Title:	**Airway Hyperresponsiveness and Bronchial Mucosal Inflammation in T-Cell Peptide-induced Asthmatic Reactions in Atopic Subjects**

Running title: AHR, T Cells and Late Asthmatic Reactions

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Abbreviations

AHR, airway hyperresponsiveness; BAL. bronchoalveolar lavage; FEV_1, forced expiratory volume in one second; histamine $PC_{20,}$ histamine provocative concentration causing a 20% fall in FEV_1; LAR, late asthmatic reaction; LT, leukotriene; PEFR, peak expiratory flow rate; PG, prostaglandin; TARC, thymus- and activation-regulated chemokine

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ABSTRACT

Background: Allergic asthmatics develop isolated late asthmatic reactions after inhalation of allergen-derived T-cell peptides. Animal experiments have shown that airway hyperresponsiveness is $CD4^+$ cell-dependent. We hypothesise that peptide inhalation produces increases in non-specific airway hyperresponsiveness (AHR) and a T-cell dominant, bronchial mucosal inflammatory response.

Methods: Bronchoscopy, with bronchial biopsies and bronchoalveolar lavage (BAL), was performed in 24 cat-allergic subjects 6 hours after aerosol inhalation of short overlapping peptides derived from Fel d 1, the major cat allergen. Biopsies and BAL were studied using immunohistochemistry and ELISA.

Results: Twelve of the 24 subjects developed an isolated late asthmatic reaction without a preceding early- (mast cell/histamine-dependent) reaction characteristic of whole allergen inhalation. These responders had significant between-group differences (responders vs non-responders) in the changes (peptide vs diluent) in AHR (p= 0.007) and bronchial mucosal $CD3^+$ (p=0.005), $CD4^+$ (p=0.006) and $TARC^+$ (p=0.003) but not $CD8^+$ or $CD25^+$ cells or eosinophils, basophils, mast cells and macrophages. The between-group difference for neutrophils was p=0.05 but with a non-significant within group value (peptide vs diluent, responders, p=0.11). In BAL there was a significant between-group difference in TARC (p=0.02) but not in histamine, tryptase, basogranulin, C3a or C5a, $LTC_4^/D_4^/E_4$, PGD_2 or $PGF_{2\alpha}$.

Conclusions: Direct activation of allergen-specific airway T cells by peptide inhalation in atopic asthmatics leads to increased airway hyperresponsiveness with local increases in CD3+ and CD4+ cells and TARC but no significant changes in eosinophils or basophil/mast cell products thereby supporting previous animal experiments which showed a CD4+ dependence for airway hyperresponsiveness.

INTRODUCTION

We have previously shown that allergen-derived T-cell peptide epitopes, administered by either intradermal injection (1) or by inhalation (2), induce late asthmatic reactions (LAR) in a proportion of atopic asthmatics ("responders"), but not in others ("non-responders"). These reactions peaked between 3 and 9 hours after peptide inhalation and had a similar time-course of onset and resolution to LARs induced by whole allergen extract. They are termed "isolated" late reactions since, unlike whole allergen challenge, there was no early (immediate) asthmatic reaction, as the peptides were too short to cross-link IgE on mast cells and did not release histamine from blood basophils (1). Thus our model has the potential to provide information on the T-cell component of allergic airway inflammation, independently of initial mast cell activation.

The classical studies of Cockcroft et al (3) and Cartier et al (4) demonstrated that after inhalation of whole allergen there were increases in the degree of non-specific airway hyperresponsiveness in subjects who develop dual (early- and late-) asthmatic reactions, but not in those who have only a single early response. Compared to single reactors, dual reactors tend to have elevated allergen-specific serum IgE concentrations (5) suggesting that the degree of mast cell sensitisation may determine the development of the late phase reaction and subsequent airway hyperresponsiveness. The ability of an anaphylactic anti-IgE to induce late phase skin reactions supports this notion (6). On the other hand there are several animal studies showing that AHR can be adoptively transferred by $CD4^+$ T cells (7,8,9) and the role of the T cell in chronic asthma is now firmly established (10). For these reasons we have used our model of peptide-induced isolated LAR to test the hypothesis, in man, that direct activation of allergen-specific airway T cells, independent of the mast cell, produces increased airway hyperresponsiveness. Thus, in the present study we show, for the first time, that LARs elicited in atopic subjects by mucosal (inhalational) challenge of allergen-derived T-cell peptides induced increases in non-specific AHR that were associated with a dominant $CD3^+$ and $CD4^+$ bronchial mucosal inflammatory cell response as well as heightened expression of the T-cell chemoattractant, TARC. In contrast there was no evidence of an involvement of eosinophils, mast cells, basophils or their products in the airways in these peptide-induced LARs (i.e. in responders) supporting the view that part of the asthma process has a dominant T cell component. As a further control we measured the same variables in age and sex-matched, cat allergic, non-responders, where we found no increases in AHR, CD3+, CD4+ cells or TARC. Thus, in the present study we have measured the PC_{20} histamine, as an index of AHR, as well as changes in inflammatory cells, tissue mast cells and also soluble mediators in bronchial biopsies and broncholveolar lavage in both responders and non-responders after the inhalation of either Fel d 1–derived peptides or diluent control.The pharmacological mediators included known bronchoconstricting agents (e.g.histamine and ecosanoids), the histamine-releasing, complement-derived anaphylotoxins, C3a and C5a (11) as well as markers of mast cell degranulation (i.e. tryptase and basogranulin). We also measured IL-13 in lavage fluid since this cytokine is known to be associated with increased AHR (12). IL-10, a regulatory cytokine, was also assayed to determine whether it was altered in responders compared to non-responders.

METHODS

Subjects & Study Design

Cat-allergic asthmatic volunteers were recruited by advertisement and characterised clinically as defined previously (1,2). The study was approved by the Royal Brompton and Harefield NHS Trust Ethics Committee. All volunteers gave written informed consent. All subjects demonstrated a PC_{20} to histamine of ≤16mg/ml at screening, evidence, during the previous 12 months, of more than 15% reversibility of the FEV_1 or peak expiratory flow rate (PEFR), either spontaneously or after inhaled β_2-agonists and a clear history of wheezy breathlessness with, or without, cough on exposure to cats. Beta$_2$-agonists were withheld on the study day, and inhaled corticosteroids were discontinued 2 months before entering the study. Subjects were excluded if they had received oral corticosteroids in the previous 2 months or Fel d-1-derived peptides in the preceding 6 months. Subjects were non-smokers and had no history of current illness or clinically significant abnormalities in routine haematology, biochemistry or urinalysis.

A randomised, placebo-controlled, crossover study design was used. Seven days after screening (visit 1), subjects received either nebulised diluent (0.9% saline) or 5µg Fel d 1-derived peptide (twelve overlapping peptides from chains 1 and 2 of Fel d 1). In all instances subjects were unaware of whether they were inhaling peptides or diluent. The challenge was postponed if the baseline FEV_1 fell below 80% predicted on any study day. To exclude significant worsening of an individual's hyperresponsiveness the nebulised peptide challenge was administered only if the subject did not exhibit a decrease in FEV_1 ≥10% to an initial inhaled control (diluent) challenge. The FEV_1 was then recorded at 0, 15, 30 and 60 minutes and hourly thereafter for five hours at which time bronchoscopy with bronchial biopsies (BB) and bronchoalveolar lavage was performed. Seven days later (visit 3) the histamine PC_{20} was measured. On visit 4 (minimum of four weeks after visit 2) volunteers again inhaled either diluent or peptide (i.e. the opposite of what was given on visit 2) and bronchoscopy with biopsies and lavage was again performed. One week later (visit 5) the histamine PC_{20} was repeated.

Thirty-one people entered the study but only 12 subjects developed an isolated late asthmatic reaction (greater than 20% reduction in FEV_1) to peptide. These responders completed both the control and peptide study days. The first 12 subjects who showed no clinical response (non-responders) to peptide also underwent both challenges and bronchoscopies. The remaining seven non-responders were not investigated since equal number of subjects in each investigational group (12 responders and 12 non-responders) had been attained.

Peptide Synthesis and Validation

Twelve overlapping peptides from chains 1 and 2 of Fel d 1
(*Chain 1*: EICPAVKRDVDLFLTGT, LFLTGTPDEYVEQVAQY, EQVAQYKALPVVLENA, KALPVVLENARILKNCV, RILKNCVDAKMTEEDKE, KMTEEDKENALSLLDK, KENALSVLDKIYTSPL, *Chain 2*: LTKVNATEPERTAMKK,TAMKKIQDCYVENGLI, SRVLDGLVMTTISSSK, ISSSKDCMGEAVQNTV, AVQNTVEDLKLNTLGR) were synthesised and dispensed as described (13). These were previously shown not to release histamine from peripheral blood basophils.

Inhalational Challenge

The peptides solution was diluted to 1 ml with 0.9% saline and delivered through the Pari LC Star nebuliser plus filter and Pari Boy compressor (Pari Medical Ltd., West Byfleet, UK) for 10 min (2).

Fibreoptic Bronchoscopy

Fibreoptic bronchoscopy with BAL and bronchial biopsies was performed 6 hours following inhalational challenge of either diluent control or Fel d 1 peptides, as described in detail elsewhere (14). BAL fluid and cells and biopsies were also processed as described previously (14).

Immunohistochemistry

Cryostat sections (6 µm) were freshly cut from biopsies, mounted on 0.1% poly-L-lysine coated slides and air dried overnight at room temperature. Monoclonal antibody staining was detected by alkaline phosphatase anti-alkaline phosphatase method, as previously described (15). Normal human serum (10%) was used to prevent non-specific binding of the second and third layer antibodies. A mouse IgG_1 myeloma protein was used as a negative control. The monoclonal antibodies used were CD3, CD4, CD8, CD68, neutrophil elastase (NE), human mast cell tryptase (Dako, High Wycombe, UK); CD25 (Becton Dickinson, Cowley, Oxford, UK); major basic protein (MBP) (BMK 13; in-house); TARC (R&D Systems, Minneapolis, USA) and BB1, a mAb recognizing a human basophil granular protein (a gift from Dr. A.F. Walls, University of Southampton, UK). Polyclonal rabbit anti-mouse immunoglobulin and APAAP reagents were purchased from DAKOPATTS.

For TARC staining cryostat sections (5 µm) were mounted on Superfrost Plus slides (VWR, Leicester, UK) and air dried overnight at room temperature. TARC was identified using a Vectastain ABC Kit from Vector Laboratories (Peterborough, UK). The reaction was visualized using Fast Red Substrate (Vector Laboratories). The TARC antibody used was raised in the goat (R&D Systems, Oxford, UK). The numbers of positively stained cells were counted in a zone 250 µm deep along the entire length of epithelial basement membrane, as defined by a squared eyepiece graticule. Cell counts were performed in a blinded fashion and expressed as the number of positive cells per mm^2 (15).

Differential BAL Cell Counts

Cells counts were performed in a blinded fashion. Unfixed cytospins of BAL cells were stained using Kwik-Diff®. The slides were rinsed in PBS and air-dried. In all cases a minimum of 400 nucleated cells were counted per slide.

Histamine Measurement

Histamine content of BAL supernatant and following stimulation of whole blood by BAL was measured by means of a commercially available competitive ELISA kit (Immunotech, Marseille, France), according to the manufacturer's instructions.

Eicosanoid Assays

BAL supernatants were frozen immediately and stored at –80°C until further analysis. Thawed BAL supernatant (5 ml) was purified and concentrated 10-fold on a C18 column. The recovery of prostaglandins and leukotrienes was determined by the addition of ^{3}H-PGD$_2$ (10,000 cpm; Amersham Bioscience UK Ltd, Bucks, UK) and ^{3}H-LTC$_4$ (6,000cpm; NEN Life Science Products, Hounslow, UK), respectively, to each 5 ml aliquot. LTC$_4$, LTE$_4$ and 11β-PGF$_{2\alpha}$ in the concentrated samples were measured by enzyme immunoassay (Cayman Chemical Co, Ann Arbor, Michigan, USA). PGD$_2$ in the concentrated samples was converted to PGD$_2$-methoxime, a stable derivative, and PGD$_2$-MOX was measured by enzyme immunoassay (Cayman Chemical Co). The detection limit of the assays are as follows: LTC$_4$ 10 pg/ml, LTE$_4$ 25 pg/ml, 11β- PGF$_{2\alpha}$ 5.5 pg/ml, PGD$_2$-MOX 3.6 pg/ml.

The specificity of the LTC$_4$ antibody is 100% for LTC$_4$ and LTC$_5$ and the antibody cross-reacts with LTD$_4$ (46%) and with LTE$_4$ (7%). The specificity of the LTE$_4$ antibody is 100% for LTE$_4$ and LTE$_5$, with cross-reactivity with LTC$_4$ (10%) and LTD$_4$ (7%). The specificity of the antibody to 11β-PGF$_{2\alpha}$ is 100% for 11β-PGF$_{2\alpha}$, 10% for 2,3-dinor-11β-PGF$_{2\alpha}$ and <0.01% for leukotrienes, thromboxane B$_2$ and other prostaglandins. The antibody to PGD$_2$-MOX is specific for PGD$_2$-MOX (100%), with cross-reactivity with PGD$_2$ (0.2%) and other prostaglandins (<0.01%).

ELISAs for Cytokines

Aliquots of 10 ml BAL supernatant were thawed and immediately concentrated 10 times using Amicon Centriplus kit (Millipore, Billerica, MA, USA), according to the manufacturer's instructions. IL-10 and IL-13 levels in the concentrated BAL supernatants were determined by ELISA (PeliKine Compact kit, CLB, Amsterdam, The Netherlands). The sensitivity of the assays are 1-3 pg/ml for IL-10 and 0.5-1.5 pg/ml for IL-13.

Tryptase and Basogranulin Measurements

Tryptase measurement was performed using a modification of a previously described ELISA method (16) that detects both pro-tryptase and the mature forms (with a sensitivity of 0.5 ng/ml). Basogranulin assay was performed using a dot blotting procedure with monoclonal antibody BB1 as described previously (17). Concentrations were expressed as milli-units per ml, where 1 unit was taken as the amount of basogranulin in a standard preparation of purified basophils.

C3a and C5a Assays

C3a/C3a des-Arginine and C5a/C5a des-Arginine levels in BAL supernatants were determined by ELISA using BD Biosciences Pharmingen kits (San Diego, USA) according to the manufacturer's instructions. In order to prevent ex-vivo complement activation, FUT-175 (Futhan, final concentration 5μg/ml) (BD Biosciences; # 552035) was added to each thawed sample. Samples were assayed undiluted, 1:3, 1:6, 1:12 as previously described (11). The sensitivities of the assays are 7.3 pg/ml for C3a and 0.06 ng/ml for C5a.

TARC Assay

TARC level in BAL supernatants was determined by ELISA (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. The sensitivity of the assay was 7 pg/ml.

BALF Assays -Protein Correction Factor

BALF samples were corrected for variable dilution using protein as an internal reference standard. Protein determination in BAL and concentrated BAL supernatants was performed using bicinchoninic acid protein assay kit (Sigma, St. Louis, USA) according to the manufacturer's instructions. The protein concentrations in all samples were normalised to the sample with the lowest protein concentration and all values obtained in the assays were corrected accordingly.

Statistical Analyses

Statistical analyses were performed with the aid of commercial software packages including GraphPad Prism® and significance was accepted if $p \leq 0.05$. Data from BALF supernatant assays, BAL cytospins, bronchial biopsy immunohistochemistry and *in situ* hybridization analyses are expressed as medians, with minimum to maximum ranges shown. Statistical comparisons of diluent and peptides inhalation (within-subject comparisons) were performed using non-parametric two-tailed Wilcoxon signed-rank tests. Between-group comparisons of the change from diluent to peptide in responders and non-responders were performed using the non-parametric Mann-Whitney test. The Mann-Whitney test was also used for unpaired data. Correlations with clinical characteristics were performed using Spearman's rank coefficient of correlation. FEV_1 data were summarised over time for each subject for the control (diluent) day and the peptide day. Areas under each curve (AUC) for the FEV_1 measured over 6 hours were calculated using the trapezoidal rule. Differences in the AUC between the control day and peptide day were analysed by paired t-test.

RESULTS

Peptide Challenge Results in Airway Hyperresponsiveness

At baseline, responders and non-responders were well matched for gender, age, FEV_1, histamine PC_{20} and total serum IgE concentration (Table 1). The changes in FEV_1, following inhalation of either peptide or diluent, in responders and non-responders, are shown in figures 1A and 1B, respectively. On the peptide day responders had an average decrease in their FEV_1 at 6 hours of approximately 33% (p<0.001). On the diluent day there was virtually no change,

Table 1. *Clinical characteristics of the cat-allergic asthmatics.*

	Responders	**Non-Responders**
Total no. of subjects (n)	12	12
Ratio M:F	4:8	6:6
Age (yr)	27 (26-34)	28 (22.5-28.5)
Baseline FEV_1 (% pred)	93.8 (87.5-102.7)	92.8 (86.1-95.6)
PC_{20} Histamine (mg/ml)	3.99 (1.67-12.5)	3.99 (1.93-4.63)
Total IgE (IU/ml)	134 (96-248.5)	256.5 (197.5-674.5)
Cat RAST (IU/ml)*	29.2 (6.17-43.9)	3.49 (1.98-7.07)

*p=0.002.   Number or median (inter-quartile range)

between baseline and 6-hour FEV_1 values, in responders, or on diluent or peptide days, in non-responders. There was a highly significantly elevated cat-specific serum IgE (p=0.002) (despite a non-significantly lower concentration of total IgE) in responders compared with non-responders (Table 1). Changes in AHR are shown in Figure 1C. There was a significant decrease in the histamine PC_{20} in responders (diluent vs. peptide, p=0.002) which was not observed in non-responders (diluent vs. peptide, p=0.52). The between group difference in the changes was p=0.007. All subjects were mild cat-allergic asthmatics with a history of cat-

induced wheeze on entering the study. At screening they all had a PC_{20} histamine of <16 mg/ml. However in two of the subjects the values changed to >16 mg/ml during the course of the study (Fig 1C).

Isolated Late Asthmatic Reactions Are Associated with Recruitment of CD3+CD4+ T cells

In responders there were significant increases (diluent vs. peptide) in the numbers of $CD3^+$ (p=0.05, Fig 2A and Table 2,) and $CD4^+$ cells (p=0.03; Fig. 2B and Table 2), with decreases in non-responders (p=0.03 for $CD3^+$, and p=0.09 for $CD4^+$ cells (Table 2). The between group differences in the changes were p=0.005 for $CD3^+$ cells and p=0.006 for $CD4^+$ cells (Fig 2A and 2B and Table 2). Using a non-paired test the numbers of CD3+ and CD4+ cells were also significantly higher in responders compared to non-responders following peptide challenge (p=0.004 and p=0.04 respectively), but not following inhaled diluent (Fig 2A and 2B). A representative photomicrograph of $CD4^+$ immunostaining in bronchial biopsies from responders and non-responders is shown in Fig 3. In responders (diluent vs peptide) there was only a trend for increases in MBP^+ eosinophils (p=0.07) (Table 2). Although there was no significant within group increase in elastase$^+$ neutrophils (p=0.11) in responders (diluent vs peptide) the between group value just reached significance (p=0.05). There were no significant changes in $CD8^+$ and $CD25^+$ cells or in $BB1^+$ basophils or tryptase$^+$ mast cells (Table 2). Eosinophil and neutrophil counts in lavage fluid showed no significant change in either responders or non-responders (diluent vs peptide). The p values for the between group differences were 0.44 and 0.37 for eosinophils and neutrophils, respectively (data not shown).

Table 2. *The effect of peptide inhalation on lymphocyte, macrophage, eosinophil, neutrophil, basophil and mast cell counts in bronchial biopsies in responders and non-responders.*

Cellular Markers	Responders (R)			Non-Responders (NR)			R vs NR
	Diluent Median (range)	Peptide Median (range)	P value	Diluent Median (range)	Peptide Median (range)	P value	P value
CD3	64 (29.3-198.0)	96 (40.0-236.0)	0.05	86.7 (34.4-218)	55.5 (34.7-116)	0.03	0.005
CD4	36.6 (14.0-101.3)	58.9 (16.0-129.3)	0.03	53.4 (15.2-93.3)	31.6 (8.0-85.0)	0.09	0.006
CD8	34.2 (18.9-72.0)	38.7 (18.7-72.0)	0.85	47.2 (10.0-87.0)	38.2 (13.3-69.3)	0.23	0.54
CD25	38.7 (14.7-96.0)	46.7 (12.0-88.0)	0.38	41.3 (11.2-84.0)	40.7 (12.0-84.0)	1.03	0.6
CD68^{+} macrophages	28.1 (15.3-62.0)	28.1 (9.8-67.0)	0.85	35 (16.0-84.0)	34.6 (5.6-66.7)	0.68	0.58
MBP^{+} eosinophils	29 (18.4-61.3)	40.2 (18.4-160.0)	0.07	33.3 (14.0-52.0)	20.3 (12.8-81.6)	0.79	0.09
Elastase[+] neutrophils	44.4 (20.5-98.0)	60.7 (32.0-108.0)	0.11	47.3 (22.4-72.0)	37.7 (28.7-68.0)	0.38	0.05
BB1[+] basophils	53 (18.5-94.0)	45.1 (18.7-128.0)	0.73	39.3 (20.6-100.0)	59.8 (20.0-94.0)	0.2	0.31
Tryptase^{+} mast cells	45.8 (22.0-72.0)	49 (22.7-84.0)	0.73	41.6 (22.5-76.0)	41 (20.0-66.0)	0.62	0.75

The results are expressed as the number of positive cells (median and range) per mm^2 beneath the basement membrane. Statistical comparisons of diluent and peptides inhalation (in-group comparisons) were performed using two-tailed Wilcoxon signed-rank tests. Between-group comparisons of the change from diluent to peptide in responders and non-responders were performed using the non-parametric Mann-Whitney test.

Table 3. *The effect of peptide inhalation on the concentrations of mast cell- and basophil-associated pharmacological mediators in BAL from responders and non-responders.*

| | BAL | | | | | | |
| | Responders (R) | | | Non-Responders (NR) | | | R vs NR |
Mediators	Diluent Median (range)	Peptide Median (range)	P value	Diluent Median (range)	Peptide Median (range)	P value	P value
Histamine	4.8 (2.0-9.9)	7.9 (2.7-17.3)	0.02	3.8 (0.7-8.1)	5.5 (1.7-8.7)	0.18	0.24
Tryptase	0.6 (0-2.3)	0.6 (0-6.3)	0.76	0 (0-0.6)	0.2 (0-1.5)	0.08	0.98
Basogranulin	39.9 (0-995.0)	49.3 (2.7-160.1)	0.52	47.0 (8.2-194.4)	29.3 (3.2-117.0)	0.06	0.13
*C3a	5.4 (4.0-8.7)	5.9 (3.2-9.0)	0.76	8.8 (5.4-11.1)	7.5 (4.6-9.3)	0.31	0.21
LTC_4/D_4	5.5 (2.2-17.7)	8.2 (1.4-19.5)	0.73	3.2 (1.1-12.1)	3.7 (1.2-26.0)	0.09	0.51
LTE_4	6.0 (1.2-34.6)	6.7 (2.5-27.8)	0.38	7.0 (2.3-14.1)	5.8 (2.7-25.6)	0.79	0.62
PGD_2	0.95 (0-8.2)	2.7 (0-9.8)	0.92	0.9 (0-9.5)	2.9 (0-21.2)	0.09	0.16
$PGF_{2\alpha}$	2.1 (0.7-3.4)	1.6 (0.6-4.6)	0.73	1.4 (0.6-4.0)	1.4 (0.49-4.1)	0.97	0.93

Statistical comparisons of diluent and peptide inhalation (in group comparisons) were performed using two-tailed Wilcoxon signed tank tests. Between group comparisons of the change from diluent to peptide in responders and non-responders were performed using the non-parametric Mann-Whitney test.

All values are expressed as pg/ml of BAL fluid with the exception of histamine, C3a and tryptase (ng/ml) and basogranulin (mU/ml).

*C5a was not detected in any sample.

Isolated Late Asthmatic Reactions Are Associated with Elevated Expression of the Th2 Chemokine TARC

There was a significant increase in the concentration of TARC in BAL (diluent vs. peptide) in responders (p=0.004), but not non-responders (p=0.97) with a between group difference of this change of p=0.02. (Fig 4A). However as shown by the non-paired test the concentrations of TARC were significantly higher in non-responders compared to responders following diluent (p=0.03), but not peptide challenge (Fig 4A).

The results for TARC$^+$ immunoreactive cells in bronchial biopsies are shown in Fig 4B. Thus, there was a significant increase in TARC$^+$ cells (diluent vs. peptide) in responders (p=0.005), but not non-responders (p=0.68) with a between group difference of this change of p=0.003. However unlike BAL there was no significant difference in TARC$^+$ cells after diluent challenge but a significant increase after peptide (p=0.02) (Fig 4B).

TARC$^+$ cells included eosinophils, neutrophils and mononuclear cells. There was also increased positive TARC staining in epithelial cells in responders but this was not formally quantified. There was a positive correlation between the magnitude of the LAR (expressed as area under the curve) and the delta changes (diluent vs peptide) in the 12 responders for TARC$^+$ cells in biopsies (r=0.59, p< 0.05) . A representative photomicrograph of TARC$^+$ immunostaining in bronchial biopsies from responders and non-responders is shown in Fig 3.

Pharmacological Mediators, IL-13 and IL-10 Are Not Elevated in BAL

The concentrations of various mast cell and basophil-associated pharmacological mediators in BAL are shown in Table 3 . There were no significant differences (responders vs. non-responders, diluent vs. peptide) in histamine, tryptase, basogranulin, $LTC_4/LTD_4/LTE_4$ PGD_2, $PGF_{2\alpha}$, and fragments cleaved from C3 (C3a). C5a was not detectable in any sample.
There were also no significant increases(diluent vs peptide) in the concentrations of IL-13 or IL-10 in BAL from responders or non-responders (data not shown).

DISCUSSION

The important, novel, observation of this study is that, in asthmatic subjects, selective activation of allergen-specific T cells by inhalation of allergen-derived peptides is sufficient to induce increases in non-specific airway hyperresponsiveness (a cardinal feature of asthma) which, in turn, is accompanied by a predominantly T cell, bronchial mucosal inflammatory response. Thus, in responders but not non-responders, inhalation of peptides produced increased AHR (measured 7 days after peptide provocation) as well as a late asthmatic reaction (which peaks at 6 hours after challenge) as previously described (1,2,13).

The elevated numbers of CD3^{+}, CD4^{+} and TARC^{+} cells in biopsies supports the view that increased AHR is linked to T cell activation and are in keeping with previous animal studies. For example adoptive transfer experiments, in Brown Norway rats, have shown that AHR can be transferred by allergen-specific CD4^{+} T cells (8,9). Moreover in mice, using a combination of anti-T-cell monoclonal antibody, T-cell transfer and bone marrow transplantation, it was shown that T cells enhanced genetically-determined AHR (7). Also depletion of murine CD4^{+} T lymphocytes prevented antigen-induced airway hyperresponsiveness and pulmonary eosinophilia (18,19).

In a previous study (14) we examined bronchial biopsies and bronchoalveolar lavage fluid from responders and non-responders 6 hours after *intradermal* injection of allergen-derived peptides, i.e. when airway narrowing was maximal. Surprisingly we found no changes (diluent versus peptide challenge) in numbers of eosinophils, neutrophils, basophils, mast cells, T cells and T-cell subsets, macrophages, Th2 cytokines, histamine, histamine-releasing factors, or eicosanoids. However, as the challenge route was systemic, T-cell activation may have occurred in perivascular tissue distal to bronchoscopic sampling. In the present study peptides were delivered via the inhaled route, using a method previously reported (2) and therefore we anticipated that with direct airway challenge we were more likely to observe mucosal infiltration of inflammatory cells and changes in concentrations of pharmacological mediators. In fact, with the exception of increases in airway T cells, our present findings, using the inhaled route, were very similar to that observed with LARs provoked by intradermal challenge (14). For example no increases were observed in BAL fluid, between diluent and peptide, in either responders or non-responders, in the concentrations of histamine, tryptase, basogranulin, C3a, C5a or $LTC_4/D_4/E_4$, PGD_2, $PGF_{2\alpha}$. With the exception of basogranulin these mediators have all been shown to be elevated in BAL following whole allergen challenge.

Although there was a an increase in neutrophils and a trend for eosinophils there were no significant changes in the numbers of basophils or macrophages. This is in contrast to changes induced in the airways after challenge with whole allergen. For example in our previous study, in which we measured cells and mediators in the airways 6 hours after whole allergen challenge, there were increases in bronchial eosinophils, neutrophils, T cells, histamine and LTC_4 in dual, but not single early, responders (20). Others have also observed whole allergen-induced airway eosinophilia in mild asthmatics at the 6-hour time point (21,22). These studies, taken together, support the view that eosinophil infiltration is more likely to be secondary to mast cell rather than T cell activation and therefore might not occur following T cell peptide inhalation since there a lack of IgE cross linking on mast cells. Furthermore, the role of the eosinophil in allergen-induced late reactions has been cast in doubt since depletion of this cell with anti-IL-5 had no effect on either late phase asthmatic (23) or skin reactions (24). Nevertheless we cannot completely exclude the role of eosinophils and neutrophils in peptide-induced LARs especially as the study population was small. However taken together with our previous biopsy and lavage study our data would suggest they are not crucial cells in the pathogenesis of the peptide-induced late phase reaction.

An unexpected finding was that the concentrations of TARC were significantly higher in non-responders compared to responders following diluent challenge (Fig 4A), as shown by a non-paired test (p=0.03). In fact this was in large part due to two subjects who had a particularly high response to diluent challenge. The fact that the more meaningful paired, within group, and between group data, showed significance with BAL and that there was a very clear TARC immunoreactive positive cell response at the level of the bronchial mucosa (Fig 4B) suggests changes in this Th2 chemokine is central and relevant in our model of asthma.

A further novel finding was that at baseline, i.e. before challenge, the responders had significantly raised serum cat-specific IgE compared to non-responders. It was previously shown that dual responders i.e those who develop early and late asthmatic reactions and subsequent increased AHR after whole allergen challenge, tended to have raised allergen-specific IgE compared to single early responders (5). This observation may help to predict why some cat-sensitive individuals, and not others, develop a LAR after the Fel d 1 peptide challenge. Thus elevated allergen-specific serum IgE may reflect high IgE density on airway dendritic cells and/or the frequency of allergen-specific T cells in the airway mucosa. Further experiments will be required to test these hypotheses. It should be emphasised that the present study supports the view that increased AHR occurs after a LAR irrespective of whether this is induced by whole allergen (and therefore preceded by a mast cell-dependent early response) or is an isolated peptide-induced LAR as in the present study. In both situations, i.e. whole allergen- or peptide-challenge, the subjects who develop a LAR have elevated serum allergen-specific IgE. Thus T-cell activation rather than mast cell activation may be more crucial for the development of increased AHR as previously observed in animal studies (7-9).

In summary, the following scenario is proposed: Inhaled T-cell peptide epitopes bind to MHC class II molecules expressed in the airways leading to activation of peptide-specific resident effector memory T cells. Activation of these cells enhances local production of TARC from antigen-presenting cells, structural cells and accessory cells. Elevated TARC expression leads to recruitment and activation of $CD3^+/CD4^+$ T cells, production of inflammatory cytokines (although this has yet to be demonstrated) and, ultimately, increased AHR. Our inability to demonstrate cells $mRNA^+$ for Th2 cytokines in 6-hour biopsies may have been related to the small numbers of IL-4, IL-5 and IL-13 positive cells at this time point (as opposed to 24 hours where previously we have found elevations after whole allergen challenge (25)). The mechanism of the late-phase reaction itself remains uncertain but in a recent report (26) we showed markedly elevated expression of calcitonin gene-related peptide (CGRP) in epithelial cells, infiltrating CD3+/CD4+ cells and smooth muscle in responders (but not non-responders) after peptide challenge.Since CGRP is a potent vasodilator this raises the possibility that airway wall oedema may be an important component of peptide-induced late reactions. In any event the present data supports the view that T-cell activation alone is capable of triggering these events, observations which may be of importance to unravelling events in chronic severe asthma where the role of the T cell is now well established.

ACKNOWLEDGMENTS

Competing Interests

ABK and ML have shares in Circassia Holdings.

LEGENDS TO FIGURES

Figure 1

Late asthmatic reactions and airway hyperresponsiveness in peptide responders and non-responders. The changes in FEV_1 after peptide challenge (closed circles) in 12 responders (A) and 12 non-responders (B) is shown, as well as the effect of diluent challenge (open circles). Differences in the AUC between the control day and peptide day were analysed by paired t-test.

Changes in airway hyperresponsiveness in responders and non-responders 7 days after inhaled challenge are shown in C. D=Diluent inhalation and P= peptide inhalation. The bars represent the medians. Comparisons of AHR after diluent and peptides inhalation, (in-group comparisons) were performed using two-tailed Wilcoxon signed-rank tests. Between-group comparisons of the change from diluent to peptide in responders and non-responders were performed using the non-parametric Mann-Whitney test.

Figure 2

The effect of peptide inhalation on the numbers of CD3+ cells (A) and CD4+ cells (B) in bronchial biopsies from responders and non-responders. Statistical comparisons of diluent and peptides inhalation, (within-group comparisons) were performed using two-tailed Wilcoxon signed-rank tests. Between-group comparisons of the change from diluent to peptide in responders and non-responders were performed using the non-parametric Mann-Whitney test. The Mann-Whitney test was also used for unpaired data.

Figure 3

Photomicrographs of CD4+ cells and TARC+ cells in bronchial biopsies from responders and non-responders. An example of CD4+ cells immunostaining from responders after peptide challenge is shown in A and in a non-responder in B. A photomicrograph of TARC+ cells after peptide challenge in a responder is shown in C and in a non-responder in D. The arrow heads show examples of immuno-positive cells.

Figure 4

The effect of peptide inhalation on (A) the concentration of TARC in BAL and (B) the numbers of $TARC^+$ $protein^+$ cells in bronchial biopsies from responders and non-responders.

Statistics were performed as in Figure 2.

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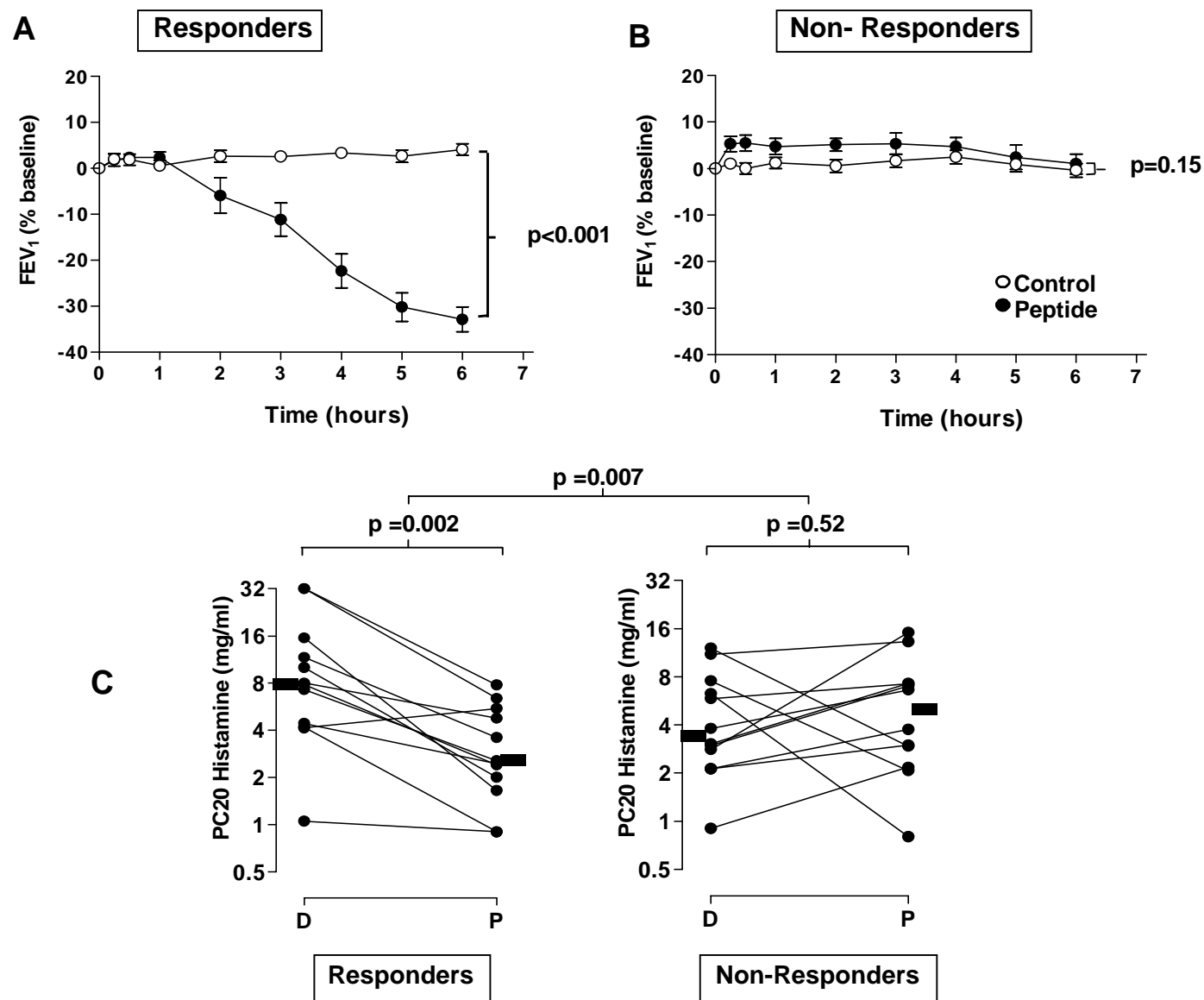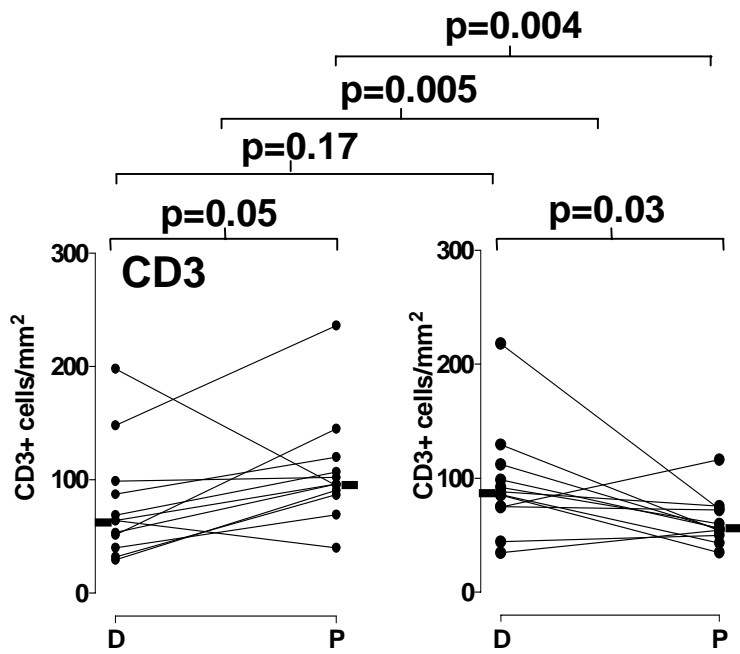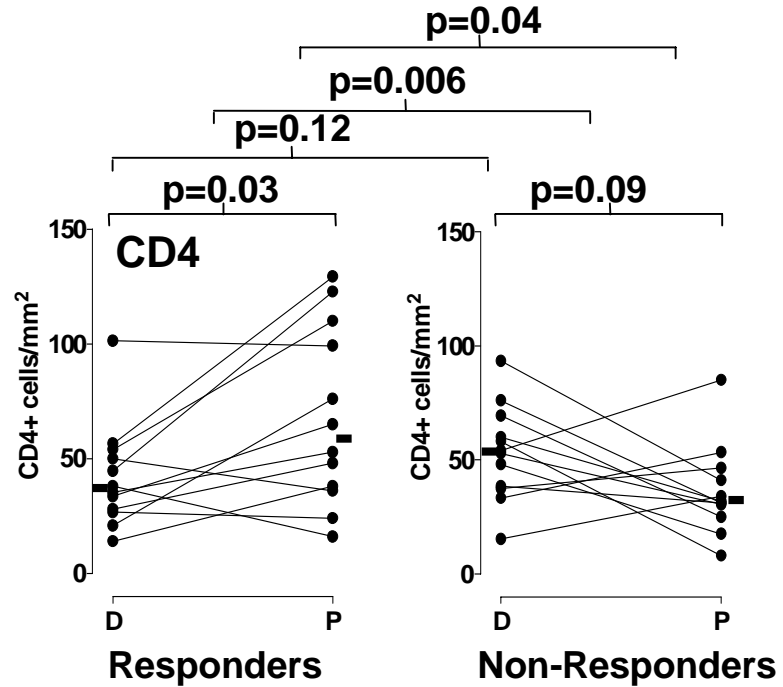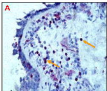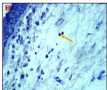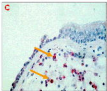
Fig. 1

Fig. 2

Fig. 3

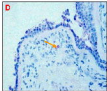| CD4 Responders | CD4 Non-Responders |
| TARC Responders | TARC Non-Responders |

Fig. 4

A

BAL

p=0.004

p=0.03

p=0.02

p=0.89

p=0.97

B

IHC/BB

p=0.005

p=0.20

p=0.003

p=0.02

p=0.68

Responders

Non-Responders