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Cigarette smoking increases bronchial mucosal IL-17A expression in asthmatics, which acts in concert with environmental aeroallergens to engender neutrophilic inflammation

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1 **Cigarette smoking increases bronchial mucosal IL-17A expression in asthmatics,**
2 **which acts in concert with environmental aeroallergens to engender neutrophilic**
3 **inflammation**

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15 Cigarette smoking, Asthma, Endobronchial biopsies, Interleukin-17A, Aeroallergen

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19

20 **Abstract:**

21 **Background**

22 Mild asthmatics who smoke cigarettes may develop unstable disease and neutrophilic
23 infiltration of the airways, features more usually associated with severe asthmatic
24 disease. The mechanisms giving rise to this response remain unclear.

25 **Objective**

26 To address the hypothesis that smoking increases bronchial mucosal production of
27 IL-17A which acts on bronchial epithelial cells directly and in concert with other
28 environmental stimuli to induce the production of IL-6 and neutrophil chemotaxins.

29 **Methods**

30 IL-17A, IL-8, IL-6, neutrophils and eosinophils was detected and quantified by
31 immunohistochemistry in endobronchial biopsy sections from smoking and non-
32 smoking asthmatics. Human tracheal epithelial cells (HTEpC) were cultured with IL-
33 17A in the presence/absence of cigarette smoke extract (CSE) and aeroallergens
34 lacking intrinsic protease activity, and IL-6 and IL-8 production measured *in vitro*.

35 **Results**

36 Expression of IL-17A, IL-6 and IL-8 and neutrophil numbers were significantly
37 elevated in the bronchial mucosa of the asthmatic smokers compared to the non-
38 smokers. Expression of IL-17A correlated with that of IL-8 and neutrophil numbers.
39 In the smoking asthmatics, eosinophil numbers also correlated with expression of IL-
40 8 and IL-17A. Exposure of HTEpC cells to both CSE and IL-17A increased
41 expression of IL-6 and IL-8 in a concentration-dependent and synergistic manner.

42 Co-stimulation with CSE, IL-17A and aeroallergens further increased IL-6 and IL-8
43 production synergistically.

44 **Conclusions**

45 The data support the hypothesis that asthmatic smokers develop neutrophilic
46 inflammation of the airways propagated at least partly by smoke-induced production
47 of IL-17A which together with smoke and other environmental stimuli acts on
48 airways epithelial cells to induce neutrophil chemotaxins.

49

50 **Abbreviations:**

51	COPD	Chronic Obstructive Pulmonary Disease
52	CSE	Cigarette Smoke Extract
53	ELISA	Enzyme-linked immunosorbent assay
54	FEV1	Forced expiratory volume in 1 second
55	HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
56	HTEpC	Human tracheal epithelial cells
57	IHC	Immunohistochemistry
58	IL	Interleukin
59	IQR	Interquartile range
60	MBP	Major Basic Protein
61	NHS	National Health Service
62	OVA	Ovalbumin
63	PBS	Phosphate-buffered saline
64	PC ₂₀	Provocation concentration producing a 20% fall in forced expiratory
65		volume in one second
66	REC	Research Ethics Committee
67	ROS	Reactive oxygen species
68	SEM	Standard error of the mean
69	Th	T helper
70	TLR4	Toll-like receptor 4

71

72 INTRODUCTION

73 Unfortunately, many asthmatics smoke cigarettes [1]. There is abundant evidence that
74 smoking destabilises asthma [2, 3] and confers resistance to therapy [4, 5]. Smoking
75 in asthma is accompanied by elevated inflammatory cellular infiltration of the
76 airways, with a prominence of neutrophils [6, 7], a scenario associated with severe,
77 difficult to control disease in non-smoking patients [8].

78 Respiratory epithelial cells constitute the common environmental interface for
79 cigarette smoke, pathogens and allergens in the respiratory tract and are able to
80 respond to the latter through pattern recognition receptors independently of the IgE
81 responses which characterise atopy [9]. There is nevertheless a paucity of research on
82 the possible interactions between cigarette smoke and the airways mucosal epithelium
83 which may drive neutrophilic inflammation in mild asthma. One notable possible link
84 in this mechanistic chain is IL-17A, which has been described to stimulate various
85 airways structural cells to produce CXC chemokines [10-12] which are produced in
86 excess in the airways of patients with “neutrophilic” asthma [13]. Interestingly, IL-
87 17A production both in severe, non-smoking asthmatics [14] and in milder asthmatics
88 who smoke [15] appears to be therapy resistant. Preliminary observations [16, 17]
89 indicate that exposure of bronchial epithelial cells both to cigarette smoke and to IL-
90 17A increases their spontaneous release of CXCL8 (a key chemotactic factor for
91 neutrophils as well as “primed” eosinophils [18]) and IL-6, an “acute phase” cytokine
92 also associated with sputum neutrophilia in asthmatics [13, 19].

93 Consequently we constructed the present study to address the following hypotheses:

94 (1) Cigarette smoking stimulates the production of IL-17A, neutrophil chemotaxins

95 and granulocytic infiltration within the bronchial mucosa of mild, corticosteroid naïve
 96 asthmatics; (2) Cigarette smoke extract and IL-17A enhance the production of pro-
 97 neutrophilic cytokines by human airways epithelial cells both independently and
 98 synergistically; (3) This effect is further potentiated by non-antigen-specific (innate)
 99 interactions with environmental allergens. In order to address these hypotheses we
 100 quantified immunoreactive IL-17A⁺, IL-6⁺ and IL-8⁺ cells as well as elastase⁺
 101 neutrophils and MBP⁺ (major basic protein) eosinophils in bronchial mucosal sections
 102 from corticosteroid naïve smoking and non-smoking asthmatics. We also examined
 103 the effects of IL-17A, alone and in combination with cigarette smoke extract (CSE)
 104 and environmental aeroallergens on the production of IL-6 and IL-8 by human
 105 airways epithelial cells *ex vivo*.
 106

107 **SUBJECTS, MATERIALS AND METHODS**

108 **Subjects and fiberoptic bronchoscopy**

109 In view of the school of thought that corticosteroid therapy itself may be at least
110 partly responsible for neutrophilic infiltration of the airways in asthma [20], we
111 elected to perform a cross sectional comparison of mild, corticosteroid naïve
112 asthmatic smokers and non-smokers (London Bridge Research Ethics Committee,
113 REC approval reference 06/Q0704/175; Guy's and St Thomas' NHS Foundation
114 Trust Research and Development Department, R&D approval number RJ1 07/0069).
115 The following definitions were used when characterising participants: (i) asthma: a
116 history of typical symptoms for ≥ 6 months prior to screening and histamine PC₂₀ ≤ 8
117 mg/ml; (ii) non-smoker: no smoking within 12 months of screening and < 0.5 pack
118 year total history; (iii) smoker: currently smoking ≥ 5 cigarettes/week. The study was
119 explorative and not formally powered because at the time inception of the study there
120 were no suitable preliminary studies available to enable this.

121 Informed consent was obtained from all subjects who then underwent a full
122 assessment of their medical history, physical examination, St. George's Respiratory
123 Questionnaire, spirometry and measurement of histamine PC₂₀. Fiberoptic
124 bronchoscopy was performed in accordance with the British Thoracic Society
125 guidelines [21]. Up to 12 endobronchial biopsies were obtained from the subcarinae
126 of 3rd to 7th generation bronchi.

127 **Immunohistochemistry (IHC)**

128 Cryostat sections (6 μ m) were cut from biopsies prefixed in 4% paraformaldehyde
129 (BDH Chemicals Ltd, Dagenham, UK), mounted on 0.1% poly-L-lysine-coated
130 slides, dried overnight at 37°C then stored with silica gel (BDH Chemicals) at -80°C
131 until used. Single IHC was performed as previously described [22] using the alkaline
132 phosphatase anti-alkaline phosphatase (APAAP) technique with monoclonal
133 antibodies against neutrophil elastase (NE, 1:100, Clone: NP57, Dako, Ely, UK) and
134 eosinophil major basic protein (MBP, 1:30, Clone: BMK13, Abcam, Cambridge,
135 UK), and polyclonal antibodies against IL-6 (1:500, Abcam), IL-8 (1:500, R&D
136 Systems, Minneapolis, MN) and IL-17A (1:200, eBioscience, Hatfield, UK). Sections
137 were then counterstained with Mayer's haematoxylin solution (Sigma-Aldrich).
138 Omission or substitution of the primary antibody with an irrelevant species- and
139 isotype-matched immunoglobulin was used as a negative control. Stained cells in
140 coded sections were counted by a single observer ignorant of their provenance in the
141 entire area of the biopsy specimens with an Olympus BX40 microscope coupled with
142 a Zeiss Vision KS300 imaging system (Carl Zeiss, Hamburg, Germany), which
143 measured these areas and uniformised background pixilation automatically.
144 Endobronchial biopsies from asthmatics have a tendency to lose some or all of their
145 epithelium during the biopsy process and/or subsequent processing. In the present
146 study, 6% of biopsies had a fully intact epithelium, 53% had a partially intact
147 epithelium and 41% had no epithelium present. There was no statistical difference in
148 the frequencies of these three outcomes in the biopsies from the non-smoking and
149 smoking asthmatics ($p = 0.4926$). In view of this we elected to measure cell counts in
150 the entire biopsy sections, which were expressed as the numbers of immunoreactive
151 cells per unit area of the sections.

152 **Cigarette smoke extract (CSE) preparation**

153 Cigarette smoke extract (CSE) was prepared based on a modification of the method of
 154 Carp and Janoff [23]. Two full-strength Marlboro cigarettes (filters removed;
 155 Marlboro Red, Class A cigarette, Tar 10 mg, Nicotine 0.8 mg; Phillip Morris USA,
 156 Richmond, VA) were combusted and the smoke bubbled through 50 ml of culture
 157 medium. To accomplish this, a 50 ml syringe (Becton Dickinson, Oxford, UK) was
 158 connected to a 3-way stopcock (Becton Dickinson). One of the other 2 arms of the
 159 stopcock was connected to a cigarette in a holder, and the final arm was connected to
 160 a glass Pasteur pipette (John Poulten, Barking, UK) submerged in a beaker containing
 161 culture medium. In a fume cupboard, each cigarette was lit and cigarette smoke drawn
 162 into the 50 ml syringe (to the 60 ml mark) over 10 seconds; following this the
 163 cigarette smoke was immediately bubbled into the culture medium over 2 to 3
 164 seconds. According to the protocol of Carp and Janoff [23], this solution was defined
 165 as “100% strength”. The resulting medium was then sterilised by passing through a
 166 0.20 µm filter (Sartorius, Epsom, UK), diluted to the required strength and used
 167 within 1 hour of preparation [23].

168 **Human Airways Epithelial Cell Cultures**

169 Human tracheal epithelial cells from mixed healthy donors (HTEpC, PromoCell,
 170 Heidelberg, Germany) were cultured in accordance with the supplier’s instructions in
 171 bovine type I collagen coated 12 well tissue culture plates in Airway Epithelial Cell
 172 Medium which contained bovine pituitary extract 0.004 ml/ml, epidermal growth
 173 factor 10 ng/ml, insulin 5 µg/ml, triiodo-L-thyronine 6.7 ng/ml, holo-transferrin 10
 174 µg/ml, hydrocortisone 0.5 µg/ml, epinephrine 0.5 µg/ml and retinoic acid 0.1 ng/ml

(PromoCell) and was supplemented with 1% antibiotic-antimycotic (Gibco, Paisley, UK). 24 hours prior to stimulation the culture medium was changed to a “starvation” medium (Airway Epithelial Cell Medium without hydrocortisone, epinephrine and retinoic acid supplemented with 1% antibiotic-antimycotic and 25 mM HEPES (Sigma-Aldrich)). The cells were then exposed for 24 hours to 0.1, 1.0 and 10 ng/ml of recombinant human IL-17A (PeproTech, London, UK), 0.16% CSE, 5000 SQ-U/ml *Felis domesticus* Aquagen SQ (Alk-Abello, Horsholm, Denmark) and 5000 SQ-U/ml *Phleum pratense* Aquagen SQ (Alk-Abello), then supernatants collected for analysis [24, 25]. The concentration (0.16%) of CSE employed for these experiments was determined from prior concentration/response and time course studies to stimulate maximal cytokine production in HTEpC (data not shown). After removal of culture supernatant, cellular viability was assessed under light microscopy by trypan blue exclusion. The viability of HTEpC cells when exposed to CSE under these experimental conditions remained in excess of 90% throughout the culture period.

ELISA

Concentrations of IL-6, IL-8, IL-17A and IL-17F were measured in culture supernatants using commercial ELISA (PeproTech, London, UK) in accordance with the manufacturer’s instructions.

Statistical Analysis

Statistical analysis was performed using software embedded in Prism 5 for Mac OS X Version 5.0c (GraphPad Software Inc, La Jolla, CA). Data were summarised as the mean and standard error, or the median and interquartile range (IQR) as appropriate.

Data were compared following testing for deviance from a Gaussian distribution and for equality of variance using parametric analysis (parametric one-way analysis of variance, paired student's t-test, linear regression, Pearson's correlation) or non-parametric analysis (Mann-Whitney U test, Kruskal–Wallis one-way analysis of variance) as appropriate. Since the variances of the data from the asthmatic groups were not homogeneous, they were compared using non-parametric statistical analysis. Parametric statistical analysis was employed for all other comparisons since the data obtained satisfied the relevant statistical assumptions. In the IL-17A and CSE interaction experiments, synergistic interaction was defined as significant variation (using one-way analysis of variance) in the magnitude of CSE-induced cytokine expression in excess of spontaneous secretion at the concentrations of IL-17A tested. In the allergen, IL-17A and CSE interaction experiments, synergistic interaction was defined as a significant, further allergen-induced increase in the effects of IL-17A and CSE, either alone or in combination. One-way analysis of variance (ANOVA) was performed on the magnitude of the IL-17A concentration series-induced increases on cytokine secretion from baseline in the additional presence of CSE. Statistical significance was taken as $p < 0.05$. P values are quoted to two significant figures. For Pearson's correlations, r is the Pearson's correlation coefficient while r^2 is the coefficient of determination.

217 **RESULTS**

218 **Subject characteristics**

219 Endobronchial biopsies were obtained from 10 asthmatic non-smokers and 8
220 asthmatic smokers (Table 1). All asthmatic smokers were current smokers with a
221 median (IQR) pack year history of 4.7 (0.9 – 18.0) pack years, currently smoking 14.0
222 (6.3 – 20.0) cigarettes per day. Of those classified as asthmatic non-smokers, 2
223 subjects were ex-smokers. The ex-smokers had stopped smoking 3 and 14 years prior
224 to the study, with pack year histories of 0.125 and 0.150 pack years. Asthma severity
225 in the two asthmatic groups was equivalent, as defined by % predicted FEV₁ (p =
226 0.9355), FEV₁/FVC ratio (p = 0.6856), PC₂₀ histamine (p = 0.4543) and
227 bronchodilator reversibility (p = 0.1609). All asthmatic subjects were using only
228 intermittent short acting β 2-agonist therapy for management of their asthma.

229 **Mucosal expression of pro-inflammatory cytokines (Figures 1 and 2)**

230 The median numbers of bronchial submucosal cells expressing immunoreactive IL-6,
231 IL-8 and IL-17A were significantly elevated in the asthmatic smokers compared to
232 the asthmatic non-smokers (p = 0.03, p = 0.0008, p = 0.04 respectively).

233 **Mucosal infiltration with neutrophils and eosinophils (Figures 1 and 2)**

234 The median number of neutrophils in the submucosa of the airways of the asthmatic
235 smokers was significantly elevated compared with the asthmatic non-smokers (p =

0.019). In contrast there was only a trend for an increase in the median numbers of eosinophils in the mucosal sections from the asthmatic smokers compared to asthmatic non-smokers ($p = 0.07$).

Correlations between pro-inflammatory cytokine expression and cellular inflammatory profile (Table 2)

Neutrophil numbers correlated strongly with IL-17A⁺ cell numbers in the bronchial mucosa of both asthmatic non-smokers and smokers ($r = 0.857$, $p = 0.002$ and $r = 0.907$, $p = 0.002$ respectively). The linear regression model accounted for 74% (non smoking asthmatics) and 82% (smoking asthmatics) of the variance in the number of neutrophils attributed to the number of IL-17A⁺ cells. In addition, IL-17A⁺ cell numbers correlated with IL-8⁺ cell numbers in both asthmatic non-smokers and smokers ($r = 0.636$, $p = 0.048$ and $r = 0.850$, $p = 0.008$ respectively). The numbers of IL-8⁺ cells correlated with the numbers of neutrophils in the asthmatic non-smokers but not the asthmatic smokers ($r = 0.735$, $p = 0.02$ and $r = 0.678$, $p = 0.06$ respectively).

There were in addition strong correlations between the numbers of eosinophils and the numbers of IL-8⁺ and IL-17A⁺ cells in the asthmatic smokers ($r = 0.800$, $p = 0.02$ and $r = 0.863$, $p = 0.006$ respectively), with the linear regression model accounting for 64% and 74% respectively of the variance in the data. These correlations were not evident in the asthmatic non-smokers.

Cigarette smoke extract does not induce ex-vivo expression of IL-17A and IL-17F by respiratory epithelial cells (Figure 3)

258 The spontaneous expression of both IL-17A and IL-17F by HTEpC was low (3 to
259 5pg/ml) and not altered in the presence of a concentration series of CSE .

260 **Cigarette smoke extract synergistically increases IL-17A-induced ex-vivo**
261 **expression of IL-6 and IL-8 by human airways epithelial cells (Figure 4)**

262 IL-17A alone effected a concentration-dependent increase in spontaneous release of
263 IL-6 and IL-8 by HTEpC (ANOVA $p < 0.0001$ and $p < 0.0001$ respectively). The
264 additional presence of CSE further augmented both IL-6 and IL-8 production by these
265 cells in a concentration related, synergistic manner (ANOVA $p < 0.0001$ and $p =$
266 0.008 respectively).

267 **Aeroallergens increase the expression of IL-6 and IL-8 induced by co-stimulation**
268 **of epithelial cells with cigarette smoke extract and interleukin-17A (Figure 5)**

269 Spontaneous secretion of IL-6 by cultured airways epithelial cells *ex vivo* was
270 significantly elevated in the presence of 0.16% CSE, 10 ng/ml IL-17A and
271 commercially available cat dander and Timothy grass pollen aeroallergen extracts (p
272 $= 0.009, 0.01, 0.006$ and 0.004 respectively). The combination of CSE and IL-17A
273 increased IL-6 production to a significantly greater degree than either agent alone.
274 While neither aeroallergen significantly altered the effects of CSE or IL-17A alone
275 (cat $p = 0.06, 0.17$; grass $p = 0.27, 0.98$ respectively), both further significantly
276 increased IL-6 production by the combination ($p = 0.0004, 0.008$ respectively).
277 Similarly, spontaneous secretion of IL-8 by the epithelial cells was significantly
278 elevated in the presence of 0.16% CSE, 10 ng/ml IL-17A and the cat dander and
279 Timothy grass pollen extracts ($p = < 0.0001, 0.0002, 0.01$ and 0.01 respectively).

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280 Again, the combination of CSE and IL-17A increased IL-8 production to a
281 significantly greater degree than either agent alone. Again, while neither aeroallergen
282 significantly altered the effects of CSE or IL-17A alone (cat $p = 0.9648, 0.2791$; grass
283 $p = 0.1573, 0.1861$ respectively), both further significantly increased IL-8 production
284 by the combination ($p = 0.03, 0.01$ respectively).
285

286 **DISCUSSION**

287 Here we report a number of novel observations pertaining to understanding the impact
288 of cigarette smoking and other environmental influences on asthma. Our data are
289 clearly compatible with the hypothesis that exposure of asthmatic patients to
290 environmental cigarette smoke increases *de novo* airways mucosal expression of IL-
291 17A, which in turn acts on structural cells of the mucosa, in this case epithelial cells,
292 to induce the release of granulocyte chemoattractants with resultant influx of
293 neutrophils. Furthermore, we show that cigarette smoke may interact with
294 environmental allergens in a non-antigen-specific (innate) manner to enhance this
295 effect.

296 Our data are consistent with those of a previous study [26] in which the authors
297 reported elevated IL-17A expression in the bronchial mucosa of mild/moderate
298 asthmatics to a degree which correlated with the numbers of neutrophils in induced
299 sputum, although surprisingly not in the bronchial mucosa. The findings of our *in*
300 *vitro* experiments are also consistent with a previous study [24] showing that IL-17A
301 induced expression of IL-6 and CXCL8 in primary human airways epithelial cells.
302 We have here extended these findings by demonstrating that CSE is able to augment
303 the expression of both IL-6 and CXCL8 induced by IL-17A in a synergistic manner,
304 implying that cigarette smoke exposure supports and augments IL-17A mediated
305 inflammation of the airways. In a similar vein, Wiehler and Proud [27] have shown
306 synergy between IL-17A and human rhinoviral infection of human airways epithelial
307 cells in inducing the production of CXCL8. Our new data raise the intriguing
308 possibility that inhaled aeroallergens may also interact with cigarette smoke at the
309 airways epithelial barrier in a manner almost certainly independent of acquired

immunity and IgE production, further to augment the expression of IL-6 and CXCL8 in the presence of IL-17A and CSE. We believe that this is the first time that synergistic interaction between IL-17A, cigarette smoke and allergen exposure to induce the expression of pro-inflammatory cytokines has been demonstrated in human airways epithelial cells. It is possible to speculate that differing combinations of environmental influences in different patients conspire to destabilise asthma control through such mechanisms.

While in our study both neutrophilia and CXCL8 expression correlated with IL-17A expression in the bronchial mucosa of both the smoking and the non-smoking asthmatic patients, supporting the hypothesis that IL-17A drives CXCL8 expression in both smokers and non-smokers *in vivo*, CXCL8 expression correlated with neutrophilia only in the non-smoking asthmatic patients, suggesting that in smoking asthmatics IL-17A may induce additional granulocyte chemoattractants. These may include eosinophil, as well as neutrophil chemoattractants given that local IL-17A expression also correlated with eosinophil infiltration in the asthmatic smokers.

Possible cellular sources of these chemoattractants other than bronchial epithelial cells include endothelial cells, fibroblasts and smooth muscle cells [10-12]. We did not attempt to identify the IL-17A immunoreactive cells in the asthmatic bronchial biopsies: although contemporary studies implicate Th17 T cells as a likely source, it should be noted that other leukocytes, including $\gamma\delta$ and invariant NK T cells, innate lymphoid cells, mast cells and neutrophils are possible alternative sources.

There is considerable circumstantial evidence that IL-17A expression and neutrophilic infiltration of the airways are implicated in the pathogenesis of “difficult to control” asthma, either spontaneous [8, 14] or as a result of smoking [15]. This evidence will

remain circumstantial until mechanisms are uncovered whereby products of granulocytes regulate bronchial hyperresponsiveness, the core pathophysiological abnormality in asthma governing disease severity and stability. Chronic obstructive pulmonary disease (COPD), characterised by severe, irreversible airways obstruction and caused predominantly by cigarette smoking, is also characterised by neutrophil infiltration and elevated IL-17A expression in the airways [26, 28]. In addition to any direct effects that neutrophil products may exert on bronchial hyperresponsiveness in asthma, animal surrogates of allergic inflammation also suggest that neutrophils may play a key role in the development of Th2 type inflammation in atopic dermatitis [29] and asthma [30].

Our study has limitations. Since data on the effects of smoking on asthmatic airways are so limited, we were obliged to make some arbitrary decisions when choosing and classifying the subjects. We arbitrarily defined a smoker as someone who currently smokes ≥ 5 cigarettes per week and a non-smoker as someone not having smoked for at least 12 months prior to screening and with a < 0.5 pack year history. We deliberately avoided setting a minimum pack year smoking history as an exposure criterion for inclusion in the smoking group as this might have admitted chronic, heavy smokers vulnerable to COPD. Similarly, because of the ongoing debate about the possible influence of corticosteroid anti-asthma medication on the numbers of neutrophils in the airways [20] we elected to study mild, corticosteroid naive asthmatics. Clearly, in similar studies, smoking history, disease severity and concomitant corticosteroid therapy of asthmatics will likely modify outcomes [31, 32]. Another intrinsic limitation of the present study is the lack of a non-asthmatic, smoking control group, without which it is impossible to determine whether or not the

358 smoking-induced changes we observed in the bronchial mucosa of our asthmatic
359 patients were attributable to smoking *per se* or a possible interaction between
360 smoking and asthma. Previous studies on smokers with or without COPD have,
361 however, clearly demonstrated that smoking *per se* fails to induce IL-17A expression
362 in the bronchial mucosa of non-diseased control subjects [26, 28, 33] and that this
363 finding is also reflected in peripheral blood T cells, strongly suggesting that these
364 effects of smoking do indeed reflect an interaction between smoking and asthma [34].
365 Similarly, the properties of airways epithelial cells may, at least in theory, differ in
366 asthmatics and non-asthmatics, and smoking and non-smoking donors. When the
367 present study was performed, human tracheal, but not bronchial epithelial cells were
368 obtainable commercially (from pooled healthy donors), so we used these as a
369 surrogate. While similarities in the properties of these cells cannot be assumed with
370 certainty, we are aware of no published evidence to the contrary. Finally, we have not
371 addressed the mechanisms by which CSE, IL-17A and allergens influence the
372 function of airways epithelial cells. Montalbano and colleagues have recently
373 demonstrated [35] that both CSE and IL-17A independently increase the production
374 of reactive oxygen species (ROS) by bronchial epithelial cells. In a similar vein,
375 exposure of human bronchial epithelial cells to a variety of pollen aeroallergens has
376 also been shown to increase the production of ROS [25]. We therefore speculate that
377 one mechanism underlying these interacting effects on airways epithelial cells
378 involves the generation of ROS.

379 In summary, our data suggest that IL-17A is at least one key mediator driving the
380 neutrophilic inflammation seen in asthmatic smokers, and that structural cells of the
381 airways likely play an important role in its pathogenesis. Our data also highlight the

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382 functional significance of the airways epithelium in integrating a variety of
383 environmental influences which may cause such inflammation and influence asthma
384 control.

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401 **CONTRIBUTORSHIP STATEMENT**

402 Dr Leonard Quok Chean Siew had obtained the endobronchial biopsies, performed all
403 the in-vitro experiments and prepared the manuscript. Ms Shih-Ying Wu performed
404 all the immunohistochemistry experiments and reviewed the manuscript. Dr Sun Ying
405 and Professor Christopher John Corrigan was involved in the planning of the study
406 and the in-vitro experiments, as well as the review and editing of the manuscript.

407 **CONFLICTS OF INTEREST**

408 The authors wish to confirm that there are no known conflicts of interest associated
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411

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552

553 **FIGURE LEGENDS**

554 **Figure 1: Endobronchial Biopsy immunohistochemistry fast red staining**

555 Endobronchial biopsy sections were stained for IL-6, IL-8, IL-17A, NE and BMK
556 using monoclonal/polyclonal antibodies and fast red staining. Immunoreactive cells
557 are stained red.

558 **Figure 2: Numbers of IL-6, IL-8 and IL-17A immunoreactive cells and elastase+**
559 **neutrophils and MBP+ eosinophils in sections of bronchial biopsies**

560 ● = Asthma Non-smoker; ○ = Asthma Ex-smoker; ■ = Asthma Smoker

561 AN = Asthma Non-smoker and AS = Asthma Smoker.

562 * $p < 0.05$, *** $p < 0.001$, n.s. = not significant.

563 **Figure 3: Cigarette smoke extract does not induce ex-vivo expression of IL-17A**
564 **and IL-17F by respiratory epithelial cells**

565 HTEpC were cultured with a concentration series of CSE. Spontaneous release of IL-
566 17A (a) and IL-17F (b) was low and not altered in the presence of CSE following 24
567 hours (open bars) and 72 hours (filled bars) of culture. Mean, $n = 2$.

568 **Figure 4: Co-stimulation of HTEpC human respiratory epithelial cells with IL-**
569 **17A and cigarette smoke extract**

570 HTEpC were cultured with a concentration series of IL-17A in the presence/absence
 571 of CSE. a) and c) CSE synergistically increased IL-17A-induced IL-6 and IL-8
 572 secretion (ANOVA $p < 0.0001$ and $p = 0.0079$ respectively). b) and d)
 573 Concentration-dependent effect of IL-17A on IL-6 and IL-8 secretion in the presence
 574 of CSE (ANOVA $p < 0.0001$ and $p = 0.0079$ respectively). Dotted lines depict the
 575 mean increases in baseline expression of IL-6 and IL-8 following stimulation with
 576 CSE alone. Paired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean $\pm$ SD, $n = 5 -$
 577 8.

578 **Figure 5: Effect of cat dander and timothy grass pollen on co-stimulation of**
 579 **HTEpC with IL-17A and cigarette smoke extract**

580 Data are presented as the mean $\pm$ SD of 4 experiments. Paired t-test. * $p < 0.05$, ** p
 581 < 0.01 , *** $p < 0.001$, **** $p < 0.0001$.
 582

583 **Table 1: Characteristics of the populations studied**

Characteristics	Asthma Non smoker	Asthma Smoker
No.	10	8
Age, yr	27 (24 – 32)	22 (18 – 36)
Height, m	1.77 (1.64 – 1.84)	1.79 (1.60 – 1.92)
Weight, kg	72.6 (66.5 – 81.8)	87.3 (68.9 – 101.5)
Male/Female	6/4	6/2
Never smoker / ex-smoker / current smoker	8/2/0	0/0/8
Pack-years *	0.125, 0.150	4.7 (0.9 – 18.0)
Atopy, No. (%)	10/10 (100)	8/8 (100)
PC ₂₀ histamine, mg/ml	0.809 (0.440 – 2.01)	1.021 (0.744 – 2.89)
FEV ₁ , % predicted	93.7 (80.7 – 107.9)	91.4 (78.3 – 112.5)
Pre-bronchodilator FEV ₁ /FVC, %	77.6 (65.9 – 83.1)	79.3 (69.9 – 84.3)
Bronchodilator response, %	7.5 (3.4 – 14.9)	9.5 (9.0 – 18.9)
St. George's Respiratory Questionnaire Score	13.48 (4.47 – 19.54)	15.59 (9.29 – 21.03)

584 † $p < 0.05$ compared with asthma non smoker. Mann-Whitney test, Median and IQR.

CIGARETTE SMOKING IN ASTHMATICS INCREASES IL-17A EXPRESSION

585 ** Two subjects in the asthma non-smoker groups were ex-smokers; pack year*
586 *histories for these subjects are presented individually.*

587 **Table 2: Correlations between pro-inflammatory cytokine expression and**
 588 **cellular inflammatory profile in sections of bronchial biopsies from asthma non-**
 589 **smokers and asthma smokers**

Correlation	Asthma non-smoker			Asthma smoker		
	NE+	MBP+	IL-17A+	NE+	MBP+	IL-17A+
IL-8+	0.735†	0.564	0.636†	0.678	0.800†	0.850§
	(0.541)	(0.318)	(0.405)	(0.460)	(0.639)	(0.722)
IL-17A+	0.857§	0.373	...	0.907§	0.863§	...
	(0.735)	(0.139)		(0.822)	(0.744)	

590 *Pearson's r values, r^2 values in parenthesis, significant correlations in bold type. † p*
 591 *< 0.05; § p < 0.01.*

Figure 1: Endobronchial Biopsy immunohistochemistry fast red staining
Endobronchial biopsy sections were stained for IL-6, IL-8, IL-17A, NE and BMK using monoclonal/polyclonal antibodies and fast red staining. Immunoreactive cells are stained red.

Figure 1
297x420mm (300 x 300 DPI)

Figure 2: Numbers of IL-6, IL-8 and IL-17A immunoreactive cells and elastase+ neutrophils and MBP+ eosinophils in sections of bronchial biopsies

● = Asthma Non-smoker; ○ = Asthma Ex-smoker; ■ = Asthma Smoker
AN = Asthma Non-smoker and AS = Asthma Smoker.
* p < 0.05, *** p < 0.001, n.s. = not significant.

Figure 2
170x118mm (300 x 300 DPI)

Figure 3: Cigarette smoke extract does not induce ex-vivo expression of IL-17A and IL-17F by respiratory epithelial cells

HTEpC were cultured with a concentration series of CSE. Spontaneous release of IL-17A (a) and IL-17F (b) was low and not altered in the presence of CSE following 24 hours (open bars) and 72 hours (filled bars) of culture. Mean, $n = 2$.

Figure 3

240x350mm (300 x 300 DPI)

Figure 4: Co-stimulation of HTEpC human bronchial epithelial cells with IL-17A and cigarette smoke extract

HTEpC were cultured with a concentration series of IL-17A in the presence/absence of CSE. a) and c) CSE synergistically increased IL-17A-induced IL-6 and IL-8 secretion (ANOVA $p < 0.0001$ and $p = 0.0079$ respectively). b) and d) Concentration-dependent effect of IL-17A on IL-6 and IL-8 secretion in the presence of CSE (ANOVA $p < 0.0001$ and $p = 0.0079$ respectively). Dotted lines depict the mean increases in baseline expression of IL-6 and IL-8 following stimulation with CSE alone. Paired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mean $\pm$ SD, $n = 5 - 8$.

Figure 4
167x146mm (300 x 300 DPI)

Figure 5: Effect of cat dander and timothy grass pollen on co-stimulation of HTEpC with IL-17A and cigarette smoke extract

Data are presented as the mean $\pm$ SD of 4 experiments. Paired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 5
161x118mm (300 x 300 DPI)