [24–26]. Thus, the increased number of infiltrating mast cells observed in the low dose allergen study model could be considered as an early event since the subjects did not develop asthma symptoms or BHR. This is the first to show an increase in TNF- α expression in the bronchial biopsies following low dose allergen exposure associated with the parallel increase in the mast cells. Earlier studies of asthmatic bronchial biopsies have co-localized TNF- α to mast cells [24]. One of the pitfalls of our study was co-localizing the TNF- α to mast cells; however, the finding of a positive correlation between the sub-mucosal mast cell numbers and cells staining positive for TNF- α suggests that mast cells are most likely to be responding low dose allergen exposure by increasing their level of TNF- α expression. The increase in TNF- α in the bronchial biopsies after low dose allergen exposure was also associated with a parallel increase in the expression of VCAM which has been shown in other studies too [27,28]. Subjects who had a greater increase in TNF- α expressing cells had greater change in the expression of adhesion molecules which points to a likely role for TNF- α in adhesion molecule expression. The low dose allergen exposure did not cause an increase in eosinophils in the airways but this has been reported in the past where, in low dose allergen, challenge leads to increase in markers of eosinophil activation viz. eosinophilic cationic protein (ECP) and IL-5 with no significant increase in eosinophils [22,29].

The low dose allergen exposure model has its drawbacks too. The absence of standardization of the dose, the duration of allergen exposure, and the possibility of facilitating an acute asthma attack all limit its usefulness. The study per se involves a lot of input from the participants with several visits and hence has its limitations. This reflects on the sample size which was small and this could have had an effect on the power of the study. Furthermore, the model could not be extended to evaluate the inflammatory responses in patients with moderate to severe asthma; so, an in vitro model involving bronchial explants had to be employed.

Development of the bronchial explant culture system has been extremely useful to assess the involvement of particular cytokines in the inflammatory response in asthma. We have shown that co-stimulation by CD80 and CD86 is essential for allergen induced cytokine production in explants from mild asthmatic subjects [16,30]. Thus, these models display disease-severity related differences and allow the testing of potentially damaging environmental agents which could not be delivered in vivo, as well as novel therapies. As found in the low dose allergen challenge model, exposure to *Der p* in vitro caused an increase in the number of cells expressing TNF- α in the explant tissue and also increased adhesion molecule expression. TNF- α is known to upregulate its own expression in mast cells via an autocrine loop [31] and to increase adhesion molecule

expression including ICAM-1 and VCAM to promote inflammation [27,28]. Although no causal relationship could be inferred from the in vivo studies, blocking the effects of TNF- α in vitro using a monoclonal antibody resulted in a decrease in TNF- α and adhesion molecules in the explants, indicating that endogenously produced TNF- α was likely to be driving the responses we observed in vitro. Even though *Der p* exposure did not significantly increase the release of soluble TNF- α detectable in the explant culture supernatants, and the levels of endotoxins were very low (0.5 EU/mL [endotoxin unit] as per the manufacturers) in the house dust mite allergen to provoke a non-specific response, the ability of CDP-870 to block the allergen-induced increase in TNF- α positive cells within the explant tissue suggests that low levels of soluble TNF- α are produced in response to allergen stimulation and that these act locally to auto-induce TNF- α expression within mast cells. Alternatively, the antibody may have ligated membrane-bound TNF- α on the mast cells to induce signals that suppress TNF- α production via mechanisms similar to those proposed in inflammatory bowel disease [32,33]. One of the drawbacks of our explant study is that we did not co-localize the TNF- α positive cells which could have thrown light on the source of TNF- α . Furthermore, looking at the effects of CDP-870 on the medium could identify the effects of this molecule on the cytokine downstream. Despite these shortcomings, the explant model does show that blocking TNF- α downregulates ICAM-1 in the bronchial biopsies on moderate asthmatics.

In addition to its pro-inflammatory properties, TNF- α also plays a role in the induction and potentiation of BHR. TNF- α regulates the function of airway smooth muscle not only by inducing a contractile phenotype by enhancing calcium signaling through different pathways but also by modulating the secretion of other cytokines and the level of expression of adhesion molecules [34]. Since mast cells have been localized to airway smooth muscle in patients with asthma [35,36] and asthma severity correlates with the number of mast cells in the bronchial biopsies [37], TNF- α neutralizing therapies should block TNF- α release from smooth muscle-resident mast cells, thereby preventing activation of the autocrine loop and increased expression of TNF- α that may eventually cause BHR.

Studies have shown that the inflammatory cell population in patients with severe asthma has a predominance of neutrophils [38,39] and we have recently found that, in severe asthmatics, the levels of IL-8 and neutrophils were reduced following 12 weeks of treatment with etanercept [40]. Consistent with this effect, CDP-870 also reduced the levels of IL-8 in the explant supernatants. However, as there was no significant effect of *Der p* on IL-8 levels, it would appear that the suppression of IL-8 levels by CDP-870 was due to the blockade of basal TNF- α levels which may have been produced in response to tissue injury rather than to allergen provocation. It is well known that IL-8 is an important chemokine associated with the recruitment of neutrophils, and by reducing the levels of IL-8, TNF- α blocking strategies could well inhibit the neutrophil influx into the airways of severe asthmatics.

In conclusion, we have found that subacute allergen exposure leads to accumulation of TNF- α expressing cells in the bronchial submucosa and increased expression of adhesion molecules, leaving the tissue poised for a vigorous

inflammatory response. By use of a neutralizing antibody in vitro, these responses appear to be driven by TNF- α acting by autocrine and paracrine mechanisms. Although initial studies with TNF- α blocking strategies in severe asthma have shown variable results [6,7,9–11], our studies are consistent with TNF- α acting as an important cytokine in the modulation of airway inflammation. Despite having a possible therapeutic role in the management of asthma, one should have a cautious optimism in view of the likely side effects. Asthma, being a heterogeneous disease, would be valuable to identify those subgroups, in particular, patients with severe asthma in whom TNF- α plays a central role in pathogenesis, and to direct the TNF- α blocking strategies on this group which has the largest unmet need.

Appendix A. Supplementary data

Supplementary data to this article can be found online at [doi:10.1016/j.clim.2011.03.005](https://doi.org/10.1016/j.clim.2011.03.005).

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Low Dose allergen Exposure

Bronchodilator reversibility was assessed with 200µg of salbutamol administered through a metered dose inhaler. The HDM specific IgE levels were assayed by radio-allergen sorbent test (RAST). Sensitivity to house dust-mite (HDM) was determined by skin prick test and a positive result was taken as at least 5mm average diameter above saline control. All the subjects were clinically stable and did not have any history of respiratory tract infection during the last 3 months. Methacholine response was performed by the method employed and modified from Chai et al (14). The results were interpreted as methacholine provocative concentration 20 (PC20) which was defined as the final cumulative methacholine dose required to produce a 20% decline in FEV1 from the value after saline inhalation. A standard bronchial allergen challenge test was performed with a standardized house dust mite allergen extract and the cumulative PD20 (provocation dose to cause a 20% fall in FEV1) was calculated. Bronchoscopy was performed three weeks after the bolus allergen bronchial provocation test and after the repeated low dose allergen exposure using a fiberoptic bronchoscope (Olympus, FB-XT20, Tokyo, Japan) in accordance with the standard published guidelines (15, 16). Bronchoscopy was performed only if the subjects' baseline FEV1 was $\geq 60\%$ of predicted. Six bronchial biopsies were obtained from the sub-carinae of the 2nd and 3rd generation bronchi of the right lower lobe using FB15 alligator forceps. The biopsies were then placed in acetone for GMA processing. The bronchial biopsies obtained were processed and immunostained for eosinophils, neutrophils, mast cells, macrophages, CD3, CD4, CD8 and TNF- α . The cell counting was performed by an independent observer blinded to the coded sample.

Methodology

The study design is outlined in the manuscript. After the screening visit, subjects were asked to keep a home diary to monitor their PEF three times a day for a week and indicate any symptoms (Scale 1-6) of asthma and rescue medication use after their screening visit. Methacholine provocation test was performed to assess the BHR. At the end of this week a standard bronchial allergen challenge was performed with a standardised house-dust mite allergen extract. PD20 was calculated. After a week bronchoscopy was performed to obtain bronchial mucosal biopsies.

Inhalation visits

Once eligibility was established and baseline measurements obtained, the subjects attended the department for inhalation of a small dose of a standardised dust mite extract calculated to be one tenth of the allergen provocation dose (PD20 allergen) for that subject. The extract was freshly diluted in 0.9% saline and administered through a nebuliser using a standard dosimeter (Spira, Electra 2, Spira Respiratory Care, Finland). Subjects were allowed to return home after 30 minutes. This procedure was repeated three times a week for a period of four weeks unless a clinically significant change in their asthma status was detected earlier. On each visit, spirometry was performed prior to the dust-mite inhalation dose of that day. At the end of the inhalation period methacholine provocation test was repeated followed by a repeat bronchoscopy.

Bronchial Explant Culture system

The culture medium was supplemented with HEPES buffer (10mM), L-glutamine (1mM), sodium pyruvate and 2-mercaptoethanol (2 μ M). Two biopsies were used for each culture condition to minimise the effects due to tissue heterogeneity. After 24 hours the cultures were stopped, the biopsies dried and weighed and placed in ice-cold acetone with a protease inhibitor (2mM phenyl methyl sulphonyl fluoride and 20mM iodoacetamide) for processing into GMA for immunohistochemistry analysis. The supernatants were centrifuged and stored at -80⁰ pending analysis. The doses of allergen and culture duration were based on conditions previously determined to be optimal for explant derived from mild and moderately severe asthmatics (19-21). A 24 hour culture period was chosen for monitoring cytokine production based on preliminary time course experiments by Jaffar *et al* on IL-5 production in bronchial explants.

Optimisation of the dose of anti TNF- α (CDP 870) for explant cultures

Preliminary experiments were conducted to optimise the dose of CDP 870. Due to the small number of bronchial biopsies available for explant culture, airway epithelial cells obtained from the same study subjects were also used to optimise the dose of CDP 870 to ensure complete neutralisation of any TNF- α produced in the explants. IL-8 levels were used as readout of TNF- α activation of the primary bronchial epithelial cells. As shown in Fig 1 there was an increase in IL-8 levels with increasing doses of TNF- α with significant stimulation being observed at 10ng/ml of TNF- α . BECS exposed to increasing concentrations of TNF- α for 24 hours and IL-8

release into supernatants measured by ELISA. Data are from 5 subjects and were analysed using Wilcoxon's test. A dose of 10 ng/ml of CDP 870 which was initially suggested by the suppliers and it was found to inhibit IL-8 release in the presence of 10ng/ml of TNF- α 2 (Fig 2). CDP 870 had no effect on the basal IL-8 production by primary bronchial epithelial cells. Hence, a dose of 10ng/ml was chosen to block the effects of TNF- α in the explant culture system.

Figure Legend

Fig 1: Dose response of IL-8 to TNF- α in primary bronchial epithelial monolayer cultures

Fig 2: Response of BECS to TNF- α in the presence and absence of CDP 870. BECS were exposed to control media (SFM), CDP870 and TNF- α alone or in combination with CDP870 for 24 hours. IL-8 release into controlled media was measured by ELISA (Biosource). TNF- α increased IL-8 release and this was inhibited by CDP870 (10ng/ml).

Fig 1

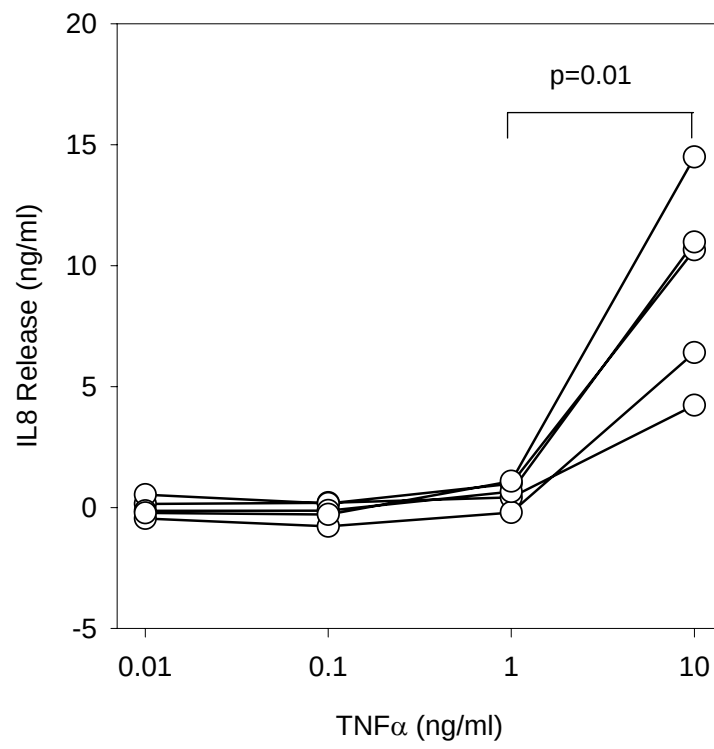
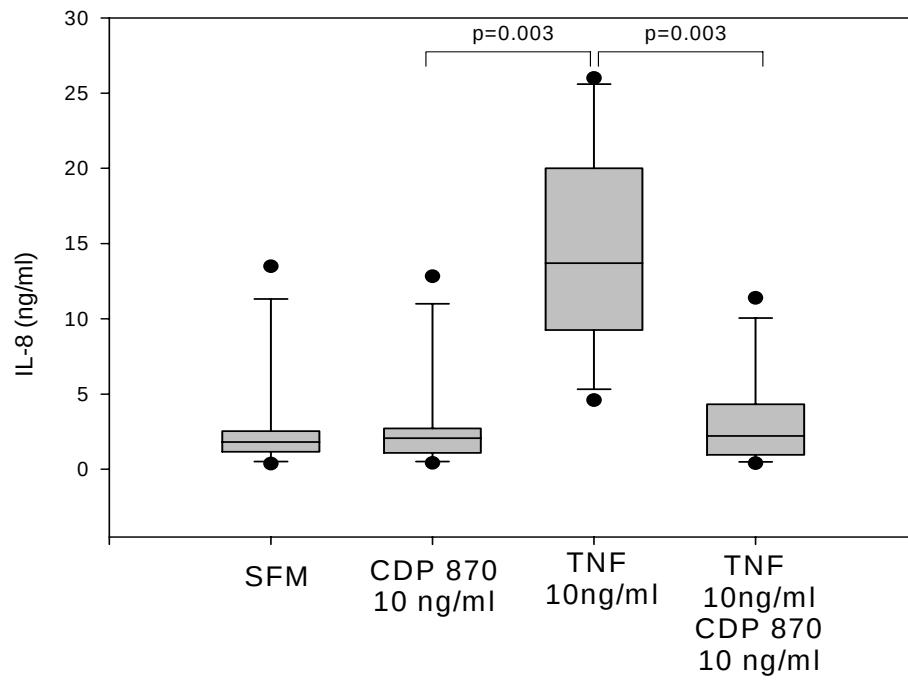


Fig 2



SFM- Serum Free Media