

CD14-dependent airway neutrophil response to inhaled LPS: Role of atopy

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Background: Inhaled endotoxin (LPS) is associated with airway neutrophilic (PMN) inflammation in both asthmatic and control subjects, with asthmatic subjects demonstrating possibly higher sensitivity. CD14 is the principal receptor mediating LPS responses *in vivo*. It is unknown whether constitutive CD14 can predict the magnitude of the PMN response after LPS inhalation and whether atopy plays a role in this response. **Objective:** We sought to examine associations between constitutive airway CD14 expression and LPS-induced PMNs after 5 µg of LPS inhalation and to examine associations between markers of atopy (eosinophils and eosinophil cationic protein) and CD14 expression and LPS-induced PMNs.

Methods: Ten atopic asthmatic subjects and 8 healthy control subjects inhaled 0.9% saline and LPS (*Escherichia coli* 026:B6, 5 µg) separated by 3 weeks. Induced sputum was collected at 24 hours before and 6 hours after inhalation. Induced sputum was analyzed for total and differential cell counts and soluble markers (soluble [s]CD14, eosinophil cationic protein, IL8, and total protein). Flow cytometry was used to analyze membrane-bound CD14 expression.

Results: Significant associations were found between the LPS-induced PMN response (PMNs per milligram of sputum) and both constitutive sCD14 ($R = 0.7$, $P = .005$) and membrane-bound CD14 ($R = 0.9$, $P = .01$). Asthmatic subjects demonstrated significantly higher levels of constitutive sCD14 compared with control subjects, and baseline eosinophils were significantly associated with baseline sCD14 ($R = 0.7$, $P = .01$) and LPS-induced PMNs ($R = 0.6$, $P = .03$).

Conclusion: Constitutive airway CD14 expression can predict the magnitude of the PMN response after inhaled LPS. Atopy appears to play a role in the level of CD14 expression and may contribute to LPS sensitivity in asthmatic subjects. (*J Allergy Clin Immunol* 2001;107:31-5.)

Key words: CD14, LPS, PMN, association with atopy

Abbreviation used

ECP: Eosinophil cationic protein

Endotoxin from gram-negative bacteria is a commonly encountered air contaminant in occupational and domestic settings that has been linked to asthma severity.^{1,2} In human subjects LPS is a potent inducer of lung inflammation that is marked by a dose-dependent increase in airway neutrophils (PMNs).^{3,4} Recent evidence suggests that asthmatic subjects may be more sensitive to the effects of LPS than nonasthmatic subjects.^{1,5-7} Allergic asthmatic subjects have been reported to have increased expression of CD14, the primary receptor for LPS, after acute allergen challenge.^{8,9} Likewise, allergen challenge in the airway enhances subsequent eosinophilic responses to LPS.^{5,10} These findings suggest that T_H2-acquired immune responses promote exacerbation of asthma caused by LPS, likely through enhancement of CD14.

Several factors are known to upregulate the expression of CD14 in both its membrane-bound (mCD14) and soluble form (sCD14) in the respiratory tract. Allergen challenge in atopic subjects⁹ and a number of cytokines in healthy subjects¹¹⁻¹³ have been reported to enhance CD14 levels. Genetic factors may also regulate CD14 expression. Recent observations suggest that a common C/T polymorphism at the -159 position of the promoter region of the *CD14* gene is involved in the human LPS response.¹⁴ TT homozygotes for this gene have significantly increased density of CD14 on blood monocytes and increased serum sCD14 when compared with those with a CT or CC genotype.¹⁴⁻¹⁷

We hypothesized that atopy may influence the airways inflammatory response to inhaled LPS through enhancement of CD14 in the airway. To test this hypothesis, we examined the relationship between airway eosinophils and eosinophil cationic protein (ECP) with CD14 and airway PMN response to inhaled LPS in a cohort of 10 atopic asthmatic subjects and 8 healthy control subjects. Likewise, we examined the relationship between constitutive CD14 levels in the airway and the inflammatory response to LPS. We also undertook preliminary experiments to explore the role of the C/T polymorphism of the *CD14* gene promoter on expression of CD14 in the airway and response to LPS.

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TABLE I. Neutrophils (cells per milligram) recovered in induced sputum

	24 h before saline	6 h after saline	24 h before LPS	6 h after LPS
All subjects (n = 18)	712 ± 163	691 ± 168	600 ± 159	2050 ± 419*†
Control subjects (n = 8)	953 ± 297	738 ± 312	700 ± 306	2127 ± 855†
Asthmatic subjects (n = 10)	519 ± 162	653 ± 186	376 ± 89	1987 ± 387*†

Data are presented as the mean ± SEM.

Significant differences ($P < .05$) are indicated as follows: *compared with baseline and †compared with saline.

METHODS

Subjects

Ten allergic subjects with mild asthma and 8 nonallergic nonasthmatic subjects between the ages of 18 and 50 years were recruited for the study. The study was approved by the Committee on the Protection of the Rights of Human Subjects, The School of Medicine, The University of North Carolina-Chapel Hill. Allergic asthmatic subjects were assessed on the basis of current National Institutes of Health guidelines¹⁸ and were otherwise healthy nonsmoking (for at least 1 year) men or women. All asthmatic subjects had positive test responses on methacholine challenge ($PC_{20} < 5$ mg/mL) and were demonstrated to be atopic on the basis of a positive skin test response to one or more aeroallergens, including house dust mite.

Study protocol

The study design consisted of 2 consecutive days, with the first day being the baseline day and the second day being the saline (0.9%) or LPS (5 µg of *Escherichia coli* #L-2262; Sigma, Inc, St Louis, Mo) inhalation challenge followed 6 hours later by postchallenge measurements (spirometry and induced sputum). This 2-day protocol was repeated 3 weeks later with the opposite inhalation challenge. Each patient underwent saline and LPS challenges, which were randomly assigned. The LPS dose of 5 µg was chosen because changes in induced sputum PMNs have been reported with this dose but not systemic symptoms (fever and chills), which have been reported at greater than 50-µg doses.³

Induced sputum

The sputum induction and processing methods are based on the technique described by Pizzichini et al¹⁹ and reported in a current publication by our laboratory.²⁰ Briefly, subjects inhaled nebulized hypertonic saline (3%, 4%, or 5%) for 21 minutes. Expectored samples from the airways were collected separately from saliva and kept on ice. Manually selected plugs were incubated in 0.1% dithiothreitol, washed in Dulbecco's PBS, and gravity filtered. Total and differential leukocyte counts (Diff-Quik stain) and cell viability (Trypan blue exclusion staining) were recorded. Supernatants were obtained and frozen at -80°C for soluble mediator analysis.

Flow cytometry and immunofluorescence staining

The flow cytometric and immunofluorescence staining methods used in this study have been described in detail in a current publication by our laboratory.²¹ Briefly, identification and gating of sputum macrophages was based on light scatter properties and relevant surface antibodies fluorescently labeled with FITC and phycoerythrin (CD45, CD14, and CD16). CD14 expression was expressed as receptor-specific mean fluorescence intensity.

Measurement of cytokine levels in sputum

Cytokines (IL-8, IL-6, IL-1β, and GM-CSF) and proteins (sCD14, total protein, ECP, and myeloperoxidase) were measured by using commercially available ELISAs (Endogen, Inc, Woburn, Mass; Bio-

Rad, Hercules, Calif; R&D Systems, Minneapolis, Minn; and Oxis International, Portland, Ore). ECP was measured by using an RIA (Pharmacia Diagnostics, Inc, Columbus, Ohio). Cytokine and protein contents were expressed as weight per milliliter of airway fluid.

Endotoxin (LPS) challenge

The method of Michel et al¹ was used to deliver nebulized inhaled LPS at a 5-µg dose.

Genotype testing

Genotype testing was performed by using a previously published method.¹⁵ Oligonucleotide primers used in this study were as follows: sense, 5'-GGTGCCAACAGATGAGGTTTACA-3', and antisense, 5'-CCGGCTTCCAGGCTTCACACTT-3'. Sequencing was performed by the University of North Carolina Lineberger DNA Sequencing Facility.

Statistics

Paired (or unpaired) parametric *t* tests were used to compare induced sputum endpoints between asthmatic and control subjects and between baseline and postexposure measurements. Simple linear regression analysis was used to determine linear relationships between study endpoints, and the Pearson correlation coefficient (*R*) was used to determine correlations.

RESULTS

Low-dose (5 µg) LPS inhalation challenge caused a significant increase in sputum PMNs compared with saline inhalation challenge and pre-LPS inhalation baseline in the entire subject cohort (Table I). No difference was observed between asthmatic and control subjects in the magnitude of the PMN response 6 hours after LPS inhalation (Table I). Cytokines and proteins measured 24 hours before and 6 hours after LPS inhalation are shown in Table II. Asthmatic subjects demonstrated significantly higher levels of total protein 24 hours before LPS inhalation than control subjects ($P = .03$) and showed a trend toward higher baseline sCD14 levels ($P = .04$, one-tailed *t* test).

Constitutive (baseline) sCD14 (in picograms per milliliter) in sputum and absolute PMNs (in cells per milligram) were recovered in 16 subjects (9 asthmatic and 7 control subjects) 24 hours before and 6 hours after LPS inhalation, respectively. A significant positive association ($R = 0.66$, $P = .005$) was observed between baseline sCD14 and LPS-induced PMNs in the combined cohort of subjects (Fig 1, A). Baseline mCD14 on sputum macrophages was measured in 10 subjects (5 asthmatic and 5 control subjects) 24 hours before LPS inhalation. A significant positive association ($R = 0.88$, $P = .01$) was observed between mCD14 expression and sputum PMNs 6 hours after LPS inhalation in these 10 subjects (Fig 1, B).

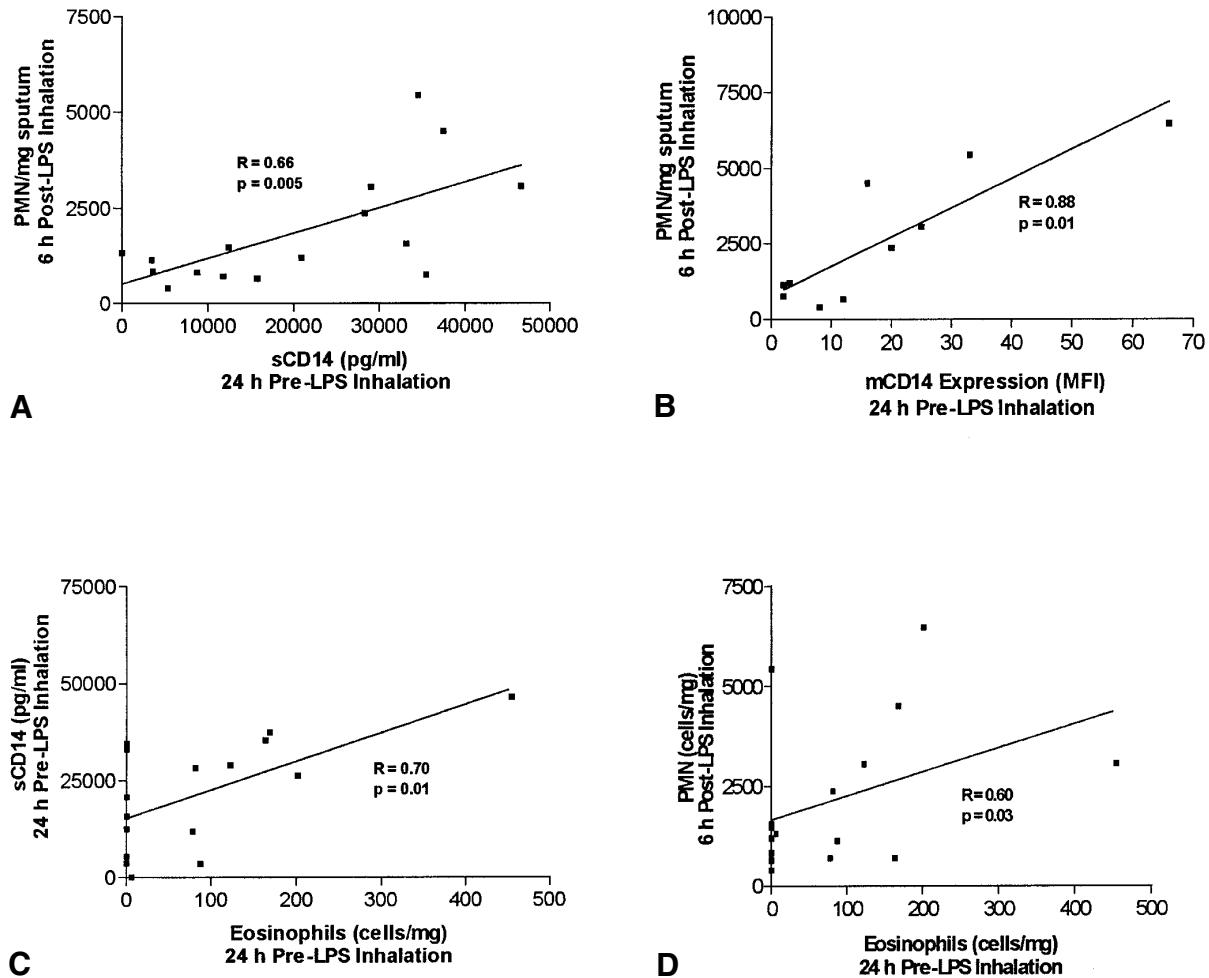


FIG 1. Simple linear regression analyses demonstrating significant positive associations between the number of sputum PMNs (cells per milligram) 6 hours after LPS inhalation challenge and baseline sCD14 (in picograms per milliliter, **A**) and sputum macrophage mCD14 (mean fluorescence intensity) expression (**B**). Significant associations are shown between the number of sputum eosinophils (cells per milligram) and baseline sCD14 (**C**) and LPS-induced PMNs (**D**).

TABLE II. Fluid-phase markers (mean $\pm$ SE) recovered 24 hours before LPS inhalation and CD14 genotype in control and asthmatic subjects

Subject	GM-CSF (pg/mL)	Total protein (mg/mL)	ECP (ng/mL)	MPO (ng/mL)	IL-6 (pg/mL)	IL-8 (pg/mL)	IL-1 β (pg/mL)	sCD14 (pg/mL)	CD14 genotype*
Control subjects (n = 8)	45 $\pm$ 42	0.96 $\pm$ 0.2	374 $\pm$ 78	328 $\pm$ 135	120 $\pm$ 25	3808 $\pm$ 1165	48 $\pm$ 28	16,112 $\pm$ 4622	TT = 1, CC = 3, C/T = 3
Asthmatic subjects (n = 10)	63 $\pm$ 28	1.63 $\pm$ 0.2 [†]	472 $\pm$ 166	188 $\pm$ 71	166 $\pm$ 36	5043 $\pm$ 1378	249 $\pm$ 210	30,008 [‡] $\pm$ 5889	TT = 4, CC = 3, C/T = 2

MPO, Myeloperoxidase.

*C/T polymorphism of the promoter region of the *CD14* gene. Seven of 8 control and 9 of 10 asthmatic subjects underwent genotyping for this polymorphism.

[†] $P = .03$ and [‡] $P = .04$ (one-tailed t test), significantly different from control subjects.

The absolute numbers of sputum eosinophils and ECP were used as markers of atopy in the airways to determine possible associations between baseline allergic inflammation and constitutive sCD14 expression and

LPS-induced PMNs. IL-8, although not a specific marker of atopic inflammation, was also used as a general marker of inflammation. A significant positive association ($R = 0.70$, $P = .01$) was observed between baseline

eosinophilia (eosinophils per milligram) and baseline levels of sCD14 in a combined cohort of subjects (Fig 1, C). A significant positive association ($R = 0.60$, $P = .03$) was also observed between baseline eosinophils and LPS-induced PMNs (PMNs per milligram) 6 hours after LPS inhalation in a combined cohort of subjects (Fig 1, D). Baseline sCD14 was significantly associated with ECP ($R = 0.60$, $P = .01$) and IL-8 ($R = 0.60$, $P = .01$), whereas ECP also demonstrated a strong association with LPS-induced PMNs ($R = 0.80$, $P = .001$).

Sixteen subjects were genotyped (9 asthmatic and 7 control subjects), and 5 were identified as TT homozygous (4 asthmatic and 1 control subjects, Table II). In these pilot observations we identified more TT homozygotes in the asthmatic subjects (44%) than expected in the healthy population (16%).¹⁴ Likewise, TT homozygotes had mean $\pm$ SE baseline sCD14 levels that were higher than a combined cohort of CC and CT subjects ($29,320 \pm 6601$ pg/mL vs $12,300 \pm 4486$ pg/mL). Despite the differences cited above, we found no differences in PMN response to LPS when subjects were stratified on the basis of the *CD14* gene promoter genotype.

DISCUSSION

It is well established that CD14 is the initial principal receptor that, in conjunction with Toll-like receptors, mediates LPS-induced inflammation in vivo. Membrane-bound CD14 is constitutively expressed on the surface of airway monocytes and macrophages,^{21,22} and sCD14 can be found in human airway fluids.^{9,12,23} Our data demonstrate that constitutive levels of mCD14 and sCD14 in the airways are positively associated with the magnitude of the airways PMN response (cells per milligram of sputum) 6 hours after LPS inhalation. Soluble CD14 likely binds to non-CD14-bearing cells and renders them responsive to LPS. Membrane-bound CD14 on airway macrophages directly responds to LPS and secretes inflammatory cytokines.²⁴⁻²⁷ Thus increased CD14 expression in the airways surface liquid confers greater airway inflammatory responsiveness to inhaled LPS.

Previous studies suggest that sCD14 in the airways arises predominantly from extravasation from the intravascular compartment^{9,12,23} and is associated with increased airways permeability.²⁶ In the current study asthmatic subjects had significantly increased protein in the airway consistent with increased permeability of the airway mucosa.

Asthmatic subjects may be more sensitive to the effects of inhaled LPS than healthy control subjects,^{6,7,28,29} and atopy may be an important mechanism. Our data suggest that atopic inflammation may play an important role in the CD14-associated responses to inhaled LPS. Sputum eosinophils and ECP correlated positively with baseline sCD14 levels and with the subsequent PMN responses 6 hours after LPS inhalation. A recent report by Virchow et al⁸ linked CD14 expression with airway eosinophilia in allergic asthmatic subjects. In that study sCD14, IL-4, and IL-5 levels in bronchoalveolar lavage fluid were significantly increased 18 hours after segmental allergen provo-

cation, and sCD14 was positively correlated with airway eosinophils and IL-13, a T_H2 -type cytokine with similar features to IL-4. Atopy may act as a mechanism to enhance sensitivity to inhaled LPS in asthmatic subjects through CD14-mediated activation of airway macrophages and granulocytes and the subsequent release of atopy-related cytokines, such as IL-4, IL-5, and IL-13.

Our exploratory genetic analysis failed to demonstrate a significant difference between genotype subgroups in the airway PMN response to inhaled LPS. However, our pilot data examining the frequency of the -159 genotype of the *CD14* gene promoter in 9 asthmatic and 7 normal subjects revealed a higher than expected frequency of TT homozygotes in the asthmatic subjects. Because this limited sample size precludes any definitive conclusion regarding the association of the TT genotype of the *CD14* gene promoter with asthma, we intend to extend this study to a larger population of asthmatic subjects (including asthmatic subjects stratified on the basis of disease severity).

Constitutive airway CD14 can predict the magnitude of the airways PMN response to low-dose (5 μ g) inhaled LPS in both allergic asthmatic and healthy control subjects. Atopy appears to play a role in CD14-mediated responses to inhaled LPS and may contribute to LPS sensitivity in atopic asthmatic subjects.

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