

Increased sputum endotoxin levels are associated with an impaired lung function response to oral steroids in asthmatic patients

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Background: Airway endotoxin might contribute to corticosteroid insensitivity in asthmatic patients.

Objective: Because cigarette smoke contains endotoxin, we tested the hypothesis that sputum endotoxin concentrations are increased in cigarette smokers and that endotoxin concentrations are associated with corticosteroid insensitivity in asthmatic patients.

Methods: Sixty-nine asthmatic patients (never smokers, smokers, and exsmokers) and 20 healthy subjects (never smokers and smokers) were recruited. Fifty-three asthmatic patients received a 2-week course of oral dexamethasone. Serum and induced sputum endotoxin and cytokine concentrations were quantified by using an enzyme immunoassay.

Results: Median (interquartile range [IQR]) sputum endotoxin concentration were not significantly different between asthmatic never smokers (184 endotoxin units [EU]/mL; IQR, 91-310 EU/mL), exsmokers (123 EU/mL; IQR, 39-207 EU/mL), and smokers (177 EU/mL; IQR, 41-772 EU/mL; $P = .703$) and healthy subjects (164 EU/mL; IQR, 106-373 EU/mL). The lung function response to oral corticosteroids decreased with increasing sputum endotoxin concentrations in the never smokers (linear regression $\alpha = .05$, Spearman $r = -0.503$, $P = .009$) but not in smokers ($\alpha = .587$, $r = -0.282$, $P = .257$), as confirmed by using multiple regression analysis. Asthmatic smokers had higher concentrations of serum endotoxin than asthmatic nonsmokers (0.25 EU/mL [IQR, 0.09-0.39 EU/mL] vs 0.08 EU/mL [IQR, 0.05-0.19 EU/mL], $P = .042$) unrelated to steroid insensitivity or serum cytokine concentrations. In the asthmatic group sputum endotoxin concentrations correlated with sputum IL-1 receptor antagonist concentrations ($r = 0.510$, $P < .001$), and serum endotoxin concentrations significantly correlated with sputum IL-6, IL-8, and chemokine motif ligand 2 concentrations.

Conclusion: Asthmatic smokers have similar sputum endotoxin concentrations compared with those of asthmatic never smokers. The association between higher sputum endotoxin levels and an impaired lung function response to oral

corticosteroids, particularly in asthmatic never smokers, suggests that airway endotoxin might contribute to corticosteroid insensitivity in asthmatic patients. (*J Allergy Clin Immunol* 2014;■■■:■■■-■■■.)

Key words: Asthma, asthma control, endotoxin, oral steroids, smokers, sputum cytokines

Corticosteroid insensitivity is an important clinical feature of asthma, particularly in patients with severe disease and smokers.¹⁻⁶ The mechanisms of corticosteroid insensitivity in asthmatic patients are poorly understood.^{3,7} Preclinical and clinical data support a role for endotoxin as a potential contributory factor to corticosteroid insensitivity in asthmatic patients. Endotoxins (bacterial LPS) are large cell-wall structural molecules from gram-negative bacteria consisting of a lipid and a polysaccharide that bind to Toll-like receptor (TLR) 4 on many mammalian cells, promoting an antibacterial response through secretion of proinflammatory cytokines. Preclinical data using a mouse model of allergic airway disease demonstrated that endotoxin inhalation exaggerates eosinophil inflammation and reduces responsiveness to corticosteroid treatment.⁸ In a further animal model of asthma, cooperative signaling between IFN- γ and endotoxin constituted a T_H1-mediated pathway for macrophage-dependent corticosteroid-resistant airway hyperreactivity.⁹ *In vitro* endotoxin enhanced the downregulation of glucocorticoid receptor expression in respiratory epithelial cells through activation of p38 mitogen-activated protein kinase, c-Jun N-terminal kinase, and cyclin-dependent kinase.¹⁰ Clinical data demonstrated that bronchoalveolar lavage (BAL) fluid endotoxin levels were increased in patients with corticosteroid-resistant asthma compared with those seen in patients with corticosteroid-sensitive asthma, and this observation was extended by demonstrating that endotoxin *in vitro* could induce a corticosteroid-resistant state in human macrophages.¹¹ Adults with neutrophilic asthma, which can be associated with corticosteroid insensitivity, have a trend toward higher sputum concentrations of endotoxin than seen in those with eosinophilic asthma.¹² In young children less than 2 years of age, BAL endotoxin concentrations correlated with airway neutrophil numbers.¹³

Asthmatic patients can be exposed to endotoxin in cigarette smoke,¹⁴ either as active smokers or through exposure to passive smoke,^{15,16} as well as in household dust.¹⁷ Certain occupations, such as cotton textile workers or farm animal workers, are associated with exposure to high concentrations of endotoxin.¹⁸ Increased bacterial load within the airways^{19,20} or increased airway permeability²¹ might also alter airway or serum endotoxin concentrations in asthmatic patients.

Our hypothesis was that cumulative exposure to endotoxin might contribute to corticosteroid insensitivity in both smokers

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Abbreviations used

ACQ: Asthma control questionnaire
 BAL: Bronchoalveolar lavage
 CCL: Chemokine motif ligand
 EU: Endotoxin units
 FEF: Forced expiratory flow
 IL-1Ra: IL-1 receptor antagonist
 IQR: Interquartile range
 TLR: Toll-like receptor

and never smokers with asthma. The main aims of the study were (1) to quantify the differences in sputum fluid and serum concentrations by smoking and asthma status and (2) to assess the relationship of endotoxin with corticosteroid responsiveness in asthmatic patients of various smoking statuses.

METHODS**Subjects and study design**

Sixty-nine smokers, exsmokers, and never smokers with stable asthma (defined as no emergency clinic or hospital visit, oral corticosteroid prescription, or change in asthma treatment in the past month) and no major additional chronic illnesses plus 20 healthy volunteers (smokers and never smokers) were recruited to a cross-sectional study. Fifty-three of the patients with asthma received a 2-week, open-label, unblinded course of oral dexamethasone.²² Treatment with up to a maximum of 2000 μg of beclomethasone (or equivalent), long-acting β_2 -agonists, and leukotriene receptor antagonists was allowed. Requirement for treatment with or the presence of conditions likely to be exacerbated by oral corticosteroids and the intention to stop smoking or become pregnant (or current pregnancy) were exclusion criteria. Current smoking was defined as 5 or more cigarettes per day and a smoking history of 5 pack years or greater. Asthmatic exsmokers were eligible if they had ceased smoking for 2 or more years from the date of recruitment, were prior daily smokers of 5 or more cigarettes, and had a smoking history of 5 or more pack years. Never smokers were required to have no history of regular smoking and to be current nonsmokers. The volunteered smoking history was validated by using consistent measures of urinary cotinine, exhaled carbon monoxide, and reduced exhaled nitric oxide levels.

Eligibility for the study required demonstration of reversible airflow obstruction (FEV_1 bronchodilator response to β_2 agonist of $\geq 12\%$ [and >200 mL]) in 53 patients or a positive methacholine test result in 16 patients. All lung function assessments met relevant international consensus guidelines.²³ Current smokers and exsmokers with asthma were required to have an FEV_1 of greater than 80% of predicted value to be eligible for methacholine testing (to reduce confounding with chronic obstructive pulmonary disease). Airway corticosteroid sensitivity was assessed by change in prebronchodilator FEV_1 to a 2-week trial of oral dexamethasone (6 mg/1.74 m^2 ; roughly equivalent to 40 mg/d prednisolone). On the day before initiation and on completion of the dexamethasone course, all subjects performed spirometry, exhaled nitric oxide measurement at 50 mL/s (fraction of exhaled nitric oxide; NIOX Flex, Aerocrine AB, Solna, Sweden), induced sputum, and completed asthma control questionnaires (ACQs).²⁴ Blood was obtained for plasma to allow measurement of cytokines before and after the dexamethasone trial. Serum cortisol was assessed on completion of the dexamethasone course, and subjects were deemed to have been compliant if their blood cortisol level was suppressed to less than 50 nmol/L.

Sputum and plasma measurements

Sputum samples were processed by using the whole sputum sample method.²⁵ Briefly, homogenization was achieved by gentle mechanical disruption in low concentrations of dithiothreitol (0.05%), which we

confirmed not to affect the immunoreactivity and recovery of exogenous cytokines spiked into dithiothreitol-treated sputum samples (data not shown). Sputum supernatants were collected and stored in aliquots at -80°C until analysis. Endotoxin concentrations in peripheral blood serum and induced sputum supernatants were measured with a kinetic high sensitivity assay (LAL Kinetic-QCL; Lonza Biologics, Slough, United Kingdom). The stability of sputum endotoxin concentrations as a putative biomarker was established by demonstrating a correlation ($r = 0.815$, $P < .001$) between replicate samples in 26 patients taken 1 month apart. Levels of the cytokines TNF- α , IL-1 β , IL-1 receptor antagonist (IL-1Ra), IL-2, IL-2 receptor, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p40/p70, IL-13, IL-15, and IL-17; interferons IFN- α and IFN- γ ; growth factor GM-CSF; and chemokines chemokine motif ligand (CCL) 2 (monocyte chemoattractant protein 1), CCL3 (macrophage inflammatory protein 1a), CCL4 (macrophage inflammatory protein 1b), CCL11 (eotaxin), CXCL9 (monokine induced by IFN- γ), CXCL10 (IFN- γ -induced protein 10), and CXCL8 (IL-8) were measured with a multianalyte bead enzyme immunoassay system (multiplex; Invitrogen, Carlsbad, Calif) by using a Luminex platform.

Statistical analysis

Statistical assessment was done with nonparametric methods (Mann-Whitney and Kruskal-Wallis tests). Correlation analysis was determined by using the Spearman rho correlation with bootstrap 95% CI after log or square root normalization, if necessary. Linear regression analysis used the least-squares method, and scatter diagrams show both the fitted linear regression line and the 95% CI for the regression line. This interval includes the true regression line with 95% probability. No correction was performed for multiple analyses because the study was viewed as exploratory for hypothesis generation. Multivariate multiple regression analysis was performed by using the least-squares method and entering all variables into the model. Sputum endotoxin levels were dependent variables, and all other data points were handled as independent variables, with smoking status as a classifier. The 95% CI with an α value of .05 as a cutoff was used as the determinant for significance throughout.

RESULTS**Baseline demographics**

Asthmatic patients had similar age and sex distribution (Table I). These patients had significantly lower FEV_1 compared with healthy subjects ($P < .001$). The asthmatic smoker and asthmatic exsmoker groups had significantly higher ACQ scores ($P < .001$) and a trend toward shorter duration of asthma ($P = .082$) compared with the asthmatic never smokers. The dose of inhaled corticosteroids was significantly higher in asthmatic exsmokers compared with asthmatic smokers and asthmatic never smokers ($P = .022$). Sputum eosinophil proportion was greater in the asthmatic patients compared with that seen in the healthy subjects ($P = .003$), but there was no difference in neutrophil proportions ($P = .717$).

Sputum endotoxin concentration

There were no significant differences in sputum endotoxin concentration (medians and interquartile ranges [IQRs]) between asthmatic patients and healthy subjects (152 endotoxin units [EU]/mL [IQR, 55-332 EU/mL] vs 164 EU/mL [IQR, 106-373 EU/mL]; $P = .833$; Fig 1, A). Among the asthmatic patients, there were no differences in sputum endotoxin levels between never smokers, exsmokers, and smokers (184 EU/mL [IQR, 91-310 EU/mL] vs 123 EU/mL [IQR, 39-207 EU/mL] vs 177 EU/mL [IQR, 41-772 EU/mL], $P = .703$; Fig 1, B). Sputum endotoxin concentrations were similar in atopic asthmatic

TABLE I. Baseline demographic, clinical, and inflammatory characteristics

	Healthy never smokers (n = 10)	Healthy smokers (n = 10)	Asthmatic never smokers (n = 29)	Asthmatic exsmokers (n = 10)	Asthmatic smokers (n = 30)
Age (y)	44.1 (4.7)	49.8 (3.8)	46.1 (2.1)	49.8 (9.0)	47.0 (1.1)
Sex (F/M)	9/1	5/5	15/14	5/5	15/15
Asthma duration (y)	—	—	30.7 (2.7)	24.6 (15.9)	22.0 (2.7)
BMI (kg/m ²)	21.7 (19.4-26.8)	24.2 (21.4-27.7)	29.2 (25.9-31.9)	32.4 (28.5-34.0)	27.4 (22.3-31.1)
Pack years smoked	—	18.3 (3.9)	—	28.5 (15.9)	28.6 (2.7)
Atopic status (yes/no)	4/6	2/8	23/4	4/6	20/9
ACQ score (0-7)	—	—	1.4 (0.2)	2.3 (0.7)	2.2 (0.2)
FEV ₁ (% predicted)	103.8 (7.5)	93.7 (4.6)	75.5 (2.8)	79.7 (24.1)	69.8 (3.2)
FEV ₁ BD response	—	—	17.2 (7.3-33.4)	11.8 (8.2-28.4)	13.7 (10.0-18.1)
Inhaled corticosteroids (beclomethasone equivalent [μ g/d])	—	—	898 (139)	1280 (551)	927 (106)
Sputum eosinophils (% of total), median (IQR)	0.00 (0.00-0.48)	0.06 (0.00-0.24)	0.33 (0.00-2.00)	1.00 (0.10-6.00)	0.40 (0.00-1.88)
Sputum neutrophils (% of total), median (IQR)	36.2 (13.7-51.1)	36.9 (16.9-56.7)	24.0 (11.0-48.0)	37.0 (20.0-65.0)	33.2 (21.0-57.9)

Data are presented as means (SDs), except where indicated.

BD, Bronchodilator; BMI, body mass index; F, female; M, male.

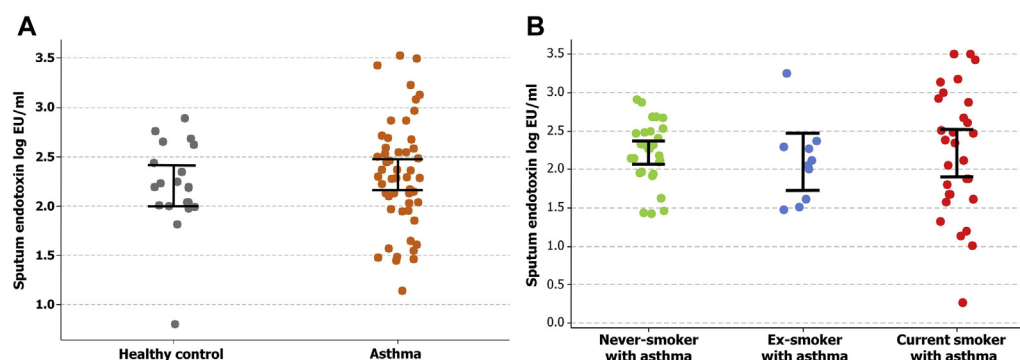


FIG 1. Endotoxin concentrations in sputum from asthmatic patients and healthy subjects (**A**) and never smokers, exsmokers, and current smokers with asthma (**B**) are shown. There were no significant differences in sputum endotoxin concentrations between asthmatic patients and healthy subjects (median, 152 EU/mL [IQR, 55-332 EU/mL] vs 164 EU/mL [IQR, 106-373 EU/mL], $P = .833$). Bars = 95% CI of the mean. There were no differences in sputum endotoxin levels between smokers, never smokers, and exsmokers (median, 177 EU/mL [IQR, 41-772 EU/mL] vs 184 EU/mL [IQR, 91-310 EU/mL] vs 123 EU/mL [IQR, 39-207 EU/mL], $P = .703$). Bars = 95% CI of the mean.

patients compared with those seen in nonatopic asthmatic patients (210 EU/mL [IQR, 86-344 EU/mL] vs 196 EU/mL [IQR, 133-1374 EU/mL], $P = .301$). The sputum fluid endotoxin concentration did not correlate with prebronchodilator or postbronchodilator percent predicted peak expiratory flow, FEV₁, forced vital capacity, FEV₁/forced vital capacity ratio, 25% to 75% forced expiratory flow (FEF₂₅₋₇₅), 25% FEF (FEF₂₅), and 75% FEF (FEF₇₅; data not shown) or with reversibility of FEV₁ after bronchodilator ($r = -0.039$, $P = .790$).

Response to oral corticosteroid and association with sputum endotoxin concentration

Asthmatic nonsmokers (never smokers and exsmokers) made a significant within-group improvement in lung function in response to oral dexamethasone (FEV₁ change, 0.202 L; 95% CI, 0.042-0.362 L; $P = .015$). This was in contrast to current smokers with asthma, who did not make a significant improvement (0.031 L; 95% CI, -0.116 to 0.178 L; $P = .657$). There was a marked reduction in plasma cortisol levels in both the nonsmoker and smoker groups from a median of 345 nmol/L (IQR, 256-486 nmol/L) and 294 nmol/L (IQR,

250-363 nmol/L), respectively, to less than the detection level of 28 nmol/L, providing evidence of endogenous corticosteroid suppression and therefore good adherence with dexamethasone.

The precorticosteroid sputum endotoxin and cytokine concentrations were assessed as determinants of lung function response to oral corticosteroids. In the combined asthmatic nonsmoker and smoker groups, the percentage change in FEV₁ after oral dexamethasone decreased with increasing sputum endotoxin concentration ($r = -0.329$ [95% CI, -0.570 to -0.036], $P = .029$, Fig 2). This negative association was observed primarily in asthmatic nonsmokers ($r = -0.388$ [95% CI, -0.674 to -0.0009], $P = .05$) and not in asthmatic smokers ($r = -0.137$ [95% CI, -0.568 to 0.352], $P = .588$). In the combined asthma group the percentage change in FEV₁ after oral dexamethasone correlated positively with the sputum eosinophil proportion ($r = 0.542$ [95% CI, 0.311 - 0.712], $P < .001$) and with a trend toward a negative correlation with the sputum neutrophil proportion ($r = -0.344$ [95% CI, -0.567 to -0.073], $P = .054$).

A statistical regression and correlation analysis of the combined asthmatic smoker and nonsmoker group (Table II) demonstrated that the sputum endotoxin concentration did not correlate with age, sex, body mass index, asthma duration, inhaled

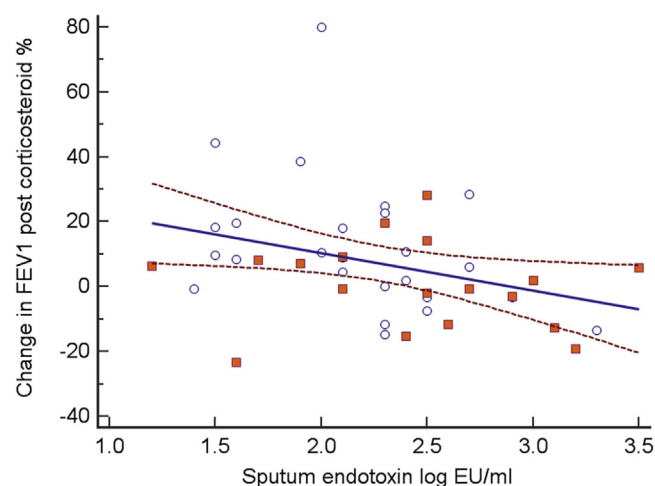


FIG 2. Association of percentage change in FEV₁ after oral corticosteroids with sputum endotoxin concentrations in asthmatic patients. The linear regression line (solid line) with the true 95% CI (dashed lines) of nonsmokers (open circles) and smokers (red squares) is shown. In the combined asthma group the percentage change in FEV₁ after oral dexamethasone decreased with increasing sputum endotoxin concentration (Spearman rho: $r = -0.329$ [95% CI, -0.570 to -0.036], $P = .029$).

corticosteroid dose, ACQ score, FEV₁ percentage reversibility, pack years smoked, fraction of exhaled nitric oxide, and exhaled carbon monoxide. However, the sputum endotoxin concentration was associated with the change in FEV₁ after oral corticosteroid treatment (linear regression $P = .029$, Spearman correlation $P = .005$), as well as with smoking status ($P = .028$ and $P = .029$, respectively). The latter finding highlighted the relevance of smoking status, and therefore substratification and reanalysis with statistical regression and correlation analysis (Table III) demonstrated that the association between sputum endotoxin concentration and poststeroid change in FEV₁ is significant in nonsmokers and not in smokers. This observation is supported by modeling these variables by using a multivariate multiple regression analysis. This demonstrated that sputum endotoxin levels do not cross-correlate with all the variables in a model across the combined asthmatic smoker and nonsmoker group (model score $P = .702$, Table IV) and across the asthmatic smoker group (model score $P = .954$, Table V), whereas within the nonsmoker model (model score $P = .201$), poststeroid change in FEV₁ and dose of inhaled corticosteroid are correlation factors for the concentration of sputum endotoxin ($P = .005$ and $P = .021$, respectively), demonstrating the significance of these parameters in an overall multiple regression model of corticosteroid-treated patients.

The sputum endotoxin concentration correlated with the sputum IL-1Ra concentration in the combined asthma group ($r = 0.510$ [95% CI, 0.273-0.689], $P < .001$; Fig 3). This association was significant for nonsmokers ($r = 0.633$ [95% CI, 0.359-0.806], $P < .001$) but not for smokers ($r = 0.359$ [95% CI, -0.099 to 0.692], $P = .120$). Sputum endotoxin concentrations did not correlate with concentrations of other sputum cytokines, including IL-1 β , in the combined asthma group ($r = -0.049$, $P = .733$) and in the nonsmoker ($r = -0.152$, $P = .416$) and smoker ($r = -0.054$, $P = .825$) categories. There was no correlation between sputum IL-1Ra and sputum IL-1 β concentrations in the asthmatic patients ($r = 0.223$, $P = .120$) and in the nonsmoker ($r = 0.077$, $P = .683$) and smoker ($r = 0.288$, $P = .232$) categories.

TABLE II. Linear regression analysis of sputum endotoxin concentration compared with baseline data from all asthmatic patients

Independent variables	All asthmatic patients					
	Regression				Correlation*	
	<i>R</i> ²	Residual SD	F ratio	<i>P</i> value	Coefficient	<i>P</i> value
Change in postcorticosteroid FEV ₁ (%)	0.108	0.511	5.097	.029	−0.416	.005
Age	<0.001	0.556	0.004	.952	−0.064	.656
Sex	<0.001	0.556	<0.001	.997	0.005	.970
BMI	0.001	0.556	0.061	.806	−0.027	.854
Smoking	0.095	0.529	5.144	.028	0.306	.029
Pack years smoked	0.038	0.546	1.934	.171	0.145	.310
Asthma duration	0.003	0.555	0.130	.720	0.023	.872
Inhaled corticosteroid dose	0.001	0.556	0.069	.795	−0.089	.535
ACQ score	0.003	0.555	0.159	.692	−0.065	.651
FENO	0.007	0.551	0.327	.570	−0.140	.353
Exhaled CO	0.042	0.555	2.035	.160	0.279	.052
Serum endotoxin	0.002	0.592	0.046	.832	0.054	.777
Bronchodilator reversibility (%)	0.031	0.564	1.471	.231	−0.127	.388

Values in boldface indicate statistical significance.

BMI, Body mass index; FENO, fraction of exhaled nitric oxide.

*Spearman rank correlation.

Serum endotoxin concentration

Serum endotoxin concentrations were equivalent in asthmatic patients and healthy subjects (0.18 EU/mL [0.07-0.30 EU/mL] vs 0.11 EU/mL [0.06-0.41 EU/mL], $P = 1.000$). Among asthmatic patients, smokers with asthma had higher concentrations of serum endotoxin than never smokers with asthma (0.25 EU/mL [0.09-0.39 EU/mL] vs 0.08 EU/mL [0.05-0.19 EU/mL], $P = .042$), and a similar trend was observed in the healthy subjects (0.26 EU/mL [0.10-0.41 EU/mL] vs 0.06 EU/mL [0.06-0.75 EU/mL], $P = .180$). Serum endotoxin concentration correlated with pack years smoking history ($r = 0.493$ [95% CI, 0.065-0.767], $P = .044$). There was no correlation between serum and sputum endotoxin concentrations ($r = 0.041$ [95% CI, -0.274 to 0.348], $P = .832$). In asthmatic patients the serum concentration of endotoxin did not correlate with the lung function response to oral corticosteroids ($P = .073$), in either the nonsmoker ($P = .456$) or smoker ($P = .196$) categories. The serum endotoxin concentration did not correlate with the proportion of blood neutrophils ($r = 0.138$, $P = .474$) or with the concentration of any of the cytokines measured in this study, and this also applied to the nonsmoking and smoking categories (data not shown). The serum endotoxin concentration did not correlate with demographics, lung function, or biomarkers, except for a correlation with the sputum cytokines IL-6 ($r = 0.481$ [95% CI, 0.147-0.717], $P = .007$), IL-8 ($r = 0.495$ [95% CI, 0.164-0.726], $P = .005$), and CCL2 ($r = 0.542$ [95% CI, 0.226-0.755], $P = .002$; Fig 4).

DISCUSSION

In this study we determined whether airway concentrations of endotoxin are increased by cigarette smoking and whether sputum endotoxin concentrations are associated with reduced

TABLE III. Linear regression analysis of sputum endotoxin concentrations compared with baseline data from asthmatic smokers and nonsmokers

Independent variable	Asthmatic nonsmokers						Asthmatic smokers					
	Regression			Correlation*			Regression			Correlation*		
	<i>R</i> ²	Residual SD	F ratio	<i>P</i> value	Coefficient	<i>P</i> value	<i>R</i> ²	Residual SD	F ratio	<i>P</i> value	Coefficient	<i>P</i> value
Change in postcorticosteroid FEV ₁ (%)	0.151	0.438	4.255	.050	−0.503	.009	0.019	0.617	0.307	.587	−0.282	.257
Age	0.060	0.425	1.866	.183	−0.314	.085	0.070	0.649	1.360	.259	0.342	.140
Sex	0.039	0.429	1.180	.286	−0.217	.242	0.038	0.660	0.703	.413	0.191	.420
BMI	0.001	0.438	0.034	.856	−0.048	.799	0.060	0.652	1.155	.297	0.211	.371
Pack years smoked							0.164	0.616	3.518	.077	0.382	.097
Asthma duration	0.024	0.433	0.724	.402	−0.116	.534	0.015	0.668	0.274	.607	0.224	.342
Inhaled corticosteroid dose	0.002	0.438	0.065	.800	−0.128	.491	0.056	0.654	1.073	.314	−0.222	.348
ACQ score	0.041	0.429	1.246	.274	−0.283	.123	0.007	0.671	0.134	.718	0.009	.969
FENO	0.005	0.431	0.146	.706	0.130	.501	<0.001	0.698	0.000	.989	−0.119	.649
Exhaled CO	0.006	0.444	0.176	.678	0.162	.393	0.012	0.688	0.213	.651	−0.164	.503
Serum endotoxin	0.027	0.421	0.306	.591	−0.110	.720	0.001	0.700	0.010	.921	0.098	.708
Bronchodilator reversibility (%)	0.002	0.456	0.064	.803	−0.102	.606	0.015	0.668	0.277	.605	−0.092	.700

Values in boldface indicate statistical significance.

BMI, Body mass index; FENO, fraction of exhaled nitric oxide.

*Spearman rank correlation.

TABLE IV. Multivariate multiple regression analysis of sputum endotoxin concentration and baseline data from all asthmatic patients

Independent variable	All asthmatic patients (<i>P</i> = .702)*						Zero-order correlation coefficient (<i>r</i>)
	Coefficient	SE	<i>r</i> _{partial}	<i>t</i> Statistic	<i>P</i> value		
(Constant)	3.550						
Change in postcorticosteroid FEV ₁ (%)	−0.010	0.006	−0.293	−1.62	.116		−0.294
Age	−0.019	0.014	−0.244	−1.333	.193		−0.072
Sex	−0.004	0.201	−0.004	−0.019	.985		0.008
BMI	−0.014	0.024	−0.115	−0.614	.544		−0.162
Pack years smoked	0.012	0.008	0.263	1.441	.161		0.101
Asthma duration	0.005	0.007	0.143	0.767	.450		0.089
Inhaled corticosteroid dose	<0.001	0.001	0.072	0.381	.706		−0.090
ACQ score	−0.179	0.147	−0.225	−1.22	.233		−0.197
FENO	0.002	0.003	0.101	0.535	.597		−0.064
Exhaled CO	−0.003	0.014	−0.047	−0.251	.804		0.061
Bronchodilator reversibility (%)	0.001	0.002	0.134	0.715	.480		−0.120

BMI, Body mass index; FENO, fraction of exhaled nitric oxide.

*Model score *P* value.

corticosteroid responsiveness in asthmatic patients. Cigarette smoke contains high endotoxin concentrations, and the levels of endotoxin in the immediate atmosphere increase after active cigarette smoking¹⁴ and exposure to passive smoke.^{15,16} Despite increased exposure to endotoxin in cigarette smoke, sputum endotoxin concentrations in active smokers were similar to those in never smokers in both the asthma and healthy groups.

Despite similar median sputum endotoxin concentrations between asthmatic patients and healthy subjects, there was a 2 log-order range of airway fluid endotoxin concentrations among members of the asthmatic group. The wide variation in sputum endotoxin concentrations was not associated with the distribution of several potentially related factors, including demographics, asthma control, lung function, or sputum cytology or cytokine concentration, other than IL-1 receptor antagonist (IL-1Ra), which is a natural cytokine inhibitor of IL-1, which has an important proinflammatory role in innate immunity.²⁶ In our study sputum IL-1Ra concentrations were associated with higher sputum endotoxin concentrations. Because endotoxin is reported

to increase the expression of IL-1Ra on bovine PBMCs,²⁷ we speculate that the presence of endotoxin in the airways might promote local production of IL-1Ra by lung mononuclear cells. Because it has an anti-inflammatory function, this suggests that endotoxin-associated increased IL-1Ra concentrations might have a homeostatic function to maintain normal concentrations of proinflammatory cytokines, including IL-1β, in an environment in which endotoxin concentrations are increased.

One potential source of airway endotoxin might be from gram-negative Proteobacteria identified in the bronchial²⁸ and induced sputum²⁹ asthma microbiome. Increased colonization of the airways has been reported in subgroups of patients with poorly controlled asthma, bronchial hyperresponsiveness,^{19,20} and corticosteroid insensitivity,³⁰ which might result in increased airway endotoxin concentrations, especially where alveolar macrophage function has been compromised by smoking or severe disease causing reduced bacterial clearance. The airway microbiome should be examined in future studies to investigate this hypothesis along with macrophage functional phenotypes.

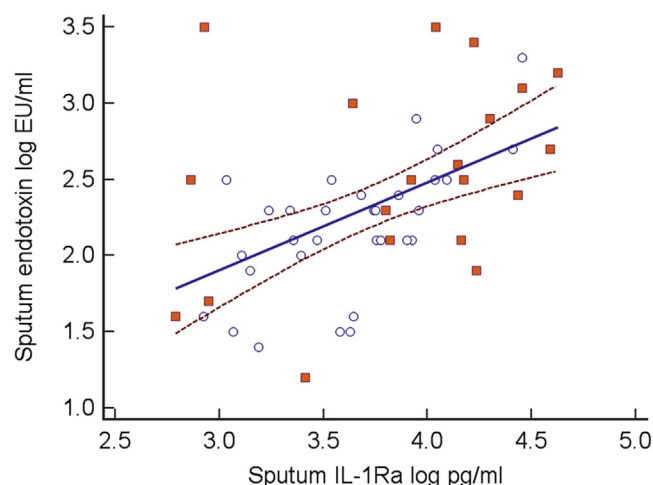
TABLE V. Multivariate multiple regression analysis of sputum endotoxin concentrations and baseline data from asthmatic nonsmokers and smokers

Independent variable	Asthmatic nonsmokers ($P = .201$)*						Asthmatic smokers ($P = .954$)*					
	Coefficient	SE	r_{partial}	t	P value	r	Coefficient	SE	r_{partial}	t	P value	r
(Constant)	1.985						19.050					
Change in postcorticosteroid FEV ₁ (%)	−0.020	0.006	−0.686	−3.402	.005	−0.387	−0.140	0.134	−0.518	−1.049	.371	−0.019
Age	−0.005	0.013	−0.111	−0.404	.693	−0.165	−0.160	0.195	−0.428	−0.821	.471	0.040
Sex	−0.356	0.207	−0.432	−1.725	.108	−0.208	−1.380	1.613	−0.443	−0.856	.455	0.264
BMI	<0.001	0.021	−0.001	−0.004	.997	0.003	−0.365	0.337	−0.531	−1.084	.358	−0.310
Pack years smoked	−0.015	0.012	−0.332	−1.269	.227	−0.097	0.006	0.047	0.079	0.137	.899	0.061
Asthma duration	0.000	0.007	−0.014	−0.051	.960	−0.077	0.026	0.035	0.394	0.742	.512	0.315
Inhaled steroid dose	0.001	0.000	0.587	2.617	.021	0.090	−0.001	0.001	−0.472	−0.929	.421	−0.360
ACQ score	0.034	0.140	0.068	0.244	.811	−0.241	1.465	1.541	0.481	0.95	.412	−0.326
FENO	0.004	0.003	0.423	1.682	.117	0.065	0.018	0.046	0.218	0.387	.724	−0.144
Exhaled CO	0.050	0.113	0.122	0.441	.666	0.090	−0.137	0.114	−0.569	−1.198	.317	−0.276
Bronchodilator reversibility (%)	0.002	0.002	0.248	0.922	.374	−0.036	0.015	0.015	0.500	1	.391	−0.092

Values in boldface indicate statistical significance.

BMI, Body mass index; FENO, fraction of exhaled nitric oxide.

*Model score P value.

**FIG 3.** Association of sputum endotoxin concentrations with sputum IL-1Ra concentrations in asthmatic patients. The linear regression line (solid line) with the true 95% CI (dashed lines) of nonsmokers (open circles) and smokers (red squares) is shown. In the combined asthma patient group sputum endotoxin concentrations correlated with the sputum IL-1Ra concentrations (Spearman rho: $r = 0.510$ [95% CI, 0.273–0.689], $P < .001$).

It is possible that differences in environmental exposure to endotoxin might account for the variability between subjects in sputum endotoxin concentrations. Sources of endotoxin associated with a high level of exposure include household dust, particularly in association with low household income, dog ownership, and household cleanliness.¹⁷ None of the participants provided a history of occupations, such as cotton textile workers or farm animal workers, associated with exposure to high endotoxin concentrations.¹⁸

Severe asthma and asthma in cigarette smokers are both associated with corticosteroid insensitivity, although the mechanism responsible for this phenomenon is still to be conclusively established.^{3,7,31} Preclinical and clinical data support a role for endotoxin as a potential cause of corticosteroid insensitivity in asthmatic patients.^{8–12,32,33} Previous studies reported increased BAL fluid endotoxin levels

in corticosteroid-resistant asthma compared with corticosteroid-sensitive asthma in nonsmokers¹¹ and with a trend for higher concentrations in patients with neutrophilic asthma.¹² Using linear regression and correlation analysis, we found that the sputum endotoxin concentration correlated with an impaired lung function response to oral corticosteroids and with smoking status. This observation is confirmed by means of multiple regression analysis showing that the sputum endotoxin concentration correlated with postcorticosteroid change in FEV₁ only in asthmatic nonsmokers, providing support for a role for endotoxin in the corticosteroid-resistant inflammatory response. The narrow range of response to corticosteroids in asthmatic smokers might prevent the identification of a significant association with sputum endotoxin concentrations. Alternatively, endotoxin might not influence corticosteroid sensitivity in this group. Potential mechanisms for endotoxin-induced corticosteroid insensitivity include downregulation of glucocorticoid receptors on airway epithelial cells¹⁰ or induction of noneosinophilic airway inflammation by endotoxin.^{9,34,35}

Circulating endotoxin concentrations are influenced by absorption of endotoxin from the gastrointestinal tract, with increased levels after a high-fat diet.^{36–38} We found that serum endotoxin concentrations were increased in asthmatic smokers compared with those seen in asthmatic nonsmokers. The explanation for this difference is not known. Body mass index was similar between the asthmatic groups, but differences in dietary fat intake cannot be excluded. Previous antibiotic treatment might alter the gut microbiota and reduce endotoxin absorption from the gut,³⁹ and possibly a variation in previous antibiotic use might alter serum endotoxin concentrations. An additional source of circulating endotoxin from the lung caused by increased airway permeability in smokers is also possible.⁴⁰

Chronic circulating endotoxemia induces low-grade systemic inflammation.^{38,41} In asthmatic patients we found that serum endotoxin concentrations were associated with sputum IL-6, IL-8, and CCL2 concentrations. Further studies are required to determine whether this is a causal relationship and, if so, whether circulating endotoxin-induced systemic inflammation affects the airways or whether the lungs act as a source of systemic inflammation. Interestingly, a high-fat diet augments airway

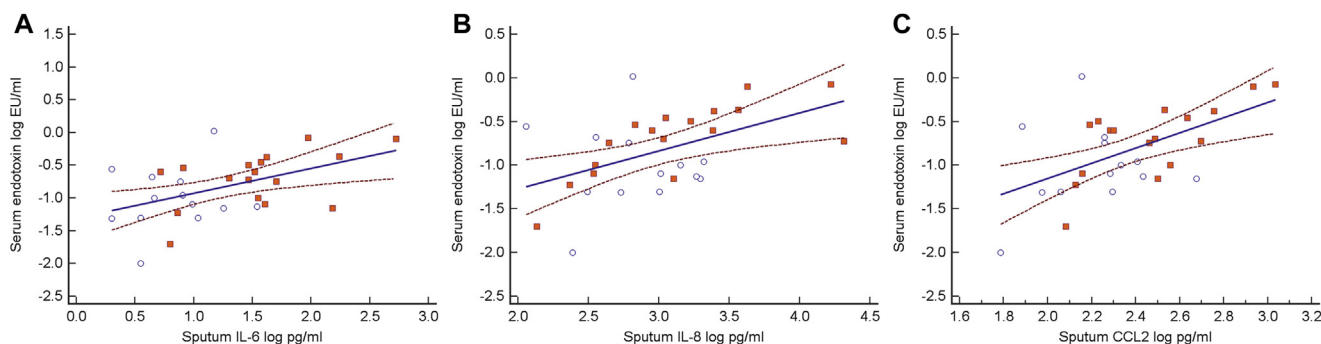


FIG 4. Association of serum endotoxin concentrations with sputum IL-6 (A), IL-8 (B), and CCL2 (C) concentrations in asthmatic patients. The linear regression lines (solid lines) with the true 95% CIs of nonsmokers (open circles) and smokers (red squares) are shown. In asthmatic patients serum endotoxin concentrations correlated with sputum concentrations of the cytokines IL-6 ($r = 0.481$ [95% CI, 0.147-0.717], $P = .007$), IL-8 ($r = 0.495$ [95% CI, 0.164-0.726], $P = .005$), and CCL2 ($r = 0.542$ [95% CI, 0.226-0.755], $P = .002$).

inflammation in asthmatic patients,⁴² including increased expression of TLR4 in sputum cells, which raises the possibility that an increase in circulating endotoxin concentration induced by a high-fat diet contributes to airway inflammation.

If the association between increased sputum endotoxin concentrations and reduced response to oral corticosteroids in asthmatic patients is causal, which remains to be established, it might open up new lines of therapy for managing corticosteroid resistant airway disease, such as decreasing exposure to endotoxin through house dust mite avoidance or reducing production of endotoxin through reduced bacterial colonization of the airways or gut with antibiotic therapy. An alternative approach might include treatment with antagonists of TLR4 receptors that are activated by endotoxin, although this could be complicated by increased susceptibility to infection.

We recognize that there are limitations in this study. The cross-sectional study design might have missed variations in airway and circulatory endotoxin concentrations between patient groups over time. The cause of the wide variation in sputum endotoxin concentrations was not determined, and future studies should examine environmental exposures to endotoxin and individual sensitivity to endotoxin.⁴³⁻⁴⁵ The mechanism by which endotoxin might impair corticosteroid sensitivity will require further investigation. The analysis of these data was exploratory, involving multiple statistical tests, and as such, the results will require confirmation by further studies.

In conclusion, we have found that neither cigarette smoking nor asthma is associated with increased endotoxin concentrations in sputum, although there is a wide variation in sputum endotoxin concentrations between asthmatic patients. The association between higher sputum endotoxin concentrations and an impaired lung function response to oral corticosteroids, particularly in asthmatic nonsmokers, suggests that airway endotoxin might contribute to corticosteroid insensitivity in asthmatic patients. Serum endotoxin concentrations are increased in asthmatic smokers compared with those seen in asthmatic nonsmokers, and levels correlate with proinflammatory cytokine concentrations in sputum and with reduced lung function. Further research should examine whether increased circulating endotoxin concentrations adversely affect the airways in asthmatic patients.

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Clinical implications: The association between higher sputum endotoxin concentrations and an impaired lung function response to oral corticosteroids, particularly in asthmatic never smokers, suggests that airway endotoxin might contribute to corticosteroid insensitivity in asthmatic patients.

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