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A CEACAM6-High Airway Neutrophil Phenotype and CEACAM6-High Epithelial Cells Are Features of Severe Asthma

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Severe asthma represents a major unmet clinical need; understanding the pathophysiology is essential for the development of new therapies. Using microarray analysis, we previously found three immunological clusters in asthma: Th2-high, Th17-high, and Th2/17-low. Although new therapies are emerging for Th2-high disease, identifying molecular pathways in Th2-low disease remains an important goal. Further interrogation of our previously described microarray dataset revealed upregulation of gene expression for carcinoembryonic Ag cell adhesion molecule (CEACAM) family members in the bronchi of patients with severe asthma. Our aim was therefore to explore the distribution and cellular localization of CEACAM6 using immunohistochemistry on bronchial biopsy tissue obtained from patients with mild-to-severe asthma and healthy control subjects. Human bronchial epithelial cells were used to investigate cytokine and corticosteroid in vitro regulation of CEACAM6 gene expression. CEACAM6 protein expression in bronchial biopsies was increased in airway epithelial cells and lamina propria inflammatory cells in severe asthma compared with healthy control subjects. CEACAM6 in the lamina propria was localized to neutrophils predominantly. Neutrophil density in the bronchial mucosa was similar across health and the spectrum of asthma severity, but the percentage of neutrophils expressing CEACAM6 was significantly increased in severe asthma, suggesting the presence of an altered neutrophil phenotype. CEACAM6 gene expression in cultured epithelial cells was upregulated by wounding and neutrophil elastase. In summary, CEACAM6 expression is increased in severe asthma and primarily associated with airway epithelial cells and tissue neutrophils. CEACAM6 may contribute to the pathology of treatment-resistant asthma via neutrophil and airway epithelial cell-dependent pathways. *The Journal of Immunology*, 2017, 198: 3307–3317.

Asthma is an important cause of morbidity and mortality, affecting 300 million people worldwide (1, 2). Approximately 10% of asthma patients have severe disease that is resistant to current therapies, and suffer from ongoing symptoms and exacerbations (3, 4). In addition, ~50–60% of asthma healthcare costs have been attributed to this severe group (5, 6). There is therefore a considerable unmet clinical need, and novel approaches to therapy are required urgently.

Asthma is a complex and heterogeneous immunopathological disorder characterized by the presence of airway inflammation, airway remodeling, and airway hyperresponsiveness. Severe asthma is characterized by persisting eosinophilic inflammation in a subset of patients, excessive airway remodeling with both glandular hyperplasia and increased airway smooth muscle mass, and the develop-

ment of fixed airflow obstruction (7–9). The molecular factors that drive these excessive treatment-resistant changes within the airway wall are poorly understood.

Unbiased transcriptomic microarray analysis of bronchial tissue obtained from asthmatic subjects and healthy control subjects can provide a wealth of information with respect to molecular airway pathology, and it is valuable for hypothesis generation. For example, we have shown that within an asthma cohort, three molecular clusters based on gene expression signatures for IL-4/13 (Th2) and IL-17A (Th17) were present: Th2-high, Th17-high, and Th17-low (10). In this study, we have undertaken further analysis of this gene expression microarray dataset (Gene Expression Omnibus repository [https://www.ncbi.nlm.nih.gov/projects/geo] accession number GSE69497) and have identified a cluster of carcinoembryonic

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Abbreviations used in this article: ALI, air–liquid interface; BTS, British Thoracic Society; CEACAM, carcinoembryonic Ag cell adhesion molecule; FEV₁, forced expiratory volume in one second; GMFI, geometric mean fluorescence intensity; HNE, human neutrophil elastase; IQR, interquartile range; MBP, major basic protein; NHBE, normal human bronchial epithelial cell; PBEC, primary bronchial epithelial cell; PC₂₀, provocation concentration producing a 20% fall; PSG, pregnancy-specific glycoprotein.

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Ag cell adhesion molecule (CEACAM) 5-, CEACAM6-, and CEACAM7-high patients with severe asthma.

CEACAMs are part of the Ig superfamily of cell adhesion molecules. The CEACAM family is composed of two branches: 7 CEACAM (CEACAM1 and CEACAM3-CEACAM8) and 11 pregnancy-specific glycoprotein (PSG; PSG1-PSG11) members (11). Proposed functions for CEACAMs to date include cell adhesion and migration (12–14), prevention of anoikis (programmed cell death after detachment from matrix), and pathogen recognition (15–17). CEACAM5-7 are GPI membrane-anchored glycoproteins that can be shed and are detectable as soluble molecules (18). CEACAM5 (also known as CEA) is best known as a biomarker of epithelial malignancy (11, 14). CEACAM6 (previous names are nonspecific reacting Ag and CD66c) can mediate intercellular adhesion through both homophilic and heterophilic molecular interactions via an IgV-like N-terminal domain (13, 19); in particular, CEACAM6 plays a role in neutrophil adhesion to endothelial cells (20, 21). CEACAM6 is also involved in cell migration (12) and inhibits anoikis, thus augmenting tissue metaplasia and tumor progression (15). It is expressed in lung-derived tumor cell lines and lung cancer tissue (12, 22), and is expressed by granulocytes from various healthy human tissues including the lung (23). In the healthy adult lung, CEACAM6 immunoreactivity was reported to be localized to both the alveolar and airway epithelium (23). Soluble CEACAM6 was detectable in bronchoalveolar lavage fluid (24), and one study reported in a limited group of patients that CEACAM6 was not associated with chronic obstructive pulmonary disease (24). However, the expression of CEACAMs in asthma has not been described.

Because CEACAM6 had been associated with neutrophil activation, as well as with epithelial metaplasia, both of which may be features of severe asthma, the aims of this study were to validate the gene expression microarray data and explore the tissue distribution and cellular localization of CEACAM6 protein expression in bronchial biopsy tissue obtained from subjects with asthma of varying severity.

Materials and Methods

Study subjects

Patients with asthma in Leicester and Belfast were recruited from local asthma specialist clinics and by advertisement. Healthy control subjects were recruited by advertisement.

In total, 73 asthmatic patients and 24 healthy volunteers were enlisted for the study of gene and protein expression. Data from the subset of these patients whose samples were used to study gene expression have been published previously (10, 25, 26). A subset of 61 asthmatic and 17 healthy subjects samples were used for immunohistochemical analysis. Patients with asthma gave a suggestive history and had objective evidence of variable airflow obstruction, as indicated by one or more of the following: 1) methacholine airway hyperresponsiveness (provocation concentration producing a 20% fall (PC₂₀) in forced expiratory volume in 1 s (FEV₁), <8 mg/ml); 2) >15% improvement in FEV₁ 10 min after 200 µg of inhaled salbutamol, and 3) peak expiratory flow (>20% maximum within-day amplitude from twice-daily peak expiratory flow measurements over a period of 14 d). Subjects underwent spirometry; allergen skin prick tests for *Dermatophagoides pteronyssinus*, dog, cat, grass pollen, and *Aspergillus fumigatus*; a methacholine inhalation test (Leicester only) using the tidal breathing method; and sputum induction (Leicester only) with incremental concentrations of nebulized hypertonic saline (i.e., 3, 4, and 5%, each for 5 min) (27). Patients with asthma in Leicester also kept a diary card for 2 wk before bronchoscopy, recording daytime and nighttime symptoms, daily short-acting β₂-agonist use, and twice-daily peak expiratory flow. Asthma severity was defined based on *British Guideline on the Management of Asthma* treatment steps (mild = step 1, β₂-agonist only; moderate = steps 2 and 3, inhaled corticosteroid ≤800 mg of beclomethasone equivalent per day ± long-acting β₂-agonist; and severe = steps 4 and 5) (28). Of the 40 patients with severe asthma at steps 4 and 5, 33 met the American Thoracic Society criteria for refractory asthma (3).

Participants were current nonsmokers with no upper or lower respiratory tract infections in the 6 wk before bronchoscopy. All asthmatic participants' symptoms were clinically stable on their usual medication at the time of

bronchoscopy. The demographic details for the combined cohort used in this study are shown in Table I.

This study was approved by the Research Ethics Committee of both institutions (Leicester: Leicestershire, Northamptonshire and Rutland Research Ethics Committee reference 04/Q2502/74; Belfast: Office of Research and Ethics Committee of Northern Ireland reference 06/NIR02/114). Written informed consent was gained from all participants before their involvement.

Bronchoscopy

Subjects underwent bronchoscopy conducted according to British Thoracic Society (BTS) guidelines (29). Bronchial mucosal biopsy specimens were taken from the right middle lobe and lower lobe carinae, fixed in acetone, and embedded in glycol methacrylate, as described previously (30). Biopsies were also placed immediately in RNA preservative (RNAlater; Ambion, Austin, TX) and submitted for microarray analysis (10, 25).

Gene expression analyses

RNA was isolated from homogenized bronchial biopsies as published previously (31). RNA was amplified (Ambion) for Agilent (Santa Clara, CA) two-color Whole Human Genome 4 × 44k gene expression microarray analysis. Universal Human Reference RNA (Stratagene, La Jolla, CA) was used for the reference channel. Probe intensities of test and reference channels were calculated by the Agilent Feature Extraction software, protocol GE2-v5_95 (Agilent). All gene expression analyses were performed using the R Project software package, version 2.10.1 (refer to <http://www.R-project.org>). Independent filtering (32) based on gene annotation was conducted; that is, genes represented by multiple array features were represented by the most variable (interquartile range [IQR]) probe. The Th2 score was calculated by using a generalized procedure (31), and the gene signature in this dataset has previously been reported (10, 25).

Immunohistochemistry

Two-micrometer glycomethacrylate sections were cut and stained as described previously (30). Two sections at least 10 µm apart were immunostained for each Ag. The following mouse primary Abs were used: anti-CEACAM6 (16.5 µg/ml) (in-house generated; Genentech, South San Francisco, CA) (33), anti-neutrophil elastase clone NP57 (0.1 µg/ml; Dako, Ely, U.K.), anti-mast cell tryptase clone AA1 (1:1000 dilution; Dako), anti-CD68 clone PG-M1 (Dako), anti-CD3 clone UCHT1 (3 µg/ml; BD Biosciences, Oxford, U.K.), anti-eosinophil major basic protein (MBP) clone BMK-13 (0.4 µg/ml; Monosan, Uden, the Netherlands), anti-MUC5AC (20 µg/ml; Millipore), anti-involucrin SY5 (Abcam), and appropriate isotype controls (Dako and BD Biosciences).

Assessment and quantification of immunohistochemical staining

Measurements and counts were performed blind to clinical characteristics. Epithelial, lamina propria, and smooth muscle bundle areas in sections were identified and measured using computer analysis system (Cell F; Olympus). Numbers of positively stained nucleated cells in epithelial and submucosal were counted and expressed per square millimeter. All counts were performed blind to clinical characteristics. Areas <0.1 mm² were considered insufficient to quantify. Cells staining in sequential sections was colocalized by using the computer analysis system. Epithelial and nonepithelial cells within the epithelium were identified by their morphology. For quantitative assessment of MUC5AC expression in the epithelium, a thresholding technique was used based on the hue saturation intensity of MUC5AC staining. The mean percentage area of MUC5AC staining in 10 areas in the epithelium from two sections was taken as the final measurement. This method has previously been validated by our group (25).

CEACAM6 ELISA

Induced sputum supernatant CEACAM6 was measured using a commercial ELISA according to the manufacturer's protocol (Sino Biological, Beijing, China). CEACAM6 standards were analyzed to assess the effects of DTT, and spiking experiments were performed with known concentrations of CEACAM6 across the range of the assay to assess protein recovery. DTT did not affect the CEACAM6 standard curve. The lower limit of CEACAM6 detection was 39.1 pg/ml, and the upper limit was 2500 pg/ml. Samples were diluted in assay diluent before ELISA.

Serum CEACAM6 was measured using commercial ELISA (R&D Systems), which had a higher limit of detection than the Sino Biological kit (20 ng/ml). The lower limit of detection was 0.625 ng/ml.

Neutrophil elastase ELISA

Induced sputum supernatant neutrophil elastase was measured using a commercial ELISA according to manufacturer's protocol (eBioscience, Cheshire, U.K.). The lower limit of neutrophil elastase detection was 0.16 ng/ml, and the upper limit was 10 ng/ml. Assay samples were diluted before ELISA.

Human bronchial epithelial cell stimulation

Primary bronchial epithelial cells (PBEs) were obtained from healthy volunteers. PBEs were cultured on collagen-coated plates in airway epithelial cell basal medium containing a supplement pack (PromoCell). Cells were plated on collagen-coated 24-well plates in media without hydrocortisone and allowed to adhere overnight. The next day cells were treated with 1 μ M dexamethasone (Sigma, Gillingham, Dorset, U.K.) over a 24-h time course. Quantitative PCR was carried using TaqMan probes to investigate CEACAM6 expression with GAPDH used as the housekeeping gene.

For further cytokine and dexamethasone experiments, 6.5-mm diameter 0.4- μ M pore density Transwell plates from Corning Life Sciences (Corning, NY) were collagen coated using 100 μ g/ml PureCol from Advanced BioMatrix (San Diego, CA). Normal human bronchial epithelial cells (NHBEs) were purchased from Lonza and were seeded in Transwells and maintained in serum-free bronchial epithelial cell growth medium (Lonza) for 96 h or until confluent. Thereafter the apical media were removed, and cells were fed basolaterally with PneumaCult complete air-liquid interface (ALI) medium (Stem Cell) and differentiated for a period of 21 d. NHBEs were cultured alone in ALI media or stimulated with rIL-13 (10 ng/ml), rIL-17 (10 ng/ml), and TNF- α (10 ng/ml) in the presence or absence of dexamethasone (1 μ M) for 24 h ($n = 1$ in triplicate).

The human mucoepidermoid lung cancer airway epithelial cell line NCL-H292 (H292; American Type Culture Collection, Manassas, VA) was cultured in RPMI 1640 media (LGC Standards, Middlesex, U.K.) supplemented with 10% FBS (Invitrogen) and antibiotic-antimycotic solution (Invitrogen). H292 cells were cultured alone in RPMI 1640 media or stimulated with recombinant neutrophil elastase (10 nM), or wounded by scratching with a 200- μ l pipette tip. Cells were analyzed for CEACAM6 mRNA at 16 h ($n = 3$) and protein at 24 and 48 h ($n = 5$). All conditions were optimized for time course and concentration (data not shown).

Quantitative RT-PCR

RNA was extracted using RNeasy Plus Mini Kit (Qiagen, U.K.), and RevertAid Kit Sigma was used for cDNA synthesis (Sigma). PCR was performed using TaqMan reagents for CEACAM6 (Hs03645554) and GAPDH (Hs03645554) on the Stratagene Mx3000P (Stratagene). Data were generated with the standard curve method and normalized to GAPDH housekeeping gene.

CEACAM6 flow cytometry

Whole blood containing EDTA was collected from asthmatic and healthy subjects. Serum components were removed by centrifugation at $500 \times g$ for 5 min three times in PBS. A total of 100,000 neutrophils was incubated with 1 μ g of human IgG for 15 min. Cells were then treated with 2.5 μ g/ml anti-CD16b-PE clone CLB-gran11.5 (BD Biosciences), 4 μ g/ml anti-CEACAM6 allophycocyanin clone 439424 (R&D Systems), or appropriate isotype controls, in a fluorescence minus one setup for 30 min. RBCs were lysed in OptiLyse C buffer (Beckman Coulter) according to the manufacturer's instructions, and leukocytes were resuspended in PBS (+0.5% BSA) containing a cell viability dye (30 nM, Sytox green; Sigma) and incubated for 30 min on ice before acquisition. Cells were acquired on a FACSCanto A flow cytometer (Becton Dickinson). Data were analyzed using FlowJo/FACSDiva software version 6. Neutrophils were identified as Sytox⁺, CD16b⁺ cells with homogenous light scatter properties. We subsequently measured CEACAM6 and corresponding isotype geometric mean fluorescence intensity (GMFI) on neutrophils and reported the degree of specific staining (Δ GMFI).

H292 cells were stained with CEACAM6 Ab (4 μ g/ml) or stained with Ig isotype control. Cells were indirectly labeled with Alexa Fluor 594 (Invitrogen). Data were analyzed using FlowJo/FACS DIVA software version 6.

Immunofluorescence

H292 airway epithelial cells were grown to confluence on fibronectin-coated chamber slides and labeled with CEACAM6 Ab (4 μ g/ml) or stained with Ig isotype control as used for flow cytometry. CEACAM6 Ab was indirectly labeled with Alexa Fluor 594 (Invitrogen). Cells were counterstained with DAPI (Sigma), and the slide was mounted using ProLong Gold antifade mountant (Invitrogen). Images were captured using a computer imaging system (Zen; Zeiss), and the intensity of CEACAM6 staining was quantified by grayscale intensity using Cell F imaging software (Olympus). The isotype control was matched for exposure time.

Statistical analysis for clinicopathological data

Statistical analysis was performed using Prism Version 6 (GraphPad, San Diego, CA). Data were tested for normality, and across-group comparisons were made using the ANOVA or Kruskal–Wallis test as appropriate, with Bonferroni correction post hoc testing where required. Reported values are shown as either mean \pm SEM or as a median value with the interquartile range. The Pearson or Spearman rank correlation tests were used to examine the presence of correlations depending on data distribution. The χ^2 test was used to compare categorical demographic data. A p value <0.05 was considered statistically significant.

Results

The clinical demographic data for the subjects are shown in Table I, and inflammatory cell counts in airway subcompartments are shown in Table II.

CEACAM6 gene expression is upregulated in asthma, predominantly in subjects with severe disease

CEACAM6 gene expression was evaluated in microarray analyses of bronchial biopsies from 51 asthmatic and 19 healthy control subjects. CEACAM6 was upregulated in a subgroup of asthmatic subjects, predominantly those with severe disease ($p = 0.038$) (Fig. 1). We have previously demonstrated mutually exclusive Th2 and Th17 inflammatory gene signatures in these asthmatic subjects (10). CEACAM6 gene expression was not related to the expression of a Th2 gene signature in asthma (Supplemental Fig. 1), but did correlate with the Th17 signature metric ($r_s = 0.41$, $p = 0.002$); expression was highest in Th17-high subjects (Supplemental Fig. 1, Table III). Blood neutrophils and FEV₁ (% predicted) correlated positively and negatively, respectively, with CEACAM6 gene expression in asthma ($r_s = 0.411$, $p = 0.030$; $r = -0.326$, $p = 0.033$, respectively) (Table III).

Immunohistochemistry for inflammatory cells and MUC5AC in bronchial biopsies

Inflammatory cell counts for a subset of the subjects studied have been published previously (25). The data for the current patients studied are summarized in Table II together with the data for MUC5AC expression. In brief, consistent with previous reports (34, 35), mast cells were elevated in the airway epithelium of mild asthmatics compared with healthy control subjects ($p = 0.0171$). CD68⁺ macrophages and MUC5AC expression were significantly increased in the epithelium of severe asthmatic subjects compared with healthy controls ($p = 0.040$, $p = 0.019$, respectively). Eosinophil counts were elevated in the bronchial lamina propria of mild asthmatics compared with healthy control subjects ($p = 0.028$). Of note, as shown in Table II, there was no difference in neutrophil counts across the asthmatic subgroups compared with healthy control subjects.

CEACAM6 immunoreactivity in bronchial tissue

We investigated the tissue distribution and cellular localization of CEACAM6 protein in the airways by performing immunohistochemistry with a previously validated, in-house (Genentech)–generated, mouse anti-human CEACAM6 mAb (33). CEACAM6 immunoreactivity was present in both the lamina propria and the airway epithelium (Fig. 2A–C) of both asthmatic and healthy control subjects, but was not seen in the airway smooth muscle. Immunostaining with an isotype control was negative (Fig. 2D).

CEACAM6 immunoreactivity is elevated in the epithelium of asthmatic patients

Suitable epithelial tissue for analysis (epithelial area >0.1 mm²) was present in bronchial biopsies from 12 healthy subjects and 35 patients with asthma (4 mild, 9 moderate, and 22 severe). Within the airway epithelium, both epithelial cells and nonepithelial cells exhibited CEACAM6 immunoreactivity. CEACAM6 expression on

Table I. Baseline demographic data for study subjects

	Healthy Control Subjects	Patients with Mild Asthma (BTS Step 1)	Patients with Moderate Asthma (BTS Steps 2 and 3)	Patients with Severe Asthma ^a (BTS Steps 4 and 5)	p Value ^b
<i>n</i>	24	12	21	40	—
Age (y), mean ± SEM	32.88 ± 2.65	34.17 ± 3.72	33 ± 2.39	45.78 ± 1.86	<0.0001
Sex (M/F), <i>n</i>	11/13	6/6	11/10	26/14	0.50
Asthma duration (y), mean ± SEM	NA	16.35 ± 3.90	17.45 ± 3.06	22.18 ± 2.42	0.33
Inhaled corticosteroid dose (μg), beclomethasone equivalents ^c	NA	0	847.5 ± 131.8	1765 ± 96.84	<0.0001^d
Subjects at BTS step 5	0	0	0	20	<0.0001
Subjects taking long-acting β-agonist	0	0	15	40	<0.0001
Exacerbations in last year, median (range)	NA	0 (0–2)	0 (0–4)	3 (0–12)	<0.0001
Smoking history (pack-year)	0.36 ± 0.26	0.78 ± 0.57	0.54 ± 0.26	0.85 ± 0.37	0.7257
Daytime symptom score, median (range)	NA	0.25 (0–1.25)	0.28 (0–1.21)	1 (0–3.00)	0.013
Daily nighttime symptom score, median (range)	NA	0.25 (0–1.14)	0 (0–0.57)	0.21 (0–3.00)	0.044
Reliever use per week, median (range)	NA	2 (0–34.50)	1 (0–70.0)	30 (0–112.0)	0.0068
Sputum eosinophil count (%), geometric mean (95% CI)	0.44 (0.27–0.73)	2.95 (1.31–6.62)	3.66 (1.42–9.41)	3.16 (1.60–6.22)	0.0012
Sputum neutrophil count (%), geometric mean (95% CI)	33.77 (15.70–72.63)	42.48 (24.10–74.85)	31.16 (16.17–60.03)	56.44 (46.34–68.73)	0.31
Blood eosinophil count (×10 ⁹ /l)	0.12 (0.087–0.17)	0.31 (0.21–0.46)	0.21 (0.15–0.29)	0.31 (0.24–0.40)	0.0021
Blood neutrophil count (×10 ⁹ /l)	3.54 (2.90–4.33)	4.02 (3.24–5.00)	3.73 (3.16–4.40)	4.69 (3.76–5.85)	0.0072
PEF amplitude (% of the mean), mean ± SEM	NA	34.82 (7.01)	24.97 (5.35)	34.77 (4.96)	0.40
FEV ₁ (l)	3.44 ± 0.26	3.38 ± 0.25	3.15 ± 0.21	2.56 ± 0.11	0.0019
FEV ₁ (% predicted)	96.85 ± 6.0	96.3 ± 2.6	86.82 ± 5.1	78.6 ± 27	0.0051
FEV ₁ /FVC ratio (%)	76 ± 4.93	79.26 ± 1.99	69.33 ± 4.17	67.25 ± 1.85	0.081
PC ₂₀ methacholine (mg/ml), geometric mean (95% CI)	> 16	0.85 (0.14–4.95)	1.14 (0.47–2.76)	0.32 (0.12–0.90)	0.080
Serum IgE (kU/l), geometric mean (95% CI)	36.33 (19.81–6.63)	86.51 (38.54–194.2)	112.8 (56.64–224.6)	234.1 (144.6–379.0)	0.13
Subjects with positive skin prick test response	9 (<i>n</i> = 15)	7 (<i>n</i> = 10)	15 (<i>n</i> = 16)	25 (<i>n</i> = 31)	0.28
Subjects with positive skin prick test response to <i>Aspergillus fumigatus</i>	0	1 (<i>n</i> = 10)	2 (<i>n</i> = 16)	8 (<i>n</i> = 31)	0.40
Biopsy gene status (no. of patients)					
Th0	NA	4	9	11	0.0810
Th2	NA	6	3	5	
Th17	NA	0	5	8	

Significant values are shown in boldface.

^a33 of 40 meet the American Thoracic Society criteria for refractory asthma.^bStatistical analysis across asthma groups unless otherwise stated.^cRatio for budesonide Turbuhaler calculated as 1.5.^dModerate compared with severe asthma.

CI, confidence interval; NA, not applicable; PEF, peak expiratory flow.

Table II. Inflammatory cell counts in the airway epithelium and airway lamina propria in healthy control subjects and patients with asthma of varying severity

	Healthy Control Subjects	Patients with Mild Asthma	Patients with Moderate Asthma	Patients with Severe Asthma
Epithelium, cells/mm ²	<i>n</i> = 12	<i>n</i> = 4	<i>n</i> = 9	<i>n</i> = 22
Neutrophil elastase	2.9 (0–17.7)	0 (0–2.5)	1.5 (0–10.0)	4.8 (0–8.3)
Tryptase	3.1 (0–6.1)	21.1 ^a (6.8–35.9)	4.7 (0.8–10.0)	3.6 (0–15.5)
MBP	0.0 (0.0–0.0)	7.7 (0–15.4)	0 (0–4.4)	0 (0–7.8)
CD68	0 (0–6.7)	10 (6.3–23.1)	2.4 (0–4.8)	20.4 ^a (1.9–31.7)
MUC5AC	0.8 (0.1–1.9)	3.8 (1.2–6.7)	1.0 (0.5–2.5)	3.1 ^a (1.1–6.5)
Lamina propria, cells/mm ²	<i>n</i> = 15	<i>n</i> = 8	<i>n</i> = 20	<i>n</i> = 33
Neutrophil elastase	10.4 (4.3–20.6)	3.3 (0.9–7.3)	6.5 (3.0–11.1)	8.1 (2.4–36.9)
Tryptase	14.4 (11.1–21.0)	12.5 (6.7–26.0)	19.7 (10.7–32.0)	10.7 (5.4–23.0)
MBP	4.5 (1.6–11.9)	11.4 ^a (10.0–14.1)	6.8 (2.4–18.2)	8.7 (3.6–24.4)
CD68	4.0 (2.3–5.1)	4.7 (3.6–6.5)	2.7 (1.4–7.2)	4.5 (2.2–13.5)

^a*p* < 0.05 compared with healthy control subjects.

epithelial cells was evident predominantly in the differentiated pseudocolumnar cells and, to a lesser extent, in basal cells (Fig. 2A). CEACAM6 epithelial cell expression was increased significantly in asthmatic patients as a whole compared with that seen in healthy control subjects (*p* = 0.0063) (Fig. 2E). Corroborating the gene expression observations, subgroup analysis demonstrated significant increases of CEACAM6 immunoreactivity within the epithelial cells of patients with severe asthma compared with healthy control subjects (Fig. 2F). There was no difference in the extent of CEACAM6 immunoreactivity in nonepithelial cells within the airway epithelium between asthmatic patients and healthy subjects (Fig. 2G, 2H). In asthmatic patients, the magnitude of epithelial CEACAM6 immunoreactivity correlated positively with CEACAM6 gene expression (*r*_s = 0.719, *p* = 0.001; Table III). It also correlated with CEACAM6 immunostaining in the lamina propria (*r*_s = 0.429, *p* = 0.016), the percentage of MUC5AC expression (*r*_s = 0.417, *p* = 0.040), and sputum CEACAM6 expression (*r*_s = 0.494, *p* = 0.037) (Table IV). Several correlations between epithelial CEACAM6 protein expres-

sion and clinical data were also observed including a positive correlation with asthma duration and reliever β₂-agonist use (Table IV).

CEACAM6 immunoreactivity is elevated in the lamina propria of asthmatic patients

Suitable lamina propria tissue for analysis was available for 15 healthy subjects and 61 patients with asthma (8 mild, 20 moderate, and 33 severe). The number of CEACAM6⁺ cells in the lamina propria was increased in the asthma group as a whole (*p* = 0.0053; Fig. 3A). Subgroup analysis showed significant increases in lamina propria CEACAM6 expression in patients with severe asthma compared with healthy control subjects (*p* = 0.0134; Fig. 3B).

There were significant correlations between CEACAM6 counts in the asthmatic bronchial lamina propria with lamina propria neutrophils (*r*_s = 0.35, *p* = 0.0098), macrophages (*r*_s = 0.54, *p* = 0.004), and sputum neutrophils (*r*_s = 0.42, *p* = 0.0046). No other significant correlations between lamina propria CEACAM6 immunostaining and clinical and/or immunopathological parameters were evident.

FIGURE 1. Bronchial biopsy CEACAM6 gene expression is elevated in bronchial biopsies from severe asthmatics (*n* = 51) relative to healthy control subjects (*n* = 19). **(A)** The overview heatmap data are Agilent log ratio data scaled and centered by row for differentially expressed genes (adjusted *p* < 0.02), and the local cluster was generated by reclustering the rows (i.e., genes) of microarray data for the 30 genes most highly correlated with CEACAM6 and ordering the columns (samples) by increasing average expression level. Clustering was performed by agglomerative algorithm with average linkage summarization. **(B)** Gene-level microarray expression data for CEACAM6 by diagnosis. The *p* value of CEACAM6 differential expression between severe asthma and healthy control subjects is from Student *t* test.

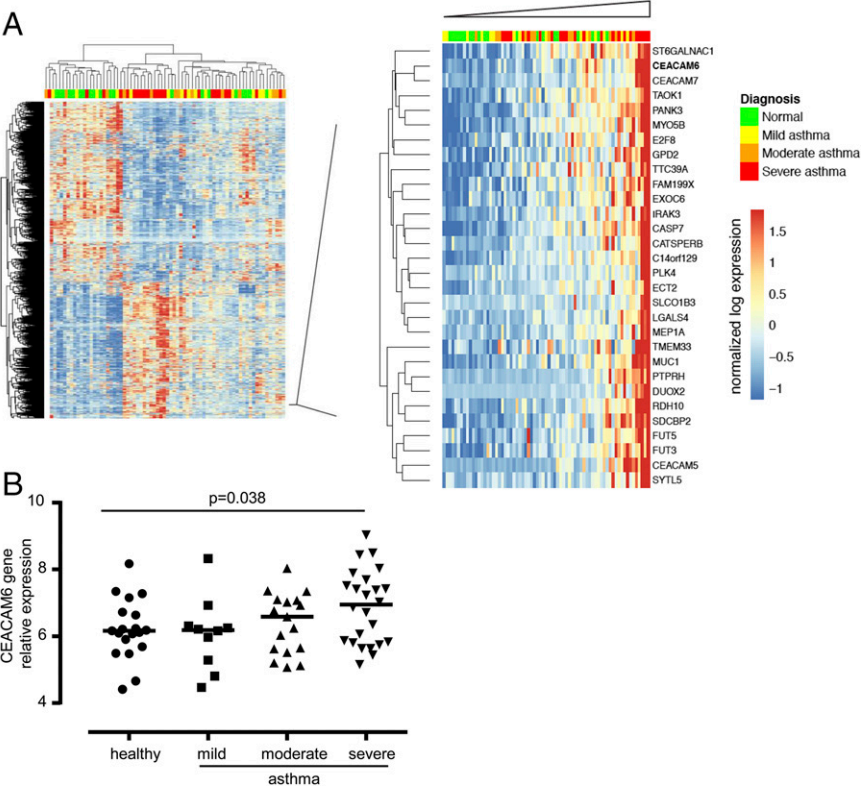


Table III. Correlations between CEACAM6 gene expression and clinicoimmunopathological variables

CEACAM6 Gene Expression	Correlation	<i>r</i>	<i>p</i> Value
Th17 gene signature metric	Pearson	0.41	0.002
Blood neutrophil count, 10 ⁹ /l	Pearson	0.411	0.030
FEV ₁ % predicted	Pearson	−0.326	0.033
CEACAM6 ⁺ epithelial cells/mm ²	Spearman	0.700	0.001
Percentage MUC5AC protein in epithelium	Spearman	0.482	0.043

CEACAM6 immunoreactivity is localized predominantly to neutrophils, eosinophils, and macrophages in the airway lamina propria

Both mononuclear and polymorphonuclear cell staining in the lamina propria was evident (Fig. 4). Colocalization of cell markers in serial 2-μm sections from a subset of six patients with severe asthma showed that approximately a median (IQR) 59.9%

(40.2–92.1%) of *CEACAM6*[−] cells in the lamina propria were neutrophil elastase⁺ neutrophils and 17.3% (2.1–29.4%) were MBP⁺ eosinophils. CD68⁺ macrophages accounted for 6.00% (0.5–11.5%) of *CEACAM6*⁺ cells. Tryptase⁺ mast cells and CD3⁺ T cells did not account for any *CEACAM6*⁺ cells (Fig. 4). The proportion of each inflammatory cell type that expressed *CEACAM6* is also shown in Fig. 4.

FIGURE 2. Epithelial *CEACAM6* immunostaining is elevated in asthmatic patients. **(A)** *CEACAM6* immunostaining in asthmatic airway epithelium; black arrows demonstrate *CEACAM6*⁺ pseudocolumnar epithelial cells and goblet cells, and red arrows highlight *CEACAM6*⁺ nonepithelial cells. **(B)** *CEACAM6* immunoreactivity in the lamina propria in asthma. **(C)** Limited *CEACAM6* immunostaining in healthy airway epithelium. **(D)** Negative isotype control staining. **(E and F)** The extent of *CEACAM6* epithelial cell-localized immunostaining in healthy control subjects and all asthmatic subjects and asthmatic subgroups, respectively. **(G and H)** The extent of *CEACAM6* immunostaining in nonepithelial cells within the airway epithelium. Immunohistology is shown at original magnification ×400 (A and B) or ×200 (C and D). At least two tissue sections >10 μm apart were immunostained for each subject (i.e., immunostained in duplicate).

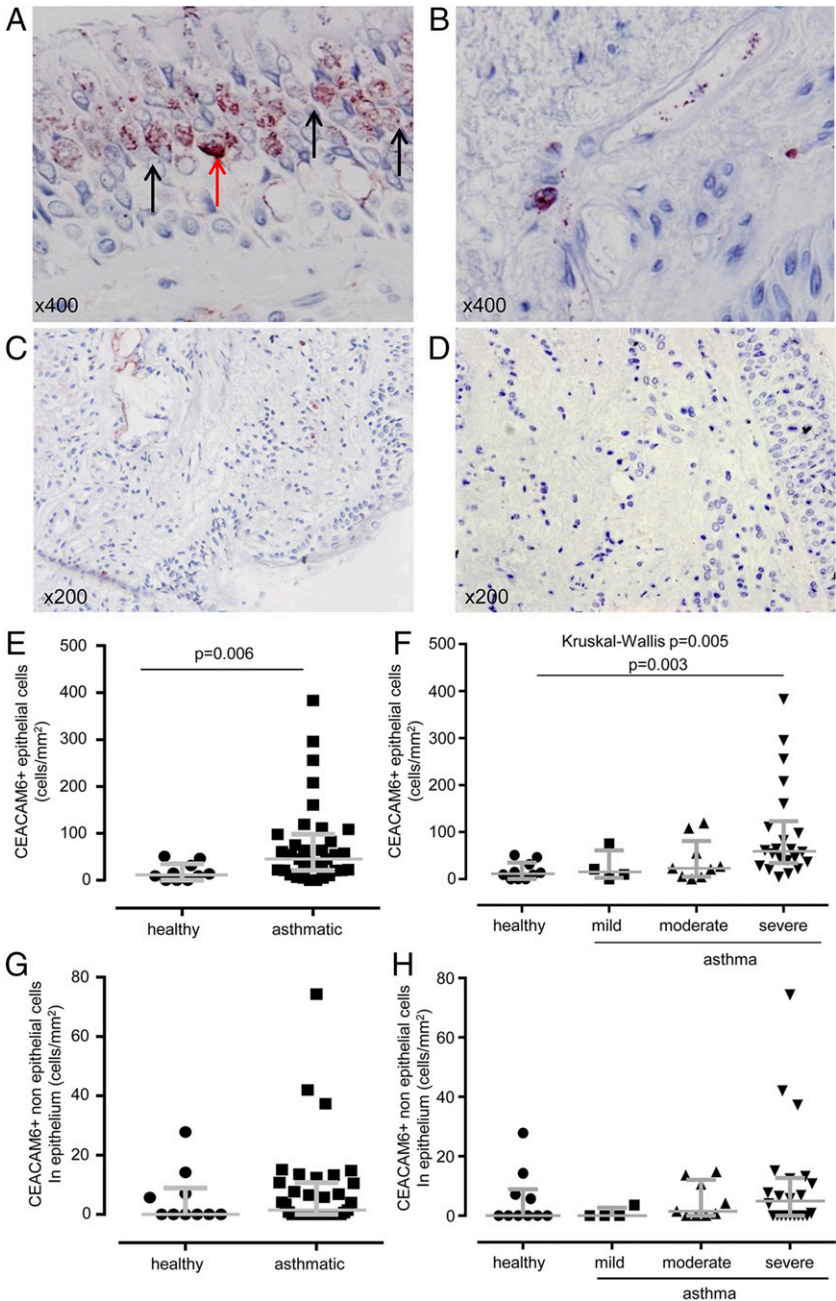


Table IV. Correlations between epithelial CEACAM6 protein expression and clinicoimmunopathological variables

CEACAM6 ⁺ Epithelial Cells/mm ²	<i>r</i> Spearman	<i>p</i> Value
CEACAM6/sputum, ng/ml	0.494	0.037
CEACAM6 lamina propria/mm ²	0.429	0.016
Percentage MUC5AC protein in epithelium	0.417	0.040
Duration of asthma history, y	0.456	0.008
Reliever β_2 agonist use	0.499	0.025
Total IgE	−0.392	0.027
Percentage historical sputum eosinophils	−0.560	0.050

Neutrophil CEACAM6 expression is increased in severe asthma

To investigate further whether the increased lamina propria CEACAM6 expression in severe asthma was related to neutrophil CEACAM6 expression, we compared the proportion of neutrophils expressing CEACAM6 among healthy control, mild asthma, moderate asthma, and severe asthma subjects using seven subjects from each group. Of note, as shown in Table II, there was no difference in epithelial or lamina propria neutrophil counts between healthy and asthma or across-asthma severity. However, the percentage of neutrophils expressing CEACAM6 was significantly increased in severe asthma compared with healthy control subjects and moderate asthma (Fig. 5).

CEACAM6 immunoreactivity does not localize to goblet cells or squamous cells in the airway epithelium

CEACAM6 epithelial expression correlated with MUC5AC expression. However, colocalization of CEACAM6 with MUC5AC

and involucrin (a marker of squamous metaplasia) in a subset of 20 patients (6 healthy control and 14 asthmatic subjects) showed that CEACAM6⁺ cells in epithelium were not goblet cells or squamous cells (Supplemental Fig. 2). Furthermore, involucrin staining was sparse and similar in healthy control and asthmatic subjects, suggesting that squamous metaplasia is not a feature of asthma and is not associated with CEACAM6 expression (Supplemental Fig. 2).

Soluble CEACAM6 in blood and sputum

CEACAM6 is shed from cell surfaces and can be measured in serum (36). To assess whether the tissue CEACAM6 signal was reflected in blood or sputum supernatant, we measured CEACAM6 in these compartments using ELISA. Sputum samples were available from 8 healthy control subjects and 35 asthmatic patients (8 mild, 10 moderate, and 17 severe). Serum samples were available from 10 healthy control subjects and 48 asthmatic patients (7 mild, 11 moderate, and 30 severe). CEACAM6 was more than the level of detection in nearly all samples. However, overall, the concentrations of CEACAM6 in sputum and serum were similar in all groups (Supplemental Fig. 3A).

Blood neutrophil CEACAM6 expression

The biopsy data demonstrate CEACAM6 is upregulated on neutrophils. Whether neutrophils express CEACAM6 in blood was assessed in blood from 11 healthy control subjects and 15 asthmatic patients. However, there was no difference in CEACAM6 expression on blood neutrophils (Supplemental Fig. 3B).

CEACAM6 epithelial expression is not upregulated by activation of Th2 or Th17 cytokines or steroid exposure

Because steroid treatment is a potential confounding factor in all asthma studies, we sought to investigate the effect of dexamethasone on epithelial CEACAM6 expression. Treatment of PBECs ($n = 2$ donors) with dexamethasone (1 μ M) had no effect on CEACAM6 mRNA expression over a time course of 2–24 h (Supplemental Fig. 4A). Furthermore, to investigate triggers for CEACAM6 epithelial expression, we stimulated NHBE cells grown to ALI with rIL-13 and rIL-17 \pm TNF- α (all 10 ng/ml), but failed to observe changes in CEACAM6 mRNA levels after 24 h ($n = 1$ performed in triplicate). Dual treatment with cytokines and steroids showed no difference from baseline expression (Supplemental Fig. 4B).

CEACAM6 expression in H292 airway epithelial cells is upregulated by wounding and by neutrophil elastase

Wounding H292 cells significantly upregulated CEACAM6 mRNA expression at 16 h ($p = 0.013$; $n = 3$ independent experiments performed in triplicate; Fig. 6A), with significant increases in cell surface protein expression analyzed by flow cytometry at 24 and 48 h ($p = 0.015$, $p = 0.035$, respectively; $n = 5$ independent experiments performed without replicates; Fig. 6B). Interestingly, total CEACAM6 analyzed by immunofluorescence was upregulated at only 48 h postwounding (Fig. 6C, 6D).

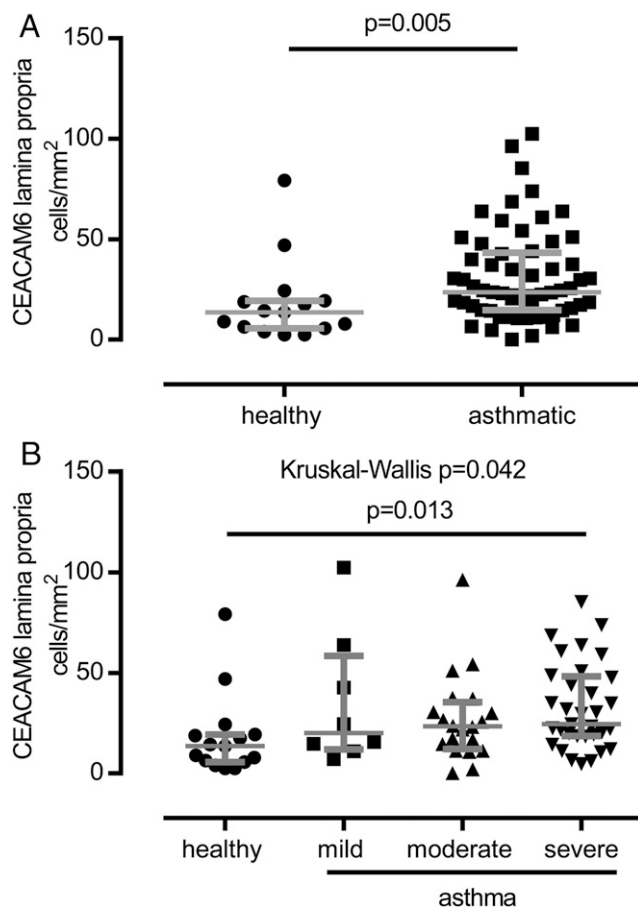


FIGURE 3. The extent of CEACAM6 immunostaining in the lamina propria in health versus asthma (A) and in health versus asthma by severity (B). Horizontal bars represent median and IQR. Immunostained in duplicate.

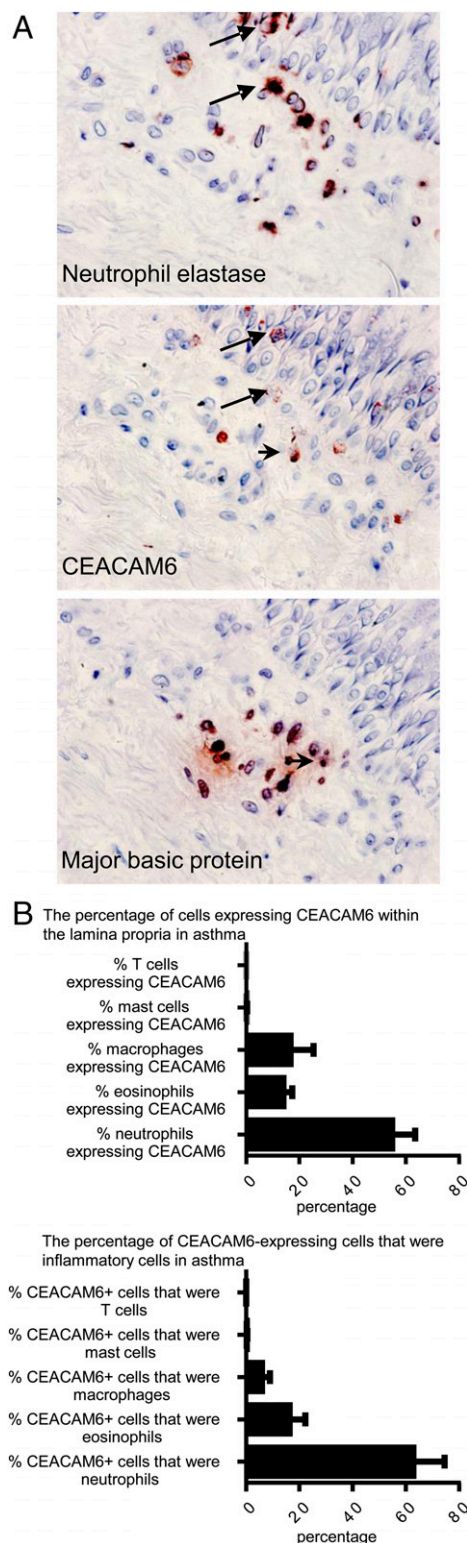


FIGURE 4. Colocalization of CEACAM6 to inflammatory cells within the lamina propria of severe asthmatic subjects. **(A)** Three sequential 2- μ m sections demonstrating colocalization of neutrophil elastase⁺ neutrophils (top panel), CEACAM6 (middle panel), and MBP⁺ eosinophils (bottom panel) shown by arrows. **(B)** The percentage of neutrophils, eosinophils, macrophages, mast cells, and T cells expressing CEACAM6 (top graph) and the percentage of CEACAM6 cells that were inflammatory cells in a subset of six patients with severe asthma (mean \pm SEM). Immunohistology is shown at original magnification $\times 400$ (A). Immunostained in duplicate.

There was no significant increase in CEACAM6 mRNA at 16 h ($n = 3$; Fig. 6A) or cell surface protein expression at 24 h ($n = 5$; Fig. 6B) after human neutrophil elastase (HNE) stimulation. However, at 48 h, both cell surface and total CEACAM6 expression were increased significantly by neutrophil elastase ($p = 0.005$, $p = 0.019$, respectively; Fig. 6B–D).

Sputum neutrophil elastase was measured in limited remaining sputum supernatants and was significantly increased in severe asthmatic patients ($n = 18$) compared with healthy control subjects ($n = 6$) ($p = 0.038$) (Supplemental Fig. 4C). However, there were no significant correlations between sputum neutrophil elastase and CEACAM6 expression in severe asthma, or between sputum elastase and sputum neutrophil numbers (data not shown).

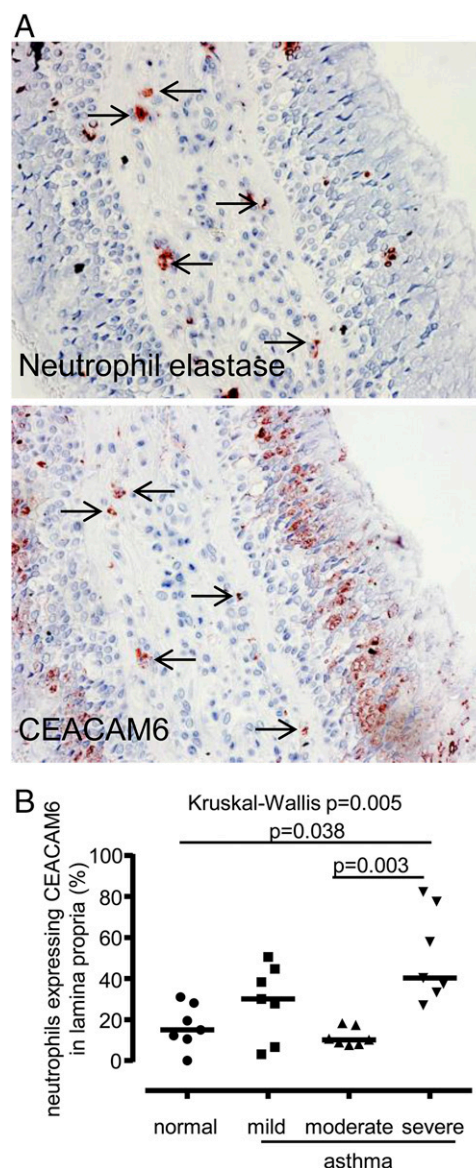


FIGURE 5. Colocalization of CEACAM6 to neutrophils within the lamina propria of healthy control and asthmatic subjects with varying disease severity. **(A)** Two sequential 2- μ m sections demonstrating colocalization of CEACAM6 to neutrophil elastase. **(B)** The graph shows the percentage of neutrophils expressing CEACAM6 in the lamina propria of healthy control subjects and the asthmatic subgroups ($n = 7$ for each). Horizontal bars represent medians. Immunohistology is shown at original magnification $\times 200$ (A). Immunostained in duplicate.

Discussion

To our knowledge, this is the first study of CEACAM6 family expression in the airways of patients with asthma. Initial analysis identified increased CEACAM5, CEACAM6, and CEACAM7 gene expression in our previously reported bronchial biopsy microarray dataset (10, 25, 26). This was increased predominantly in severe asthma. Analysis of bronchial biopsy tissue for CEACAM6 protein expression using immunohistochemistry has demonstrated that it is upregulated in both airway epithelial cells and lamina propria neutrophils in patients with severe asthma. Bearing in mind the known functions of CEACAMs, and the observed clinicopathological correlations in this study, CEACAM6 has the potential to play an important role in ongoing treatment-resistant severe asthma through neutrophil- and epithelial-dependent pathways.

CEACAM6 protein expression in airway epithelial cells was increased in severe asthma and correlated with CEACAM6 gene expression in the microarray dataset. The epithelial CEACAM6 protein expression was located predominantly in columnar epithelial cells, and although CEACAM6 was not expressed by goblet cells, there was a correlation with MUC5AC immunostaining, suggesting that upregulation of CEACAM6 arises in association with goblet cell hyperplasia. The factors that regulate CEACAM6 expression on epithelial cells are poorly understood. However, its upregulation in premalignant and malignant epithelial cells (23) indicates an association with epithelial metaplasia. In asthma, although there is frequently evidence of epithelial disruption, there are also some features of metaplasia with goblet cell hyperplasia (9, 37) and, in severe asthma, increases in epithelial area and proliferation (38). The increased expression of CEACAM6 in the airway epithelium in severe asthma in our study provides further evidence of epithelial metaplasia in asthma, although there was no difference in involucrin staining, a marker of squamous metaplasia.

CEACAM6 epithelial protein expression correlated with the length of asthma history, raising the possibility that repeated cycles of epithelial damage and repair in conjunction with chronic exposure to proinflammatory cytokines leads to CEACAM6 upregulation in columnar epithelial cells. In support of this, transgenic mice expressing human CEACAM6 upregulate CEACAM6 on alveolar epithelial cells after injury induced by diverse agents (39). To investigate the factors that might regulate CEACAM6 expression in epithelial cells, we investigated the effects of cytokines, corticosteroids, neutrophil elastase, and wounding in human epithelial cells. Cytokines (IL-17A, TNF- α , IL-13) and corticosteroids were without effect, suggesting that the expression levels present in the tissue are not a direct consequence of cytokine exposure or treatment. Both epithelial wounding and neutrophil elastase upregulated CEACAM6 expression, and neutrophil elastase was increased in asthmatic sputum. This raises the possibility that both epithelial wounding, for example, in response to allergen exposure and/or viral infection, and neutrophil activation contribute to increased CEACAM6 expression evident in the airway epithelium in tissue.

In this study, we did not observe a difference in airway mucosal neutrophil numbers in asthma when compared with healthy controls, similar to many previous studies (25, 40–43). This suggests that the idea of neutrophil-driven asthma based on sputum cell counts may be misplaced (44). However, our study suggests that the phenotype of neutrophils in the lamina propria in severe uncontrolled asthma is altered in that CEACAM6 expression is markedly increased compared with patients with moderate controlled asthma. Investigating the functional consequences of increased neutrophil CEACAM6 expression in severe asthma will be

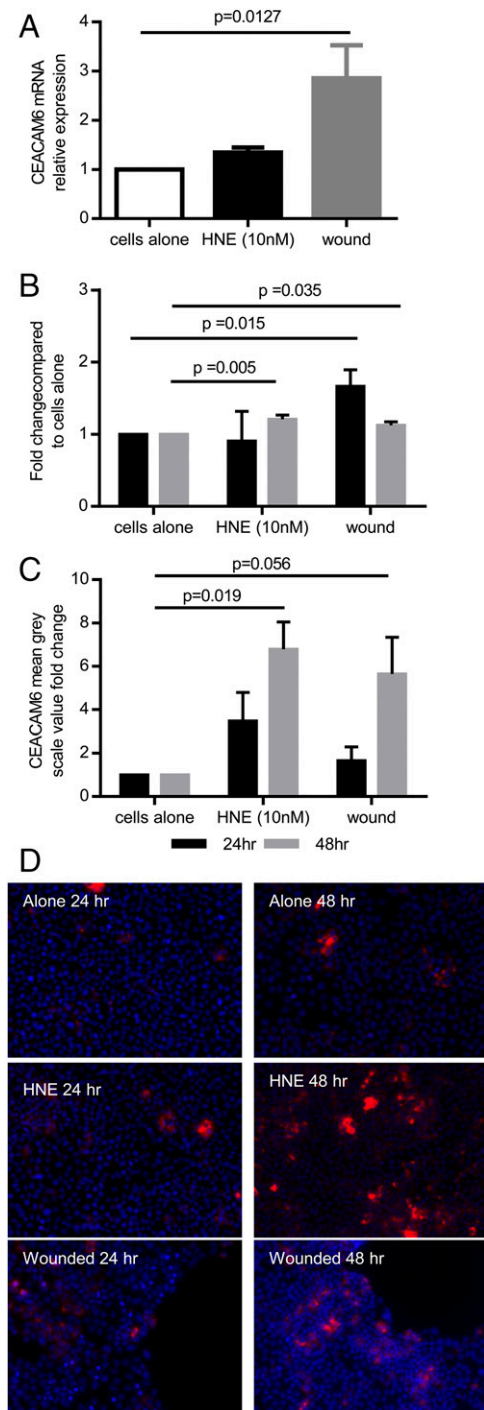


FIGURE 6. H292 airway epithelial cell CEACAM6 epithelial expression is upregulated by lung injury and HNE. **(A)** CEACAM6 mRNA expression in H292 cells at 16 h after wounding or exposure to HNE (10 nM; $n = 3$ independent experiments for each performed in triplicate). **(B)** Flow cytometry demonstrating the fold change in cell surface CEACAM6 expression after wounding or exposure to HNE (10 nM) at 24 and 48 h ($n = 5$ independent experiments for each performed once). **(C)** Quantification of immunofluorescence showing fold change in total CEACAM6 expression after wounding or HNE (10 nM) exposure at 24 (gray bars) and 48 h (black bars) ($n = 5$ independent experiments for each, performed once). **(D)** Representative immunofluorescence images from **(C)** of CEACAM6 expression (Alexa Fluor 594 red) in H292 cells (DAPI blue) at 24 and 48 h. Immunohistochemistry is shown at original magnification $\times 200$. Data are plotted as mean \pm SEM.

important in understanding the biological significance of our observations. Homophilic binding of CEACAM6 to N-domain CEACAM6 peptides can potentially lead to neutrophil activation with the generation of superoxide (13). We can therefore speculate that CEACAM6 expressed by neutrophils may form a homophilic interaction with CEACAM6 that is overexpressed on airway epithelial cells, potentially contributing to neutrophil activation and consequent epithelial damage and dysfunction. Because neutrophil numbers in airway glands in fatal asthma correlate with the extent of mucous plugging (8), and epithelial CEACAM6 expression correlates in this study with MUC5AC expression, it is plausible that CEACAM6-dependent neutrophil-epithelial cell cross-talk promotes mucous hypersecretion and contributes to the persistent airflow obstruction and exacerbations that characterize severe disease.

In contrast with tissue CEACAM6 expression, surface CEACAM6 expression on neutrophils from the whole blood of asthmatic patients showed no difference when compared with healthy control subjects, and was expressed highly in both groups. Expression of surface CEACAM6 on the neutrophil surface is increased by several stimuli including fMLF, Ca^{2+} ionophore, and PMA, as a result of mobilization from intracellular granules, and surface CEACAM6 is thus considered a marker of neutrophil activation (45). Whether the increase of the CEACAM6⁺ neutrophil phenotype in asthmatic airways occurs as a failure to downregulate expression on tissue neutrophils in asthma versus health, or results from local factors stimulating CEACAM6 expression remains unclear. However, the latter appears more likely because CEACAM6 was also increased in the epithelium in asthma. Corticosteroids did not alter CEACAM6 expression in cultured primary human bronchial epithelial cells, suggesting that the increased expression within the epithelium observed in the tissue in severe asthma is unlikely to occur because of corticosteroid treatment.

CEACAM6 gene expression levels were associated with our previously described Th17 gene metric, but were not related to the expression of a Th2 gene metric described in these patients previously (10, 25). Furthermore, there were negative correlations between epithelial CEACAM6 immunostaining with sputum eosinophil counts and total IgE, markers of Th2-high disease. IL-17 or IL-13 did not directly upregulate CEACAM6 epithelial gene expression, but understanding the factors that regulate CEACAM6 gene expression and its relationship to the activity of other cytokines will be of great interest in future studies.

In summary we have shown that CEACAM6 is upregulated in severe asthma, predominantly on airway neutrophils and airway epithelial cells. Our data suggest that although neutrophil numbers in the airway tissue remain consistent across health and asthma, their variation in CEACAM6 expression provides evidence of an altered neutrophil phenotype in severe asthma. CEACAM6 may therefore contribute to the pathology of treatment-resistant severe asthma via both neutrophil and airway epithelial cell-dependent pathways.

Disclosures

The authors have no financial conflicts of interest.

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