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Original Research

ASTHMA

Synthetic Response of Stimulated Respiratory Epithelium

Modulation by Prednisolone and iKK2 Inhibition

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Background: **The airway epithelium plays a central role in wound repair and host defense and is implicated in the immunopathogenesis of asthma. Whether there are intrinsic differences between the synthetic capacity of epithelial cells derived from subjects with asthma and healthy control subjects and how this mediator release is modulated by antiinflammatory therapy remains uncertain. We sought to examine the synthetic function of epithelial cells from different locations in the airway tree from subjects with and without asthma and to determine the effects of antiinflammatory therapies upon this synthetic capacity.**

Methods: **Primary epithelial cells were derived from 17 subjects with asthma and 16 control subjects. The release of 13 cytokines and chemokines from nasal, bronchial basal, and air-liquid interface differentiated epithelial cells before and after stimulation with IL-1β, IL-1β and interferon-γ, or Poly-IC (Toll-like receptor 3 agonist) was measured using MesoScale discovery or enzyme-linked immunosorbent assay, and the effects of prednisolone and an inhibitor of nuclear factor κ-B2 (IKK2i) were determined.**

Results: **The pattern of release of cytokines and chemokines was significantly different between nasal, bronchial basal, and differentiated epithelial cells but not between health and disease. Stimulation of the epithelial cells caused marked upregulation of most mediators, which were broadly corticosteroid unresponsive but attenuated by IKK2i.**

Conclusion: **Synthetic capacity of primary airway epithelial cells varied between location and degree of differentiation but was not disease specific. Activation of epithelial cells by proinflammatory cytokines and toll-like receptor 3 agonism is attenuated by IKK2i, but not corticosteroids, suggesting that IKK2i may represent an important novel therapy for asthma.**

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Abbreviations: ALI = air-liquid interface; ANOVA = analysis of variance; BEGM = bronchial epithelial growth medium; ELISA = enzyme-linked immunosorbent assay; IFN = interferon; IκB = inhibitory κB; IKK = IκB kinase; NF-κB = nuclear factor κB; TLR = Toll-like receptor; TNF = tumor necrosis factor

The airway epithelium is at the interface between the host and the environment, plays a critical role in normal wound repair, and is implicated as key in the immunopathogenesis of asthma.[1] Epithelial cells in vivo are in an activated state, with increased expression of chemokines such as CXCL8[2] and CCL11.[3] Structural changes observed consistently in the asthmatic epithelium include increased permeability,[4] reduced ciliary beat frequency and coordinated cilia movement, increased cell protrusion and cytoplasmic blebbing,[5] goblet cell hyperplasia,[6] increased mucin production,[7] and increased levels of epithelial proliferation and apoptosis[8] when compared with nonasthmatic epithelium.

Whether these abnormalities persist in vitro is important to determine the relative contribution of the asthmatic environment and intrinsic changes in cellular behavior in defining disease expression. Following wounding, asthmatic epithelium demonstrates aberrant repair, dyssynchronous mitosis,[9] and defective epithelial tight junctions, suggesting persistence of abnormalities in wound repair.[10] In contrast, whether asthmatic epithelial cells have an enhanced synthetic response is contentious. Some reports have found

constitutive chemokine and cytokine release by epithelial cells from subjects with asthma compared with healthy control subjects is upregulated,[11] downregulated,[12] or unchanged.[9] Similarly, both an increased[13] and deficient[14] interferon (IFN)-β response following exposure of epithelial cells from subjects with asthma to virus is reported. The phenotype of epithelial cells may also vary throughout the airway tree as demonstrated by differences in transepithelial resistance between epithelial cells from conducting airways and the nose.[13] Therefore, the synthetic function of asthmatic epithelium from different sites within the airway and their response to antiinflammatory therapy need to be further defined.

Inflammatory gene expression often involves the transcription factor nuclear factor κB (NF-κB), and this signaling pathway represents a site for antiinflammatory intervention. Phosphorylation of the inhibitory κB (IκB) proteins by the IκB kinase (IKK) 2-containing IKK complex and subsequent degradation of the IκB proteins are prerequisites for NF-κB activation. Therefore, inhibition of IKK2 would specifically prevent NF-κB transcription and signaling. One of the mechanisms of action of glucocorticosteroids also involves targeting the NF-κB pathway, and glucocorticoids are the most effective antiinflammatory treatments for asthma.[15] Although the response to these compounds has been well characterized in inflammatory cells within the airway, there is a lack of data examining the response to glucocorticosteroids in primary epithelial cells.[16]

We, therefore, hypothesized that synthetic capacity would be altered in primary airway epithelial cells from subjects with asthma vs healthy subjects and that there would be differential effects of antiinflammatory therapy. To test our hypothesis, we aimed to examine: (1) the synthetic function of airway epithelial cells from different locations in health vs disease with and without stimulation by measuring a panel of epithelial-derived chemokines and cytokines,[14,17,18] and (2) to determine the effects of corticosteroids and novel antiinflammatory therapies upon the synthetic capacity of these epithelial cells.

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Materials and Methods

Subjects

Subjects were recruited from Glenfield Hospital, Leicester, England and by local advertising. Asthma was defined according to GINA (Global Initiative for Asthma) guidelines.[19] Subject characterization included demographics, spirometry, allergen skin prick tests, sputum induction, methacholine bronchial challenge, nasal brushings, and bronchoscopy. The study was approved by the Leicestershire ethics committees, and all patients gave their written informed consent.

This study was conducted in accordance with the amended Declaration of Helsinki. The Leicestershire, Rutland, and Northamptonshire ethics committee (ethics reference 4977/project approval number 6347) approved the protocol, and written informed consent was obtained from all patients.

Epithelial Cell Culture

Epithelial cells were obtained from nasal and bronchial brushings from the second- or third-generation bronchi and were grown on 12-well tissue culture plates in bronchial epithelial growth medium (BEGM; Lonza Group Ltd), including supplement SingleQuot BulletKit (Lonza Group Ltd), 0.3% Fungizone antimycotic (Life Technologies Corporation), and 1% antibiotic-antimycotic (Life Technologies Corporation) for 2 to 7 days. Basal cells were then expanded into 75-cm^2 flasks and upon confluence seeded at 10^5 cells/cm^2 on 1.2-cm^2-diameter transwell clear inserts (Corning Incorporated) under BEGM for 2 days. All culture surfaces were collagen coated (Nutacon B.V.). After reaching confluence, the basal cell monolayer was fed on the basolateral side only with air-liquid interface media (ALI) media. This ALI media consisted of 50% BEGM and 50% hi-glucose minimal essential medium (Life Technologies Corporation) containing 100 nM retinoic acid (Sigma-Aldrich Co LLC), including supplements as previously detailed. Supplements were removed 24 h prior to experiments. Nasal and bronchial basal epithelial cells were characterized using immunofluorescence for cytokeratin 5 and 14 expression (Abcam plc). Spontaneous mucus production occurred from 14 to 21 days, suggesting the presence of goblet cells, and ciliation occurred between 25 and 35 days. There was a 100% success rate of nonasthmatic and asthmatic cultures from the initial brushing to the passage into ALI and a 58% success rate of these ALI cultures becoming ciliated. Differentiated bronchial epithelial cells were characterized using high-speed video microscopy for the presence of active cilia

Mediator Analysis

Mediators from basal and differentiated epithelial cells were analyzed using both multiplex and single enzyme-linked immunosorbent assay (ELISA) kits. We measured IL1β, tumor necrosis factor (TNF)-α, CCL2, CXCL8, CXCL10, CCL11, CCL13, CCL17, CCL22, and CCL26 (MesoScale Discovery [MSD]; Meso Scale Diagnostics, LLC). Limits of detections were 2.4 to 10,000 pg/mL. ELISA kits analyzing single mediators were used to examine CCL5 (R&D Systems, Inc) and IFN-β (Pestka Biomedical Laboratories, Inc) production from epithelial cells. Limits of detection for the CCL5 and IFN-β ELISAs were 31.2 to 1,000 pg/mL and 25 to 2,000 pg/mL.

Study Protocol

The compounds IKK2i (100 nM, 300 nM, 1 μM, or 3 μM), (gifts from GlaxoSmithKline, Stevenage, England), prednisolone (10 nM, 100 nM, 1 μM, 10 μM) (GlaxoSmithKline) were added where appropriate to cell cultures for 1 h before the addition of any stimulus. Stimuli used included Poly-IC (0.5, 2.5, 12.5, 25 μg/mL) (InvivoGen) with Poly-dIdC (0.5, 2.5, 12.5, 25 μg/mL) (Sigma-Aldrich Co LLC) as a negative control, IL-1β (10 ng/mL) or IL-1β (10 ng/mL) and IFN-γ (10 ng/mL) (R&D Systems). Nasal epithelial basal cells were used only to obtain a dose response to prednisolone and poly IC. Bronchial epithelial basal and differentiated cells were then used to examine the response to the full panel of compounds/stimuli. Epithelial basal cells were cultured on 24-well culture plates, and compounds/stimuli were added to the media. Differentiated epithelial cultures were cultured on transwells with compounds/stimuli added basolaterally. Cell culture supernatants were collected from all experiments after 24 h. Mediator concentrations were corrected for the volume of growth medium and cell count for each condition. Cell viability was assessed using trypan blue.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software, Inc). Constitutive levels of mediators were log normally distributed and, thus, expressed as geometric mean (95% CI). Therefore, stimulated levels of mediators were expressed as fold change over constitutive levels. Paired and unpaired data were analyzed using paired and unpaired *t* tests, respectively. Comparison across groups was assessed using analysis of variance (ANOVA). Differences were considered significant when $P \leq .05$. Correction for multiple comparisons was not conducted.[20]

Results

The clinical characteristics of the subjects with asthma and healthy control subjects are as shown in e-Table 1. The patterns of cytokine and chemokine expression constitutively released over 24 h by nasal, basal bronchial, and differentiated ALI culture bronchial epithelial cells were significantly different for IFN-β, IL-1β, CCL2, CCL5, and CXCL8 (e-Table 2, Fig 1). These differences between cell types were similar for cells derived from subjects with and without disease, but no differences were observed between subjects with asthma and control subjects (e-Figs 1a-1c, e-Table 3). No differences were found between subjects who were treated with corticosteroids and those who were corticosteroid naive (data not shown).

There was a concentration-dependent release of mediators by nasal basal epithelial cells in response to poly-IC activation compared with the Poly-dIdC control as illustrated in e-Fig 2. The concentration of Poly IC that induced maximal mediator release above constitutive levels in nasal basal cells was then used to stimulate bronchial basal and differentiated cells. Stimulation of the epithelial cells by poly IC, IL-1β or IL-1β, and IFN-γ significantly increased mediator levels above constitutive levels for the majority of mediators, and the pattern of mediator release was distinctive for the stimulus and epithelial cell type (Tables 1-3). Differences were only observed in four of the 111 parameters measured between nonasthmatic and asthmatic groups in any of the cell types examined (e-Tables 4-6).

Bronchial basal epithelial cell mediator release in response to Poly IC or IL-1β and IFN-γ activation were inhibited by IKK2i in a concentration-dependent manner but not by prednisolone (e-Fig 3). The addition of inflammatory stimuli and compounds did not affect cell viability. Prednisolone (10 μM) and IKK2i (1 μm) were used to assess the effect upon basal cell and ALI epithelial synthetic capacity. IKK2i reduced the release of nearly all mediators by basal and ALI epithelial cells in response to poly-IC, IL-1β or IL-1β, and IFN-γ, and the effect was greater in basal epithelial cells. Prednisolone had minimal effects upon the mediator release (e-Fig 4, Tables 4-6). Differences between efficacies of the IKK2 inhibitor and prednisolone were significant for the majority of mediators and stimuli for basal cells (e-Table 7). For poly IC-stimulated ALI epithelial cells, differences between the efficacy of the IKK2 inhibitor and prednisolone were significant for CCL11, CXCL10, CCL2, CCL4, CCL5, ($P < .001$), CCL17 ($P < .01$), and CCL26 and CCL22, ($P < .05$) (e-Table 8).

Discussion

We report here for the first time, to our knowledge, differences in the constitutive production of chemokines and cytokines in vitro between nasal basal, bronchial basal, and bronchial differentiated epithelial cells, highlighting the importance of the location of the airway epithelium in relation to its mediator production. We were unable to demonstrate differences in the constitutive release of mediators from epithelial cells derived from subjects with asthma vs healthy control subjects from any of the epithelial cell types. There was marked up-regulation in mediators in response to proinflammatory cytokines and toll-like receptor (TLR)-3 stimulation, which was greater in nasal than bronchial epithelial cells. However, we did not identify differential response to these stimuli between health and disease. This increased expression was broadly corticosteroid unresponsive in epithelial cells from both subjects with asthma and healthy control subjects, suggesting this activation of synthetic capacity is via corticosteroid-insensitive pathways that are not disease specific. In contrast, the release of these mediators was markedly attenuated by IKK2 inhibition, suggesting that this novel antiinflammatory therapy may have potential to modulate corticosteroid-unresponsive pathways in asthma.

The view that intrinsic differences persist in vitro between epithelial cells from subjects with asthma vs healthy control subjects is controversial. Here we add

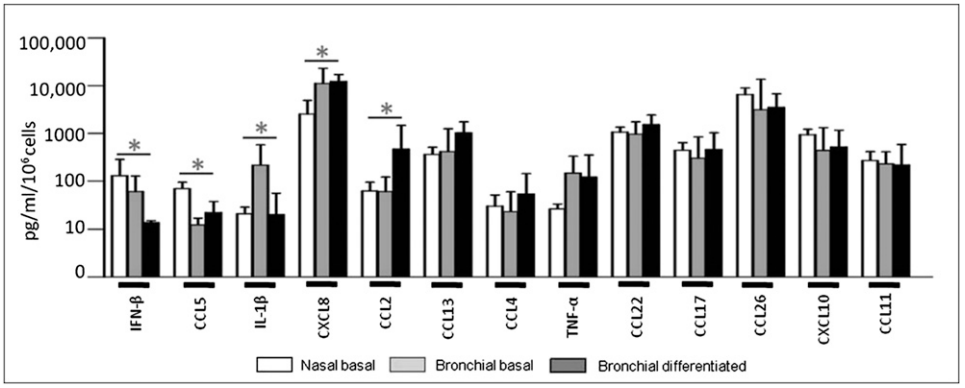

FIGURE 1. Constitutive mediator concentration between primary epithelial cell types. Constitutive expression (pg/mL/10^6 cells) after 24 h of mediators from nonasthmatic and asthmatic nasal basal (white), bronchial basal (gray), and bronchial differentiated (black) epithelial cells. Cytokines are ordered according to the statistical difference of mediators between cell types (*$P \leq .05$) (n = 6 nasal basal, n = 17 bronchial basal, and n = 21 bronchial differentiated epithelial cells). IFN = interferon; TNF = tumor necrosis factor.

to this debate, as our data were unable to identify differential expression of a broad range of chemokines or cytokines from primary epithelial cells in health compared with disease either constitutively or following stimulation.

Deficiency in type 1 and 3 IFNs following viral infection in asthma is proposed to be a fundamental impairment in the innate immune response promoting persistence of a viral infection and the development of an exacerbation of asthma.[14,21] This deficient response may be in part due to chronic airway inflammation in asthma or due to intrinsic differences in asthmatic epithelium. However, we and others[13] were unable to confirm this finding in vitro. Taken together, these data suggest that the interferon release by epithelial cells is heterogeneous, and although this abnormality in innate immunity may be important in some individuals, it is unlikely to represent a defining characteristic of asthma.

Interestingly there was a differential synthetic capacity of epithelial cells from different locations and states of differentiation. This was particularly apparent for IFN-β, CCL5, IL-1β, CXCL8, and CCL2. These may represent important functional differences in wound repair and host defense. In addition to these differences in constitutive mediator release, there

Table 1—*Mediator Concentration Between Primary Epithelial Cell Types Following Poly IC Stimulation*

	Poly IC (12.5 μg/mL) Stimulated Epithelial Cells, Fold Change Over Constitutive Levels			
Mediators	Nasal Basal (n = 6)	Bronchial Basal (n = 17)	Bronchial Differentiated (n = 17)	*P* Value
IFN-β	3.1 (1.0-9.6)[a]	4.8 (2.2-10.6)[b]	1.1 (0.9-1.5)	< .01[c]
CCL5	534.6 (274.0-1043.0)[b]	994.6 (549.3-1801.0)	42.6 (14.5-125.3)[b]	< .01[b,c,d]
IL-1β	35.5 (11.1-111.3)[b]	2.5 (1.4-4.4)[e]	2.8 (1.1-7.1)[a]	< .01[a,d,f]
CXCL8	25.9 (13.1-51.2)[b]	4.7 (2.51-8.9)	2.1 (1.5-2.7)[b]	< .01[b,c,d,f]
CCL2	52.5 (23.7-116.2)[b]	8.0 (3.0-21.2)	6.4 (2.7-15.2)[b]	.02[b,d,f]
CCL13	18.3 (10.8-30.8)[b]	5.7 (1.7-19.5)[e]	1.7 (1.3-2.2)[b]	< .01[b,c,d]
CCL4	485 0.0 (219.6-1071.0)[b]	135.1 (50.1-364.8)	18.1 (6.8-48.3)[b]	< .01[b,c,d]
TNF-α	145.3 (41.1-513.1)[b]	11.1 (6.3-19.5)	5.0 (2.4-10.3)[b]	< .01[b,d,f]
CCL22	25.0 (14.4-43.5)[b]	5.2 (2.3-11.6)	2.2 (1.7-2.9)[b]	< .01[b,c,d,f]
CCL17	65.2 (28.0-151.7)[b]	10.7 (2.7-41.9)[e]	2.9 (2.1-3.9)[b]	< .01[b,d,f]
CCL26	131.5 (29.4-588.6)[b]	4.7 (1.0-21.7)	2.5 (1.3-4.9)[e]	< .01[d,e,f]
CXCL10	376.2 (134.8-1050.0)[b]	168.9 (35.7-799.5)[b]	48.9 (21.2-112.7)[b]	.07[b]
CCL11	29.5 (15.6-55.7)[b]	10.7 (5.2-22.1)[b]	5.9 (3.1-11.5)[b]	.02[b,d]

Fold change (geometric mean [95% CI]) over constitutive levels after stimulation for 24 h. ANOVA = analysis of variance; IFN = interferon; TNF = tumor necrosis factor.

[a]$P \leq .05$ compared with constitutive levels.

[b]$P < .001$ compared with constitutive levels.

[c]Bronchial basal vs bronchial differentiated, one-way ANOVA with Tukey post hoc multiple comparison.

[d]Nasal vs bronchial differentiated, one-way ANOVA with Tukey post hoc multiple comparison.

[e]$P \leq .01$ compared with constitutive levels.

[f]Nasal vs bronchial basal, one-way ANOVA with Tukey post hoc multiple comparison.

Table 2—*Mediator Concentration Between Primary Epithelial Cell Types Following IL-1β Stimulation*

Mediators	IL-1β (10 ng/mL) Stimulated Epithelial Cells, Fold Change Over Constitutive Levels			*P* Value
	Nasal Basal (n = 6)	Bronchial Basal (n = 11)	Bronchial Differentiated (n = 10)	
IFN-β	1.2 (0.6-2.6)	0.9 (0.7-1.2)	1.0 (0.8-1.1)	.39
CCL5	1.9 (0.9-3.8)	9.2 (3.4-25.1)[a]	11.7 (3.3-41.0)[b]	.06[b]
IL-1β	N/A	N/A	N/A	N/A
CXCL8	10.0 (2.0-51.6)[c]	3.8 (1.8-7.8)[b]	1.9 (1.2-3.0)[c]	.02[c,d]
CCL2	2.0 (1.6-2.4)[a]	1.4 (0.6-3.3)	23.2 (5.4-99.5)[a]	< .01[a,d,e]
CCL13	1.8 (1.4-2.4)[b]	3.2 (0.5-21.5)	1.7 (1.1-2.5)[c]	.57[c]
CCL4	9.6 (3.6-25.5)[b]	4.2 (1.7-10.7)[b]	19.1 (4.6-79.8)[b]	.14[b]
TNF-α	4.3 (1.9-9.6)[b]	2.7 (1.9-3.8)[a]	6.4 (2.1-18.8)[b]	.19[b]
CCL22	2.0 (1.5-2.8)[b]	1.5 (1.0-2.3)	2.4 (1.8-3.2)[a]	.11[a]
CCL17	1.6 (1.3-2.0)[b]	2.1 (0.5-9.2)	2.6 (2.0-3.5)[a]	.67[a]
CCL26	2.2 (1.7-2.7)[a]	1.5 (0.1-16.0)	2.0 (0.7-5.4)	.90
CXCL10	2.1 (1.6-2.7)[a]	3.3 (0.6-18.1)	6.0 (3.6-10.2)[a]	.28[a]
CCL11	2.3 (1.9-2.8)[a]	1.4 (1.0-2.0)	5.3 (1.6-17.7)[c]	.07[c]

Fold change (geometric mean [95% CI]) over constitutive levels after stimulation for 24 h. N/A = not applicable. See Table 1 legend for expansion of abbreviations.

[a]$P < .001$ compared with constitutive levels.

[b]$P \leq .01$ compared with constitutive levels.

[c]$P \leq .05$ compared with constitutive levels.

[d]Nasal vs bronchial differentiated, one-way ANOVA with Tukey post hoc multiple comparison.

[e]Bronchial basal vs bronchial differentiated, one-way ANOVA with Tukey post hoc multiple comparison.

were key differences in release following stimulation. For example, CCL2 has been implicated in wound repair and recruitment of monocytes[22,23] and fibrocytes[24]; its release was increased constitutively, and following stimulation by IL-1β there was marked up-regulation of release by the differentiated epithelial cells compared with increased release by nasal-derived basal cells following stimulation with poly-IC. IFN-β was increased in nasal and bronchial basal cells constitutively compared with differentiated cells. Similarly, there was a generalized increase in most chemokines and cytokines in the nasal epithelial basal cells following Poly IC stimulation compared with the bronchial epithelial cells. This perhaps suggests a heightened sensitivity of these cells to TLR3 activation, which may be a consequence of epigenetic changes promoted by previous exposure to viruses. These differences have hitherto been unexplored and highlight

Table 3—*Mediator Concentration Between Primary Epithelial Cell Types Following IL-1β and IFN-γ*

Mediators	IL-1β (10 ng/mL) + IFN-γ (10 ng/mL) Stimulated Epithelial Cells, Fold Change Over Constitutive Levels			*P* Value
	Nasal Basal (n = 6)	Bronchial Basal (n = 17)	Bronchial Differentiated (n = 11)	
IFN-β	0.7 (0.4-1.2)	0.9 (0.7-1.3)	0.9 (0.7-1.1)	.60
CCL5	8.1 (2.4-27.6)[a]	46.6 (19.0-114.2)[b]	50.2 (18.5-136.1)[b]	.07[b]
IL-1β	N/A	N/A	N/A	N/A
CXCL8	30.5 (10.3-89.7)[b]	4.4 (2.4-7.8)[b]	1.9 (1.1-3.1)[c]	< .01[c,d,e]
CCL2	39.1 (15.5-98.4)[b]	63.5 (25.6-157.3)[b]	73.8 (15.9-342.3)[b]	.78[b]
CCL13	19.4 (7.8-48.0)[b]	6.4 (2.2-18.4)[a]	4.3 (2.9-6.5)[b]	.11[b]
CCL4	87.8 (38.5-68.0)[b]	42.7 (15.0-121.4)[b]	65.1 (19.7-214.5)[b]	.64[b]
TNF-α	5.3 (3.6-7.9)[b]	4.4 (3.1-6.2)[b]	5.6 (1.6-19.2)[c]	.85[c]
CCL22	14.3 (6.3-32.5)[b]	6.4 (3.3-12.4)[b]	6.9 (5.0-9.7)[b]	.18[b]
CCL17	29.7 (9.9-88.5)[b]	16.1 (5.4-48.4)[b]	13.8 (9.3-20.6)[b]	.56[b]
CCL26	28.2 (11.7-68.0)[b]	6.8 (1.8-25.7)[a]	6.9 (3.0-16.0)[b]	.23[b]
CXCL10	390.8 (122.1-1251.0)[b]	323.1 (115.5-903.3)[b]	645.8 (253.0-1652.0)[b]	.54[b]
CCL11	30.6 (11.6-80.6)[b]	13.7 (8.0-23.5)[b]	30.3 (9.9-93.0)[b]	.22[b]

Fold change (geometric mean [95% CI]) over constitutive levels after stimulation for 24 h. See Table 1 and 2 legends for expansion of abbreviations.

[a]$P \leq .01$ compared with constitutive levels.

[b]$P < .001$ compared with constitutive levels.

[c]$P \leq .05$ compared with constitutive levels.

[d]Nasal vs bronchial basal, ANOVA with Tukey post hoc multiple comparison.

[e]Nasal vs bronchial differentiated, ANOVA with Tukey post hoc multiple comparison.

Table 4—*Prednisolone and IKK2i Modulation of Poly IC-Mediated Bronchial Epithelial Cell Mediator Release*

	Bronchial Basal (n = 17)			Bronchial Differentiated (n = 11)		
	Poly IC (12.5 μg/mL)	Poly IC (12.5 μg/mL)		Poly IC (12.5 μg/mL)	Poly IC (12.5 μg/mL)	
Mediators	pg/mL/10^6 cells	+ Pred	+ Ikk2i	pg/mL/10^6 cells	+ Pred	+ Ikk2i
IFN-β	72.4 (22.5-233.1)	1.3 (1.0-1.3)	0.7 (0.4-1.0)[a]	15.8 (11.6-21.4)	0.9 (0.9-1.0)	0.8 (0.5-1.3)
CCL5	11,352 (5,614-22,956)	1.1 (0.9-1.3)	0.1 (0.1-0.3)[b]	626.0 (194.6-2,014.0)	1.0 (0.5-1.9)	0.2 (0.1-0.4)[b]
IL-1β	541.2 (313.2-935.1)	0.9 (0.7-1.1)	0.4 (0.2-0.7)[c]	60.3 (20.3-178.9)	0.9 (0.7-1.1)	1.0 (0.5-2.0)
CXCL8	53,004 (34,652-81,076)	1.0 (0.9-1.1)	0.1 (0.1-0.3)[b]	22,319 (16,126.0-30,891.0)	0.9 (0.8-1.0)[a]	0.8 (0.7-1.1)
CCL2	412.8 (180.9-941.9)	1.2 (0.9-1.1)	0.1 (0.0-0.2)[b]	1,915.0 (582.3-6,297.0)	0.9 (0.8-1.1)	0.2 (0.1-0.4)[b]
CCL13	2,287 (1,323-3,955)	1.1 (0.9-1.4)	0.2 (0.1-0.3)[b]	1,326.0 (717.2-2,450.0)	1.0 (0.8-1.3)	0.7 (0.6-1.0)
CCL4	2,912 (1,756-4,829)	1.0 (0.8-1.2)	0.0 (0.0-0.0)[b]	865.3 (304.4-2,460.0)	0.8 (0.6-1.2)	0.2 (0.1-0.6)[c]
TNF-α	1,630 (1,015-2,618)	0.7 (0.6-0.9)[c]	0.1 (0.0-0.1)[b]	540.4 (234.8-1,244.0)	0.7 (0.4-1.1)	0.4 (0.1-1.1)
CCL22	4,981 (2,502-9,916)	1.1 (0.9-1.4)	0.1 (0.1-0.3)[b]	2,621.0 (1,428.0-4,811.0)	0.9 (0.8-1.1)	0.6 (0.5-0.9)[c]
CCL17	3,143 (1,468-6,730)	1.2 (0.9-1.6)	0.1 (0.0-0.3)[b]	1,434.0 (707.0-2,910.0)	0.9 (0.6-1.5)	0.5 (0.3-0.8)[a]
CCL26	14,855 (8,918-2,4745)	1.0 (0.9-1.4)	0.2 (0.0-0.3)[b]	10,671.0 (5,806-19,614.0)	0.9 (0.7-1.3)	0.5 (0.3-0.9)[a]
CXCL10	70,950 (28,845-174,515)	1.0 (0.9-1.2)	0.0 (0.0-0.1)[b]	27,465 (10,525-71,667)	0.9 (0.6-1.5)	0.1 (0.0-0.3)[b]
CCL11	2,433 (1,376-4,302)	1.1 (0.9-1.4)	0.1 (0.0-0.2)[b]	1,209.0 (539.4-2,711.0)	1.0 (0.8-1.1)	0.4 (0.2-0.7)

Stimulated levels (pg/mL/10^6 cells) in conjunction with modulation by prednisolone (Pred) (10 μM) or iKK2 (1 μm) (fold change over stimulated levels) after 24 h. All data are expressed as geometric mean (95% CI). See Table 1 legend for expansion of abbreviations.

[a]$P \leq .05$ compared with constitutive levels.

[b]$P < .001$ compared with constitutive levels.

[c]$P \leq .01$ compared with constitutive levels.

Table 5—*Prednisolone and IKK2i Modulation of IL-1β-Mediated Bronchial Epithelial Cell Mediator Release*

	Bronchial Basal (n = 11)			Bronchial Differentiated (n = 11)		
	IL-1β (10 ng/mL)	IL-1β (10 ng/mL)		IL-1β (10 ng/mL)	IL-1β (10 ng/mL)	
Mediators	pg/mL/10^6 Cells	+ Pred	+ Ikk2i	pg/mL/10^6 Cells	+ Pred	+ Ikk2i
IFN-β	34.8 (13.1-92.8)	1.1 (0.8-1.4)	1.1 (0.8-1.3)	13.5 (11.3-16.2)	1.0 (0.8-1.4)	1.0 (0.8-1.2)
CCL5	82.6 (29.1-234.7)	1.2 (0.7-2.2)	0.2 (0.1-0.7)[a]	92.24 (25.9-328.3)	1.1 (0.9-1.3)	0.1 (0.0-0.4)[b]
IL-1β	N/A	N/A	N/A	N/A	N/A	N/A
CXCL8	51,154 (25,440-102,859)	0.9 (0.8-1.0)[a]	0.2 (0.1-0.4)[c]	17,828 (9,334-34,052)	1.0 (0.7-1.5)	1.2 (0.7-2.2)
CCL2	101.3 (31.0-330.3)	1.0 (0.8-1.2)	0.4 (0.2-0.8)[a]	1,908.0 (488.9-7,446.0)	1.1 (0.8-1.5)	0.2 (0.1-0.4)[c]
CCL13	1,140 (431.4-3,010.0)	1.0 (0.9-1.1)	0.3 (0.1-1.5)	664.4 (359.7-1,227.0)	1.1 (0.9-1.2)	0.9 (0.8-1.1)
CCL4	171.6 (41.3-713.5)	1.0 (0.6-1.5)	0.2 (0.1-0.5)[b]	351.0 (136.0-905.9)	1.1[d] (1.0-1.3)[a]	0.4 (0.2-0.8)[a]
TNF-α	919.8 (436.1-1,940.0)	1.2 (0.8-1.6)	0.3 (0.2-0.5)[c]	277.3 (100.4-765.9)	0.9 (0.6-1.2)	0.3 (0.1-0.9)[a]
CCL22	1,498 (563-3,986)	0.9 (0.7-1.1)	0.4 (0.2-1.1)	1,657.0 (875.4-3,135.0)	0.9 (0.8-1.0)	0.7 (0.6-0.9)[a]
CCL17	554.7 (203.0-1,515.0)	1.2 (0.9-1.6)	0.9 (0.6-1.3)	715.5 (383.2-1,336.0)	1.1 (0.9-1.2)	0.8 (0.6-1.0)
CCL26	1,581.0 (397.5-6,286.0)	3.1[d] (1.4-6.4)[b]	0.6 (0.1-4.0)	12,606.0 (4,420.0-35,952.0)	1.3 (0.7-2.1)	0.8 (0.4-1.6)
CXCL10	1,189.0 (367.9-3,843.0)	0.8 (0.5-1.3)	0.3 (0.1-1.2)	1,988.0 (754.4-5,237.0)	0.9 (0.5-1.5)	0.4 (0.2-0.8)[a]
CCL11	419.7 (143.3-1,229.0)	1.0 (0.7-1.4)	0.7 (0.4-1.1)	433.5 (231.6-811.5)	1.0 (0.9-1.2)	0.8 (0.6-1.0)[a]

Stimulated levels (pg/mL/10^6 cells) in conjunction with modulation by prednisolone (Pred) (10 μM) or iKK2 (1 μm) (fold change over stimulated levels) after 24 h. All data are expressed as geometric mean (95% CI). See Table 1 and 2 legends for expansion of abbreviations.

[a]$P \leq .05$ compared with constitutive levels.

[b]$P \leq .01$ compared with constitutive levels.

[c]$P < .001$ compared with constitutive levels.

[d]Significant upregulation.

Table 6—*Prednisolone and IKK2i Modulation of IL-1β and IFN-γ-Mediated Bronchial Epithelial Cell Mediator Release*

	Bronchial Basal (n = 17)			Bronchial Differentiated (n = 11)		
	IL-1β/IFN-γ (10 ng/mL)	IL-1β/IFN-γ (10 ng/mL)		IL-1β/ IFN-γ (10 ng/mL)	IL-1β/IFN-γ (10 ng/mL)	
Mediators	pg/mL/10^6 Cells	+ Pred	+ Ikk2i	pg/mL/10^6 Cells	+ Pred	+ Ikk2i
IFN-β	35.6 (14.4-87.7)	0.9 (0.6-1.5)	1.4 (0.7-2.5)	13.2 (12.2-14.2)	1.3 (0.9-1.8)	1.0 (0.9-1.1)
CCL5	1,076 (509.6-2,271.0)	1.2 (0.9-1.7)	0.0 (0.0-0.0)[a]	397.2 (144.9-1,089.0)	1.0 (0.7-1.3)	0.1 (0.1-0.2)[a]
IL-1β	N/A	N/A	N/A	N/A	N/A	N/A
CXCL8	49,032 (30,759-78,160)	1.0 (0.8-1.2)	0.1 (0.1-0.2)[a]	17,947 (11,053-29,142)	1.0 (0.9-1.1)	1.0 (0.9-1.2)
CCL2	3,841 (1,588-9,287)	1.3 (0.7-2.5)	0.2 (0.1-0.5)[b]	6,365.0 (2,222.0-18,230.0)	0.8 (0.6-1.1)	0.4 (0.2-0.6)[a]
CCL13	2,649 (1,447-4,849)	1.2 (0.9-1.7)	0.6 (0.3-0.9)[c]	1,744.0 (1,059.0-2,873.0)	1.1 (0.9-1.4)	0.8 (0.6-1.1)
CCL4	996.4 (469.8-2,113.0)	1.4 (0.8-2.4)	0.4 (0.2-0.9)[c]	1,326.0 (720.9-2,440.0)	1.2 (0.9-1.7)	0.7 (0.4-1.1)
TNF-α	648.6 (327.0-1,286.0)	1.0 (0.9-1.2)	0.3 (0.2-0.5)[a]	262.4 (110.7-621.9)	1.0 (0.6-1.4)	0.5 (0.2-1.0)
CCL22	6,206 (3,124-12,328)	1.3 (0.8-1.9)	0.6 (0.4-1.1)	4,879.0 (2,951.0-8,068.0)	1.1 (0.9-1.4)	0.8 (0.6-1.1)
CCL17	4,871 (2,392-9,917)	1.4 (0.8-2.2)	0.6 (0.3-1.1)	3,688.0 (2,239.0-6,074.0)	1.1 (0.9-1.5)	0.7 (0.5-1.1)
CCL26	21,244 (13,104-344,442)	1.1 (0.9-1.4)	0.5 (0.3-0.8)[b]	47,002.0 (23,802.0-92,816.0)	1.3 (0.7-2.1)	0.6 (0.3-1.3)
CXCL10	141,993 (84,327-239,091)	1.1 (1.0-1.2)	0.8 (0.7-1.0)[c]	222,457 (10,0350-493,146)	0.8 (0.7-0.9)[c]	0.7 (0.5-0.9)[c]
CCL11	3,145 (1,675-5,907)	1.4 (0.9-2.2)	0.7 (0.4-1.3)	2,409.0 (1,431.0-4,055.0)	1.1 (0.9-1.3)	0.7 (0.5-1.0)

Stimulated levels (pg/mL/10^6 cells) in conjunction with modulation by prednisolone (Pred) (10 μM) or iKK2 (1 μm) (fold change over stimulated levels) after 24 h. All data are expressed as geometric mean (95% CI). See Table 1 and 2 legends for expansion of abbreviations.

[a]$P < .001$ compared with constitutive levels.

[b]$P \leq .01$ compared with constitutive levels.

[c]$P \leq .05$ compared with constitutive levels.

the potential importance of epithelial cell type in the study of epithelial function in asthma.

The concentration of prednisolone used in this investigation was representative of the plasma concentration present in subjects with severe asthma taking such medication.[25] It is very interesting and an important finding that the corticosteroid used had little effect on cytokine production, in contrast to IKK2 inhibition. The observed corticosteroid epithelial relative resistance, but responsiveness toward inhibition of IKK2 in donors with and without asthma, would suggest that corticosteroid epithelial unresponsiveness is related to features of the canonical NF-κB signaling pathway. These data, therefore, do not support the view that there is an intrinsic corticosteroid unresponsiveness in asthmatic epithelium as a consequence of differential corticosteroid receptor expression, altered ligand affinity, or decreased corticosteroid binding to DNA.[26] Indeed, although IL-8 expression has been shown to be significantly repressed by dexamethasone, the induction of NF-κB-dependent transcription in primary human bronchial epithelial cells was unaffected by the application of the corticosteroid, suggesting that the targeting of NF-κB transcription by corticosteroids may represent a more minor mechanism of action than has been previously believed.[27]

There is a potential for using small molecule inhibitors of IKK2 to treat asthma. These compounds bypass problems associated with corticosteroid-resistant therapy, such as reduced corticosteroid receptor expression and translocation.[28] To date, such small molecule inhibitors have been proven to significantly inhibit NF-κB transcription and inflammatory gene expression in primary human airway epithelial cells,[27] comprehensively reduce chronic pulmonary inflammation in murine models,[29] and inhibit pathologic features of airway remodelling and ameliorate airway responsiveness in a chronic allergen exposure model of bronchial asthma in mice.[30]

The effects of the site of sampling, selection of subjects, activators, and inhibitors upon mediator release are important to consider. Our study focused upon synthetic capacity of epithelial cells in vitro to determine the persistence of abnormalities in asthmatic cells independent of the in vivo environment. However, one potential criticism is that we may have failed to observe other important functional differences. Indeed, our findings do not exclude the possibility that there remain fundamental differences in epithelial cell function in asthma. Critical differences between epithelial cells from asthma and health for other functional responses are reported for proliferation[9,31] and wound repair.[9] Asthma severity may also be an important factor, although we recruited subjects across the spectrum of asthma severity and were unable to demonstrate differences in synthetic capacity and disease severity. However, our study was underpowered to fully explore this question, and, thus, it remains a possibility that disease severity may exert an important influence upon epithelial cell function. Although the cells were stimulated basolaterally, it is acknowledged that mucus production on the apical surface may have still influenced the function of the cell culture as a whole. To reduce the interference of mucus production with the production of cytokines, mucus was removed from ALI cultures prior to simulation by gentle washing with PBS. There is also the possibility that an alternative duration of stimulation and even apical stimulation of the differentiated epithelial cultures may have produced differences between the donors with and without asthma. Indeed, there is evidence to suggest variation in apical and basolateral secretion of mediators, but this may be due to the location of receptors for certain stimuli.[32] The IFN-γ receptor is expressed basolaterally in both cultured airway epithelia and normal human airway tissue. Therefore, stimulating the ALI cultures from the basolateral surface allowed for the maximal response to be examined from the differentiated epithelium. Furthermore, in vitro conditions may also be important determinants of functional responses, such as the matrix environment may influence some of the differences observed between reports[9,11,12]; indeed, the effects of culture conditions upon the differentiation process and its dynamic during culture could have resulted in problems regarding the interpretation of data. This is unlikely to have affected our study, as we controlled carefully the culture conditions for the epithelial cells. Samples were obtained from the second- or third-generation bronchi and cultured on collagen-coated surfaces. Spontaneous mucus production occurred from 14 to 21 days and ciliation between 25 to 35 days. Those cultures that did not reach the final stages of differentiation were not used for this investigation. One further potential criticism is that we did not undertake corrections for multiple comparisons. We chose not to undertake these corrections, as there is a debate among statisticians about the validity of various methods. If a Bonferroni correction was applied, the revised P value for significance would have been adjusted from $P = .05$ to $P = .0039$.

In conclusion, the synthetic capacity of epithelial cells differs between location and the degree of differentiation, but in vitro we were unable to identify differential expression between health and disease, suggesting that activation of epithelial cells in vivo maybe largely a consequence of the asthmatic environment. Activation of epithelial cells by proinflammatory cytokines and TLR3 agonists is corticosteroid unresponsiveness independent of disease, but is sensitive to IKK2 inhibition, suggesting that IKK2 inhibitors may be important novel therapies for asthma.

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