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Severe Asthma: differential chemokine response of airway epithelial cells

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1 **TITLE PAGE**2 **Letter to the Editor**3 **Severe Asthma: differential chemokine response of airway epithelial cells**

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Keywords

Asthma, allergy, cytokine, chemokine, *Streptococcus pneumoniae*, *Dermatophagoides pteronyssinus*, Der p 1

Capsule summary

The differential chemokine response of airway basal cells of severe asthma patients to *Streptococcus pneumoniae* and *Dermatophagoides pteronyssinus* allergen may be of significance in the context of developing novel immunomodulatory therapeutic strategies for atopic asthma.

To the Editor:

Approximately 10-15% of asthmatic adults belong to a group with severe refractory asthma and suffer from debilitating chronic symptoms, despite optimal standard asthma treatment.¹ Unraveling the complex pathophysiology of severe asthma has proven to be a major research challenge.¹ There is growing interest in the role of airway epithelium and its interactions with inhaled aeroallergens and pathogens, in the pathogenesis of severe asthma.

In a study of patients with severe asthma and healthy controls, we have recently shown that profound ciliary dysfunction and marked ultrastructural abnormalities of the airway epithelium are features of severe asthma.² One potential consequence of these abnormalities is prolonged and more intense exposure of the airway epithelium to inhaled aeroallergens and pathogens. Moreover, given the marked epithelial disintegrity seen in patients with severe asthma and the ability of the proteolytically active substances such as the *Dermatophagoides pteronyssinus* allergens to cause disruption of the intercellular tight junctions, resulting in increased transepithelial permeability,³ the airway basal cells could also be exposed to inhaled allergens and pathogens. In this regard, we studied the effect (in terms of cytokine and chemokine release) of a common respiratory pathogen (*Streptococcus pneumoniae*) on primary airway basal cells of patients with atopic severe asthma and compared that to healthy controls. As a positive control, the cytokine and chemokine release in response to a common inhaled allergen (*Dermatophagoides pteronyssinus* allergen 1 [Der p 1]) by primary airway basal cells was also studied.

Detailed methodology is given in this article's Online Repository. Briefly, we studied 8 subjects with severe asthma and 6 healthy controls. Subjects with severe asthma met the American Thoracic Society criteria for refractory asthma,¹ were current non smokers and had a smoking history of less than 10 pack years. Healthy controls were non smokers, had no history of respiratory disease and had normal lung function and PC₂₀. Demographics and clinical detail were collected. All subjects underwent flexible bronchoscopy and using epithelial brushings taken from the bronchus intermedius, confluent monolayers of basal cell cultures were developed. The basal cells were

incubated with wild type *Streptococcus pneumoniae* (strain D39) at concentrations of 10^6 cfu/ml and 10^7 cfu/ml for up to 4 hours at 37°C. For the control, basal cells were incubated with 400µl bronchial epithelial base medium (BEBM) (Clonetics, UK). The supernatants were harvested at one hour and four hours after incubation and stored at -70°C. Similarly, confluent monolayers of basal cells were incubated with LoTox™ Natural Der p 1 (Indoor Biotechnologies) at concentrations of 1 µg/ml and 5 µg/ml for up to 24 hours. The supernatants were harvested at eight hours and 24 hours after incubation and stored at -70°C. Chemokines and cytokines in the supernatant were measured using a 96-well multispot assay (Meso Scale Discovery [MSD], Maryland, USA) using a high band MS6000 10 spot plate, using SECTOR Imager 6000 (MSD, Maryland, USA) according to the manufacturer's instructions. The lower limit of detection was 1 pg/ml.

The baseline characteristics of the subjects and the data on chemokine and cytokine release in response to *Streptococcus pneumoniae* and Der p 1, are given in the online repository tables E1 – E5. The release of cytokines and chemokines by airway basal cells of patients with severe asthma and healthy controls in response to *Streptococcus pneumoniae* and Der p 1 was time and dose dependent. The magnitude of release of chemokines CXCL8 (IL8), CCL11 (Eotaxin) and CCL26 (Eotaxin_3) in response to *S pneumoniae* by basal cells from healthy controls, was significantly higher ($p<0.05$), compared to that from severe asthma patients (see Figure 1). In contrast, the magnitude of release of chemokines CXCL8 (IL8), CCL11 (Eotaxin), CCL26 (Eotaxin_3) (see Figure 2); as well as CCL4 (MIP 1b), CCL5 (RANTES), CCL13 (MCP 4), CCL17 (TARC) and CCL22 (MDC) in response to Der p 1 by basal cells from patients with severe asthma, was significantly higher ($p<0.05$) compared to that from healthy controls. We observed a similar differential cytokine response (IL6 and IL1b) of basal cells from severe asthma patients and healthy controls, to Der p 1 and *Streptococcus pneumoniae* (Online repository table E4 & E5, Figure E3).

In the context of profound ciliary dysfunction and epithelial disintegrity seen in patients with severe asthma,² the differential chemokine response of severe asthma patients' airway basal cells to Der p 1 and *Streptococcus pneumoniae* that we observed in this study is of great interest due to two main

reasons. Firstly, asthma has been shown to be an independent risk factor for invasive pneumococcal disease.^{4, 5} It remains to be determined if the reduced CXCL8 release by asthmatic airway epithelium compared to that of healthy controls leads to a reduction in neutrophil influx and delayed bacterial clearance, thereby increasing the risk of invasive pneumococcal disease in patients with severe asthma. Secondly, it has been suggested that in individuals with atopic sensitization to aeroallergens, there may be an altered mucosal immune response to bacterial antigens.^{6, 7} In recent studies, using a mouse model of allergic asthma, immunomodulatory therapy with *Streptococcus pneumoniae* vaccine has been shown to attenuate both Th1 and Th2 cytokine production.^{8, 9}

In this study we did not attempt to elucidate the mechanisms underlying the basal cell response to Der p 1 or *Streptococcus pneumoniae*. It would be of interest to investigate the effect of aberrant chemokine milieu on epithelial injury-repair mechanisms and whether prior exposure of asthmatic airway epithelium to *Streptococcus pneumoniae* leads to an attenuated response to Der p 1. As we used different time points for assessing the epithelial response to *Streptococcus pneumoniae* and Der p 1, there remains the possibility that alterations in the epithelial response kinetics may be contributory to the differential response that we showed and this needs further investigation.

In summary, our study shows that airway basal cells of patients with atopic severe asthma and healthy controls are capable of releasing chemokines and cytokines in response to Der p 1 and *Streptococcus pneumoniae* in a dose and time dependent manner. Though no major conclusions may be drawn from this small pilot study, the differential response of the asthmatic epithelium is of interest and may be further explored in the context of developing novel immunomodulatory therapeutic strategies for the treatment of allergic airway inflammation.

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Figure legends

Figure 1. Release of CXCL8 (Fig 1 A & B), CCL11 (Fig 1 C & D) and CCL26 (Fig 1 E & F) by primary respiratory basal cells of patients with severe asthma and healthy controls, in response to *Streptococcus pneumoniae* (D39) at 10^6 cfu/ml and 10^7 cfu/ml. A, C & E- CXCL8 response of basal cells at 1 hour post exposure; B, D & F- CXCL8 response of basal cells at 4 hours post exposure. Data expressed as median (IQR).

† $p < 0.01$ compared to corresponding values for severe asthma.

Figure 2. Release of CXCL8 (Fig 1 A & B), CCL11 (Fig 1 C & D) and CCL26 (Fig 1 E & F) by primary respiratory basal cells of patients with severe asthma and healthy controls, in response to LoTox Der p 1, 1µg/ml and 5µg/ml. A, C & E- response of basal cells at 8 hours post exposure; B, D & F- response of basal cells at 24 hours post exposure. Data expressed as median (IQR).

† $p < 0.01$ compared to corresponding values for healthy controls.

Online Repository

Methods

1. Subjects

8 subjects with severe asthma were recruited from Glenfield Hospital, Leicester, United Kingdom. 6 healthy controls were recruited from staff and by local advertising. Asthma was diagnosed based on presence of typical clinical features and objective measures of airway hyperresponsiveness and variable airflow obstruction as described previously.¹ Subjects with severe asthma met the American Thoracic Society criteria for refractory asthma² and belonged to the Global Initiative for Asthma (GINA) treatment steps IV-V.³ Healthy controls were non smokers, had no history of respiratory disease and had normal lung function and PC₂₀. Subjects with severe asthma were current non smokers, had a smoking history of less than 10 pack years and had been free from infections and asthma exacerbations requiring antibiotics and/or systemic corticosteroids for at least 6 weeks at the time of collection of bronchial epithelial samples. The study protocol was approved by the Leicestershire and Rutland Regional Ethics Committee and written informed consent was obtained from all subjects.

2. Measurements

Demographics, including age, gender and current medications were recorded. All subjects had their plasma IgE measured and all underwent spirometry and methacholine challenge using the tidal breathing method.⁴ Subjects with asthma underwent allergen skin prick tests to house dust mite, measurement of exhaled nitric oxide concentration (flow rate 50ml/s)⁵ and sputum induction.¹ All subjects underwent flexible bronchoscopy conducted according to the British Thoracic Society guidelines⁶ and epithelial brushings were taken from the bronchus intermedius.

3. Culture of primary bronchial epithelial cells

3.1 Collagen coating

PureCol solution of collagen was prepared as a 1% w/v solution in phosphate buffered saline (500µl in 50ml phosphate buffered saline). A sufficient volume of PureCol solution was added to completely cover the surface of plates, flasks, glass-slides and wells used in bronchial epithelial cell cultures and experiments involving primary bronchial epithelial cells. After incubating for 5 hours at room temperature, they were washed with nano pure water, left to air dry and then stored in a sealed bag at room temperature until used.

3.2 Bronchial brushing

Flexible bronchoscopy was conducted according to the British Thoracic Society guidelines.⁶ An Olympus (BC-202D-1210) cytology brush was passed through the channel in the bronchoscope and epithelial brushings were taken under direct vision by brushing the bronchial epithelium in a forward and backward direction. Typically, a good biopsy yielded about 1mg of ciliated tissue. Each tissue strip contained rows of 10-50 ciliated cells. The epithelial strips obtained were then dislodged by agitating in 2ml of 20mM Hepes buffered medium 199 (pH 7.4), containing penicillin (100 IU/ml), streptomycin (100 µg/ml) and fungizone (2.5 µg/ml), and kept in the fridge overnight. This allowed time for the antibiotics and fungizone to work and clear any potential deep cellular infections.

3.3 Primary bronchial epithelial cell culture

An adaptation of a previously described method was used to grow primary bronchial epithelial cells.⁷ The brush biopsy contained a heterogeneous population of both differentiated and undifferentiated bronchial epithelial cells. The contents of the brush biopsy (unknown number of cells) was placed in a collagen-coated well from a 12 well plate in 1ml of bronchial epithelial growth medium (BEGM), containing penicillin (100 IU/ml), streptomycin

(100 µg/ml) and fungizone (2.5 µg/ml), at 37°C. The bronchial epithelial cells were fed every other day by removing the medium and replacing it with 1ml BEGM. When the cells were 90 to 100% confluent (after 7-10 days) the entire medium was removed and the cells were washed with 0.3ml Trypsin/EDTA solution (0.5g porcine trypsin and 0.2g EDTA per liter of Hanks' Balanced Salt Solution with phenol red) and left at room temperature for about 2-3 minutes, occasionally agitating the tray. As soon as the cells began to detach, they were suspended by pipetting the Trypsin/EDTA solution over the cell surface. Cells were then placed in a 15ml tube containing 1ml BEGM to inactivate the trypsin. The well surface was then washed with 3ml of BEGM (to recover all the cells) and this also was placed in the 15ml centrifuge tube. The cells were then centrifuged (2,000 X *g* for 10 min) and the supernatant was removed. The pellet was re-suspended in 1ml BEGM, making sure that there were no clumps, by vigorous pipetting. The cell suspension was then added to a T80 (80cm²) collagen-coated flask containing 14ml BEGM. When the cells in the T80 flask were 90 to 100% confluent (up to 4-7 days), the entire medium was removed and the cells were washed with 1.4ml Trypsin/EDTA solution and left at room temperature for about 2-3 minutes, agitating the flask occasionally. As soon as the cells detached, 8ml BEGM was added and the cells were suspended by pipetting the medium over the cell surface. The cell suspension was pipetted into a 15ml tube and the cells were centrifuged for 5 minutes at 4,000*g*. The supernatant was removed and the pellet re-suspended in 1.8ml BEGM. This cell suspension was used for bronchial epithelial cell culture studies. 60 µl of the cell suspension was added to each well of 8-well collagen coated glass chamber slides. The bronchial epithelial cells in the 8-well glass chamber slides were fed with fresh BEGM (400µl) every other day, until confluent monolayers were obtained. Confluent monolayer in the glass chamber slides contained ~ 10⁵ cells.

3.4 Immunohistochemical characterization of basal cells

Confluent basal cells grown in glass chamber slides, were fixed with 4% w/v paraformaldehyde in phosphate buffered saline for 10 minutes at room temperature. The cells were then washed with 200µl phosphate buffered saline for 20 minutes with three buffer changes. The last wash was replaced with 1ml 3% w/v BSA (Bovine serum albumin) in phosphate buffered saline and left for 10 minutes at room temperature and then washed three times with phosphate buffered saline. Cells were stained for 2 hours with 200µl mouse anti-cytokeratin peptide 14 (CK14) monoclonal antibody (Sigma, UK.C8791), at a dilution of 1:200 in 1% w/v BSA in PBS at 37°C.⁸ After three washes in phosphate buffered saline, FITC-Goat anti-mouse IgG, A, M (Zymed laboratories, 65-6411) was diluted 1:50 in 1% w/v BSA in phosphate buffered saline and was added for 2 hours at 37°C. During the final 10 minutes, 1:1000 Hoechst stain (Sigma Aldrich, UK, H6024) was added to stain the nuclei. After 3 washes with PBS, the chamber was removed and a few drops of mountant (80% v/v glycerol, 3% w/v n-propyl gallate in phosphate buffered saline) was placed onto the slide, covered with a size 1.5 coverslip and sealed with nail varnish. The cells were then visualised using a Nikon eclipse TE2000-U microscope. Over 98% of the basal cells in the primary cultures expressed CK-14. (Figure E1 & E2)

4 Preparation of *Dermatophagoides pteronyssinus* allergen 1

LoToxTM Natural Der p 1 (Indoor Biotechnologies Ltd, Witshire, United Kingdom) with cysteine protease activity >200 RFU at 25 µg/ml concentration and an endotoxin content of ≤ 0.01 EU/µg was used. LoToxTM Natural Der p 1 at concentrations of 1 µg/ml and 5 µg/ml were prepared and incubated with the reducing agent Dithiothreitol 1mM to make it enzymatically active, before addition to the bronchial epithelial cell monolayer.

5 Preparation of suspensions of *S pneumoniae*

Streptococcus pneumoniae (D39), a wild type laboratory strain (serotype 2) from National Collection Type, Culture 7466, London, UK was used. *Streptococcus pneumoniae* was grown on blood agar plates in a CO₂ jar (BBL GasPak system, USA). A candle was lit and placed inside the jar, the lid tightly closed and incubated at 37°C overnight. The candle was placed to create anaerobic conditions by eliminating any oxygen present. Colonies from the blood agar plate were used to inoculate 100ml brain heart infusion broth (BHI) which was then incubated overnight at 37°C. The next day the OD₅₀₀ was adjusted by the adding BHI broth until it was between 0.8-1.0 (equivalent to mid exponential phase). The culture was then separated into Eppendorf tubes (1ml per tube) containing 10% v/v glycerol and frozen at -70°C until required. Numbers of viable bacteria in the stock were determined by colony counting on blood agar plates. Viable counts were calculated as average colony forming units (cfu) formed from duplicate 50 µl volumes plated onto an appropriate agar plate,⁹ following ten-fold serial dilutions in sterile phosphate buffered saline. The cfu/ml was determined by the following equation; $\text{cfu/ml} = y \times 10^d \times 20$, where y is the average colony count in 50µl and d is the dilution factor.

Before use, frozen stocks were thawed at room temperature and the bacteria were then sedimented (4,000 X g for 10 min) and re-suspended in 1ml bronchial epithelial basal medium (BEBM, Lonza, UK) and diluted to the required concentrations (10⁶ cfu/ml and 10⁷ cfu/ml).

6 Cytokine and chemokine assay

Chemokines and cytokines were measured using a 96-well multi spot assay (Meso Scale Discovery [MSD], Maryland, USA) according to the manufacturer's instructions. Briefly, the assay employs a sandwich immunoassay format where capture antibodies are coated in a single spot, or in a patterned array, on the bottom of the wells of a multi-spot plate (Figure E4). Samples or standards are incubated in the multi-spot plate, and each cytokine binds to

its corresponding antibody spot. Following this, when a potential is applied to the electrode, bound labelled sulpho tag produces light and this is proportional to the amount of inflammatory proteins in the sample. Unknown samples are calculated by comparing their light emitted to that of a known amount of the protein on the standard curve.

Cytokines were measured using a human Th₁/Th₂ standard 10 spot plate (Catalog number N01010A-1) and human chemokines were measured using a high band MS6000 10 spot plate (Catalog number N01001B-1), using SECTOR Imager 6000 (MSD, Maryland, USA). The lower limit of detection was 1 pg/ml. Further details of the methodology may be obtained from the following link.

http://www.meso-scale.com/CatalogSystemWeb/Documents/Human_96_well_Base.pdf

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Figure legends

Figure E1: Light microscopic image of human bronchial epithelial cells in culture. Scale bar represents 20µm.

Figure E2: Immunofluorescent images showing the expression of CK-14 in basal respiratory epithelial cells. A) Basal cells stained with anti-CK-14 antibody (red) and B) Negative control showing basal cells only stained with the secondary antibody. The nuclei are stained with Hoechst (Blue). Original magnification x 600.

Figure E3. Release of IL-1b (E3A) and IL-6 (E3B) by primary respiratory basal cells of patients with severe asthma and healthy controls, in response to *Streptococcus pneumoniae* (D39) at 10^6 cfu/ml and 10^7 cfu/ml, 4 hours post exposure. Release of IL-1b (E3C) and IL-6 (E3D) by primary respiratory basal cells of patients with severe asthma and healthy controls, in response to LoTox Der p 1, 1µg/ml and 5µg/ml, 24 hours post exposure. Data expressed as median (IQR). [†]p<0.01 compared to corresponding values for healthy controls.

RESULTS

Table E1: Baseline characteristics of the subjects

Characteristic	Control (n=6)	Severe asthma (n=8)
Age (yr) [†]	29.5 [23-53]	44 [31.5-52]
Sex (no. of subjects)		
Male	2	3
Female	4	5
Age at onset of symptoms(yr) [†]	-	4.5 (2.2-30)
Positive skin prick test to house dust mite (%)	0	100
Plasma IgE (IU/ml) [†]	62.7 (43.4-120.4)	317 (105-564.8)*
FEV ₁ [†]		
Litres	3.7 (2.9-4.1)	2.4 (1.5-2.8)**
Percentage of predicted value	105 (93.5-110.3)	71.5 (64-94)**
FEV ₁ : FVC ratio (%) [†]	92 (86.5-94.5)	76 (68-90)*
Exhaled Nitric oxide (ppb) [†]	-	58.4 (18.2-102)
Sputum measurements [†]		
Eosinophils (%)	-	2.6 (0.2-21.3)
Neutrophils (%)	-	69.6 (43.6-89.3)
PC ₂₀ (mg of methacholine/ml) [†]	>16	0.1 (0.06-0.31)
Inhaled steroid dose in µg (BDP equivalent) [†]	0	1800 (1600-2000)

† Median (IQR). **p<0.01 and *p<0.05 compared to control group, BDP = Beclomethasone dipropionate

Table E2. Chemokine release by basal cells from healthy controls and patients with severe asthma, in response to *Streptococcus pneumoniae*

Chemokine	Healthy Controls						Patients with severe asthma					
	1 hour			4 hours			1 hour			4 hours		
	Control	10 ⁶ cfu/ml	10 ⁷ cfu/ml	Control	10 ⁶ cfu/ml	10 ⁷ cfu/ml	Control	10 ⁶ cfu/ml	10 ⁷ cfu/ml	Control	10 ⁶ cfu/ml	10 ⁷ cfu/ml
CXCL8	2.3 [1.7-2.9]	21.4 [19.6-26.8]‡	71.7 [69-77.4]†	4.4 [4.0-7.5]	177 [158.8-364.5]†	915.8 [805.3-1208]**‡	2.7 [1.6-2.9]	15.2 [13.4-16.8]	50.5 [35.1-58.6]	5.1 [3.5-7.3]	108.9 [91.6-116.9]	321.3 [266.6-543.3]**
CXCL10	308 [282.3-318.8]	405.3 [398-409.9]	439.9 [430-455.3]	349.7 [340-361]	506.1 [486.9-523.5]	572.3 [545.8-682.8]**	311.3 [307.2-321.8]	417.6 [411.6-421.7]	456.7 [449.6-470.8]	354.2 [346.7-364.9]	528.1 [494-532.6]*	642.4 [596.4-664.2]***
CCL2	2.9 [0.1-3.8]	5.8 [5.4-6.3]	7.1 [7-7.5]	4.7 [4.5-4.9]	8.7 [8-9.5]	10.9 [10.2-13.3]*	3.1 [0.4-3.8]	6.4 [5.7-6.6]	7.4 [6.9-7.5]*	4.7 [4.2-4.9]	9.1 [8.4-9.3]*	12.3 [11.3-13]***
CCL4	0	1.7 [0.8-2.4]	2.4 [2-4.1]	0	5.7 [3.9-7.2]*	7.8 [4.9-11.3]**	0	1.7 [1-1.9]	2.9 [2.3-3.3]	0	5.8 [3.5-6.5]**	8.3 [4.6-9.1]***
CCL5	0	1.3 [1.2-1.7]	1.9 [1.6-2.1]	1.1 [0.7-1.3]	2.8 [2.6-3.1]	4.5 [3.7-6.7]*	0	1.4 [1.1-1.7]	2.3 [1.9-2.4]	0	2.8 [2.6-2.9]*	4.2 [3.3-6.1]***
CCL11	53.7 [52.3-63.7]	94.3 [91.7-100.3]†	105.8 [105.3-111.3]*‡	69.7 [63.6-75.1]	122.2 [116.7-123.4]*†	140.9 [134.5-158.2]**‡	57.5 [54.8-59.7]	80.4 [77 - 87.1]	85.1 [81.5-95.1]	67.3 [61.5-69.8]	102.7 [97.1-113.8]	113.1 [111.1-124.5]**
CCL13	58.9 [56.8-60.9]	92.6 [86.7-103.6]	106.7 [104.3-110.7]	77.9 [70.8-79.4]	127.2 [121.3-134.5]	144.5 [134.5-183.5]*	57.1 [42.8-65.4]	99.6 [93.6-104.2]	110.2 [101.2-113]*	72.3 [68.9-78.9]	128.7 [122.8-135.9]*	155.5 [150.3-180]***
CCL17	31.4 [27.4-39.5]	68.4 [64.2-71.2]	80.3 [74.9-85.9]	52.2 [40.4-56.1]	91.1 [87.5-109.2]	118.2 [100.9-144.2]**	32.4 [27.9-39.1]	67.7 [61.1-71.9]	78.5 [74.4-82.4]	51.2 [44.9-58.9]	90.9 [89.5-92.9]*	124.4 [116.1-136.2]***
CCL22	279.2 [259.3-285.5]	376.7 [365.1-385.8]	402.8 [374-416]	308.5 [299.4-326.6]	464.7 [447.9-507]	522.1 [476.5-607.2]*	278.9 [270.4-289.1]	376.9 [354.6-389.5]	405.3 [399.7-416.2]	305.6 [304-322]	480 [449.1-504.1]*	562.3 [518.1-584.9]***
CCL26	949.3 [688.9-1021]	1560 [1536-1579]†	1775 [1759-1802]†	1177 [880.4-1232]	2231 [2197-2298]**†	2815 [2626-3695]**†	902.9 [772.8-976.7]	1422 [1361-1468]	1685 [1647-1720]	1126 [1077-1230]	1866 [1824-1968]*	2031 [1954-2307]**

Data as Median (IQR) pg/ml. *p<0.05, **p<0.01, ***p<0.001 compared to control

† p<0.01 compared to corresponding values for patients with severe asthma

‡ p<0.001 compared to corresponding values for patients with severe asthma

Table E3. Chemokine release by basal cells from healthy controls and patients with severe asthma, in response to *Der p 1*

Chemokine	Healthy Controls						Patients with severe asthma					
	8 hours			24 hours			8 hours			24 hours		
	Control	<i>Der p 1</i> 1 µg/ml	<i>Der p 1</i> 5 µg/ml	Control	<i>Der p 1</i> 1 µg/ml	<i>Der p 1</i> 5 µg/ml	Control	<i>Der p 1</i> 1 µg/ml	<i>Der p 1</i> 5 µg/ml	Control	<i>Der p 1</i> 1 µg/ml	<i>Der p 1</i> 5 µg/ml
CXCL8	2.1 (1.7-3.4)	5.3 (4.9-11.3)*	25.0 (23.5-26.4)*†	7.8 (7.1-8.5)	16.9 (15.1-20.3)*‡	64.7 (51.3-71.7)*†‡	3.4 (2.5-4.1)	13.9 (11.7-14.9)*	38.3 (36.6-46.6)*†	6.3 (5.4-8.4)	60.6 (53.2-67.1)*‡	100.7 (82.2-165.8)*†‡
CXCL10	54.5 (51.1-62.6)	71.5 (67.7-72.4)*	77.4 (75.2-79.4)*†	67.0 (66.3-68.7)	74.1 (67.9-74.4)	89.9 (86.4-98.6)*†‡	61.1 (58.5-64.3)	71.1 (69.0-71.8)*	78.1 (73.7-79.5)*†	67.8 (63.7-68.5)	68.5 (67.2-73.7)	88.6 (85-97.2)*†‡
CCL2	5.0 (4.6-6.8)	4.9 (4.3-5.3)	6.1 (5.6-6.7)	6 (5.2-6.3)	5.1 (4.7-5.7)	6.5 (5.7-7.7)*†‡	5.3 (4.6-5.8)	4.8 (3.8-5.7)	5.3 (5.1-5.5)†	5.5 (5.1-6.1)	5.8 (4.9-6.2)	7.4 (6.4-8.3)†
CCL4	1.9 (1.0-2.2)	3.1 (3.0-3.2)*	3.2 (3.0-3.2)*	2.6 (2.6-2.7)	3.4 (3.1-3.4)*	3.6 (2.9-3.7)*	2.5 (2.3-2.6)	3.5 (3.1-3.6)*	3.9 (3.8-4.1)*†	2.8 (2.7-3.0)	4.8 (4.4-5.1)*‡	6.4 (5.4-7.1)*†‡
CCL5	0 (0-0)	0.2 (0-0.3)	0.5 (0.4-0.6)†	0 (0-0)	0.7 (0.6-0.8)‡	1.7 (0.9-2.2)*†‡	0 (0-0)	1.0 (0.9-1.1)	1.5 (1.1-2.3)†	0 (0-0)	2.9 (2.7-3.3)‡	4.5 (4-5.3)*†‡
CCL11	34.8 (33.2-36.7)	45.3 (44.6-46.1)*	67.8 (60.4-189.9)*†	41.1 (39.9-42.3)	255.8 (246.5-264)*‡	271.8 (269-275.6)*†‡	35.7 (33.6-36.9)	46.6 (45.6-47.7)*	289.9 (285.4-305.2)*†	42.3 (40.2-42.8)	339.1 (327-350.9)*‡	412.6 (398.9-428.9)*†‡
CCL13	37.3 (35.9-38.5)	47.1 (43.6-47.5)*	48.3 (48-49.4)*†	43.2 (42-44.8)	50.7 (49.3-51.5)*	52.7 (51.3-55.3)*†‡	39.6 (35.9-39.9)	56.1 (54.9-58.1)*	60.9 (59.7-63.6)*	44.5 (42.8-46.2)	57.8 (55.5-62.0)*‡	73.5 (69.5-86.1)*†‡
CCL17	28.7 (23.2-30.4)	34.3 (33.8-35.6)*	38.2 (37.6-38.3)*†	33.8 (32.1-34.6)	39.2 (38.9-39.9)*‡	40.9 (39.6-42.9)*†‡	30.4 (28.7-32.6)	36.8 (35.9-37.3)*	42.1 (39.9-42.9)*†	34.6 (32.8-34.9)	47.7 (44.7-49.3)*‡	55.6 (54.5-63.1)*†‡
CCL22	90.6 (83.1-92.6)	102.2 (102.1-103.4)*	105.4 (103.8-106.5)*†	98.7 (97.9-99.2)	107 (104.7-108.8)*‡	114.2 (109.1-118.5)*†‡	90 (85.5-93.6)	104.9 (103.6-105.9)*	116.5 (111.7-118.6)*†	100.1 (98.7-100.7)	127.8 (122.6-129.7)*‡	148.2 (141.4-155.3)*†‡
CCL26	38.9 (38.5-39.6)	50.7 (48.6-52.9)*	218.2 (55.8-243.8)*†	41.2 (38.9-42.9)	258.5 (249.8-263.1)*‡	271.1 (268.8-275.4)*†‡	39.9 (38.7-41.3)	55.6 (53.8-195.4)*	312.9 (301.7-322.5)*†	42.1 (41.2-43.5)	342.4 (325.2-350)*‡	466.6 (455.8-551.1)*†‡

Data as Median (IQR) pg/ml. *p<0.05 compared to control; †p<0.05 compared to corresponding values for *Der p 1*, at concentration of 1 µg/ml, ‡p<0.05 compared to corresponding values at 8 hours.

Table E4: Cytokine release by basal cells from healthy controls and patients with severe asthma, in response to *Streptococcus pneumoniae*

Cytokine	Healthy Controls						Patients with severe asthma					
	1 hour			4 hours			1 hour			4 hours		
	Control	10 ⁶ cfu/ml	10 ⁷ cfu/ml	Control	10 ⁶ cfu/ml	10 ⁷ cfu/ml	Control	10 ⁶ cfu/ml	10 ⁷ cfu/ml	Control	10 ⁶ cfu/ml	10 ⁷ cfu/ml
IL6	0	0	0	0	2.1 [1.7-2.9] ^{*†‡}	7.9 [4.9-10.9] ^{***†‡}	0	0	0	0	1.4 [0.8-1.6]	3.2 [2.5-4] ^{***†}
IFN- γ	0	0	0	0	0	1.6 [1.2-2] ^{**}	0	0	0	0	0	1.6 [1-1.9] ^{**}
IL10	0	0	0	0	1.8 [1.3-2.4]	4.5 [2.5-6.9] ^{**}	0	0	0	0	1.5 [0.8-2.7]	4.4 [2.9-6.6] ^{***}
IL12p70	0	0	0	0	1.6 [1.1-2.3]	3.1 [2.4-3.5] ^{**}	0	0	0	0	1.7 [1.1-1.8] [*]	3.1 [2.8-3.7] ^{***}
IL13	0	0	0	0	2.1 [1.6-3] [*]	5.5 [3.7-8.4] ^{***}	0	0	0	0	2.3 [1.8-2.4] ^{**}	5.1 [3.6-7.3] ^{***}
IL1b	0	2.5 [1.5-3.4]	5.2 [5-5.7]	0	10.3 [8.9-10.9] ^{†‡}	35 [20.3-48.9] ^{**†‡}	0	1.6 [1-3.1]	5.6 [4.1-5.7] [*]	0	7.1 [6.1-8.2]	15.9 [12.4-17.1] ^{***}
IL2	0	0	0	0	2.1 [1.6-3.1]	7 [6.2-8.2] ^{**}	0	0	0	0	2.3 [1.5-2.6] ^{**}	8.8 [4.4-10.1] ^{***}
IL4	0	0	0	0	0	0	0	0	0	0	0	0
IL5	0	0	0	0	0	1.4 [1.2-2.6] ^{**}	0	0	0	0	0	1.5 [0.9-2.4] ^{***}
TNF α	0	0	0	0	2.6 [1.7-5.8]	11.9 [6.9-17.2] ^{**}	0	0	0	0	4.3 [2.7-5] [*]	9.9 [5.9-24.2] ^{***}

Data as Median (IQR) pg/ml. *p<0.05, **p<0.01, ***p<0.001 compared to control

† p<0.01 compared to corresponding values at 1 hour

‡ p<0.05 compared to corresponding values for patients with severe asthma

Table E5: Cytokine release by basal cells from healthy controls and patients with severe asthma, in response to *Der p 1*

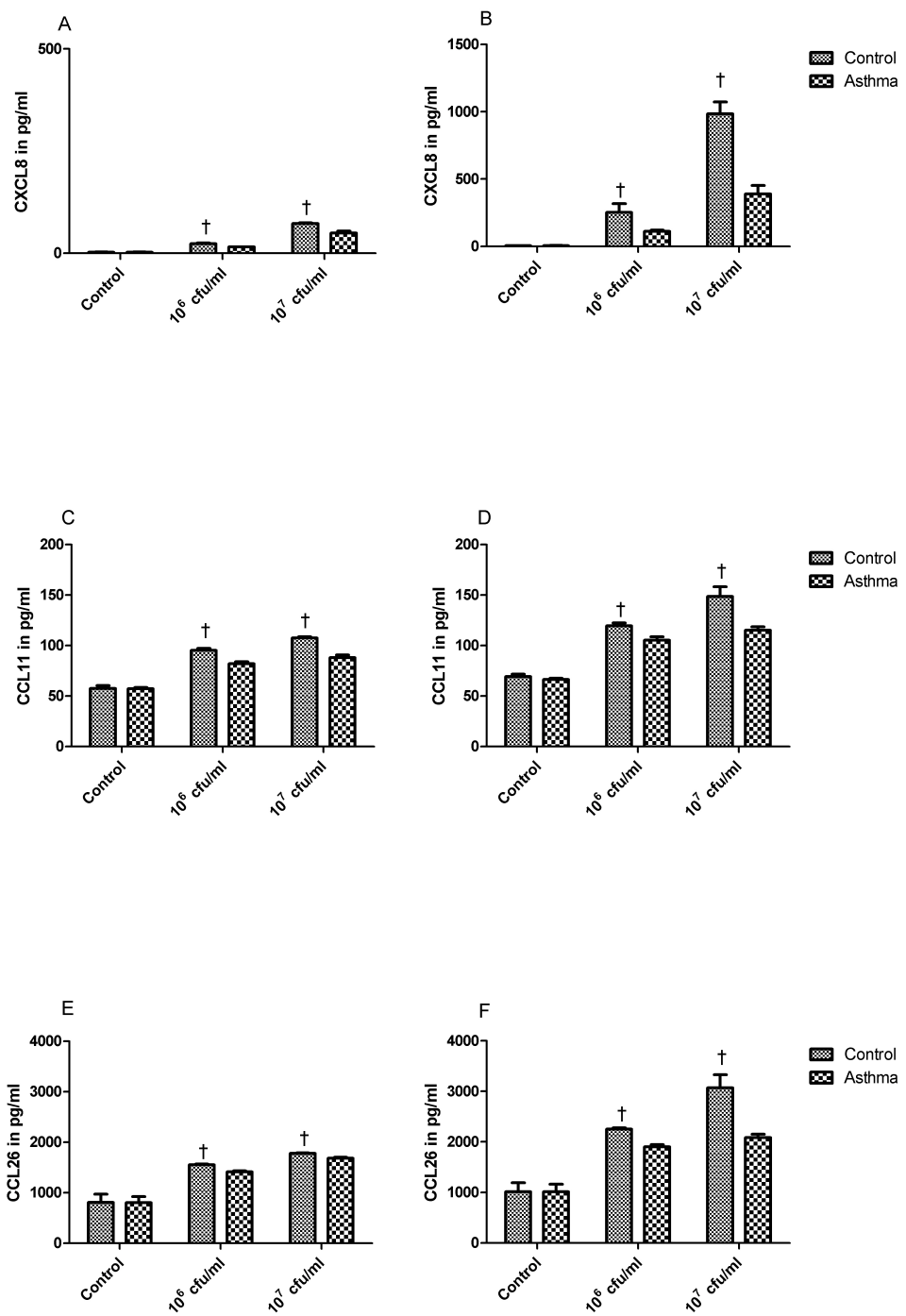
Cytokine	Healthy Controls						Patients with severe asthma					
	8 hours			24 hours			8 hours			24 hours		
	Control	<i>Der p 1</i> 1 µg/ml	<i>Der p 1</i> 5 µg/ml	Control	<i>Der p 1</i> 1 µg/ml	<i>Der p 1</i> 5 µg/ml	Control	<i>Der p 1</i> 1 µg/ml	<i>Der p 1</i> 5 µg/ml	Control	<i>Der p 1</i> 1 µg/ml	<i>Der p 1</i> 5 µg/ml
IL6	0	0	0	0	0	0	0	0	0	0	0	5.1 (4.5-7.1)** [†]
IFN-γ	0	0	0	0	0	0	0	0	0	0	1.9 (1.3-2.4)*	0
IL10	0	0	0	0	0	0	0	0	0	0	0	1.4 (1.2-2.9)** [†]
IL12p70	0	0	0	0	0	0	0	0	0	0	0	2.1 (1.7-2.8)* [†]
IL13	0	0	0	0	0	1.2 (1.0-1.2)*	0	0	1.3 (1.1-1.5)***	0	1.8 (1.7-1.9)**	3.7 (2.6-4.6)*** [†]
IL1b	0	0	1.1 (1-1.1)	0	1.2 (1.1-1.2)	2.1 (1.8-2.3)	0	1.5 (1.4-1.7)	3.7 (2.9-4.1)**	0	5.6 (4.8-7.5)*	24 (16.1-70.6)** [†]
IL2	0	0	0	0	0	0	0	0	0	0	0	2.5 (1.9-3.9)*** [†]
IL4	0	0	0	0	0	0	0	0	0	0	0	0
IL5	0	0	0	0	0	0	0	0	0	0	0	0
TNFα	0	0	0	0	0	0	0	0	0	0	1.2 (0.5-1.4)	1.9 (1.8-3.6) [†]

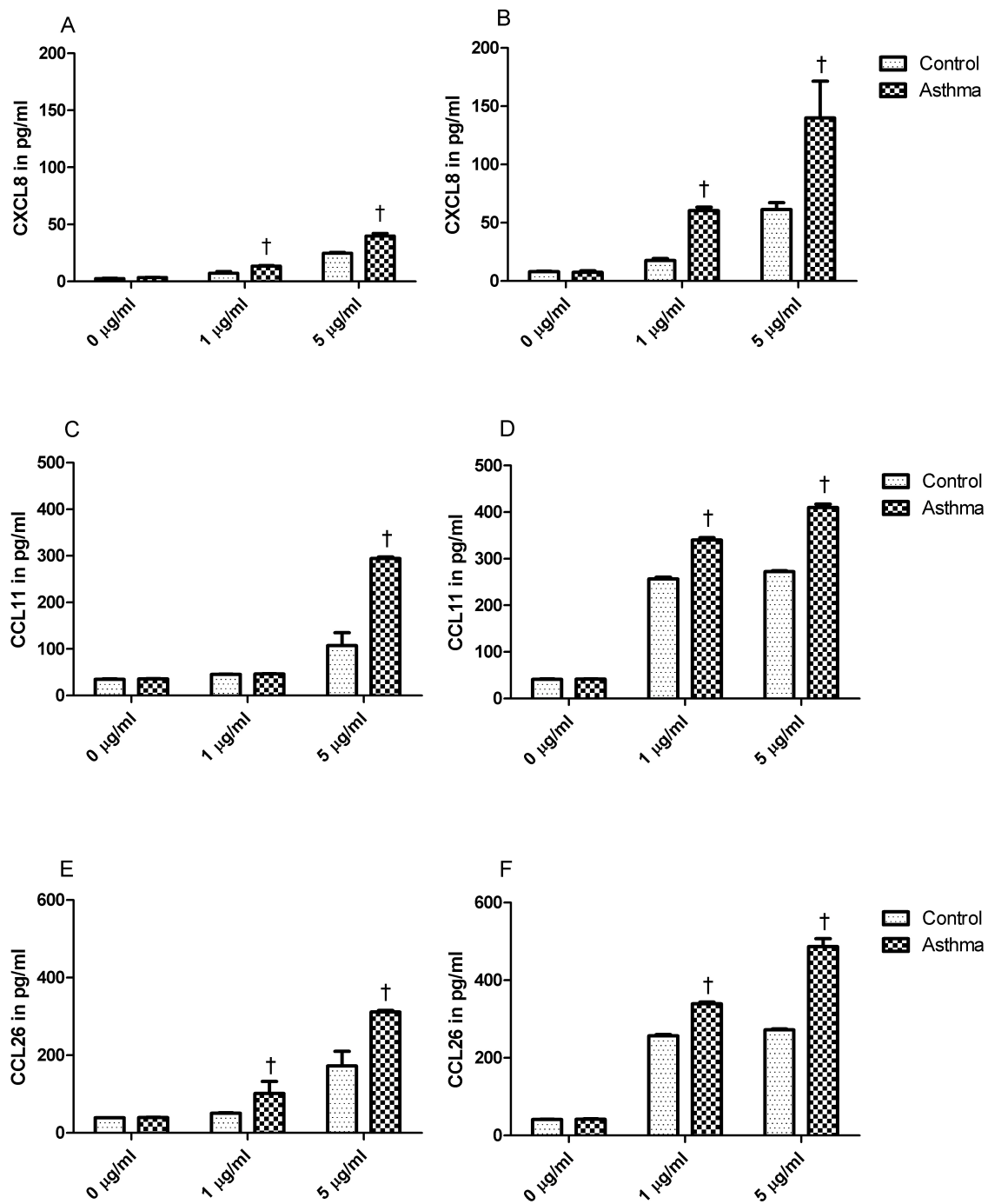
Data as Median (IQR) pg/ml. *p<0.05, **p<0.01, ***p<0.001 compared to control

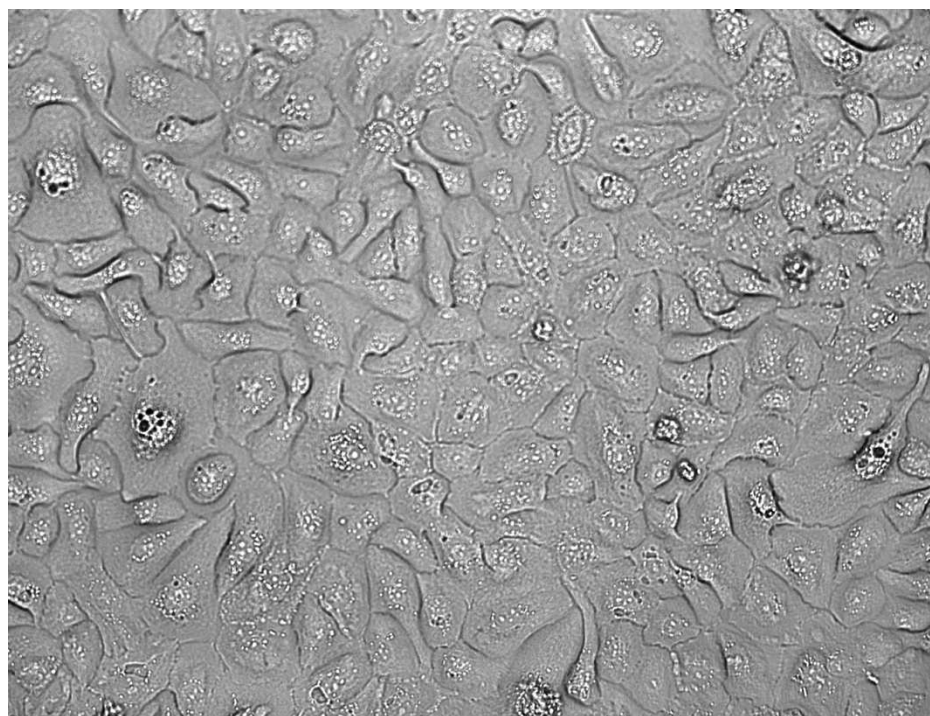
† p<0.05 compared to corresponding values for healthy controls.

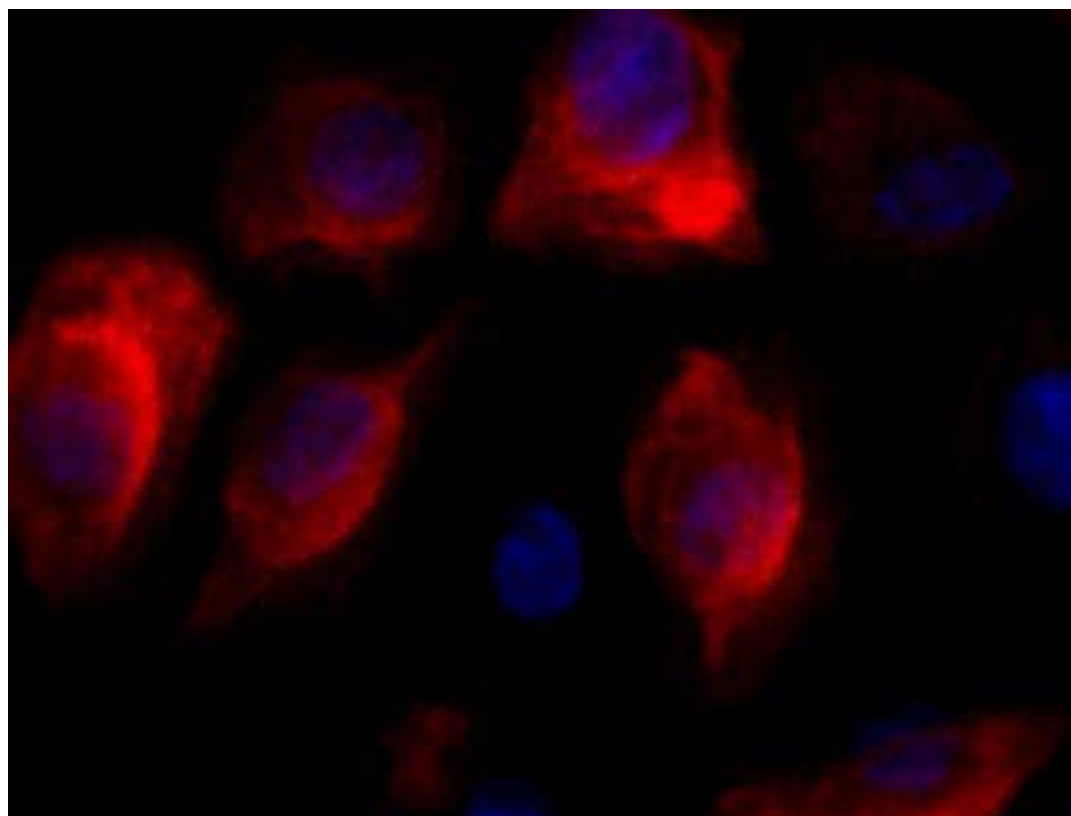
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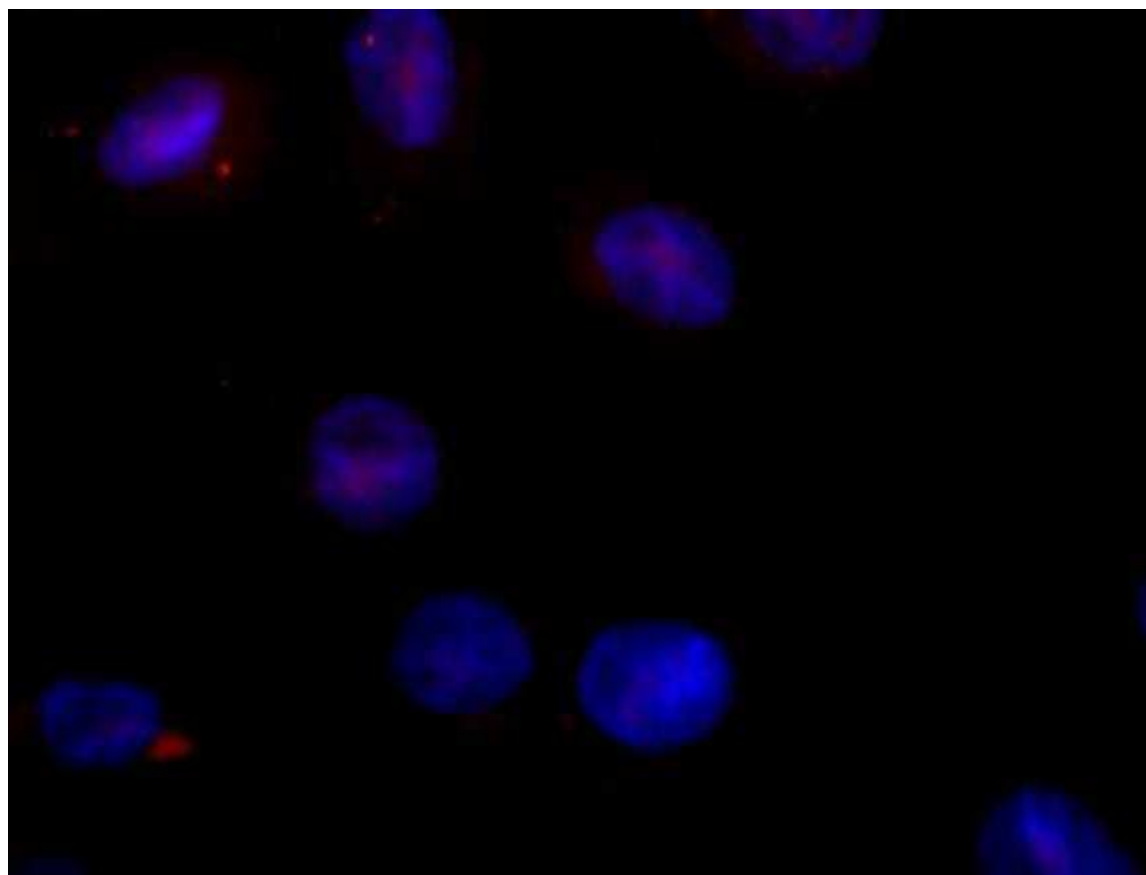
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