

Acute LPS inhalation in healthy volunteers induces dendritic cell maturation *in vivo*

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Background: We have been studying the innate immune response of airways cells of healthy human volunteers to inhaled LPS, a Toll-like receptor 4 (TLR4) ligand, and have shown that macrophage phagocytic capacity is blunted. **Objective:** Because a primary feature of dendritic cell (DC) maturation is a loss of phagocytic capacity, we sought to determine whether acute LPS inhalation in healthy volunteers promotes DC maturation *in vivo*. **Methods:** Phagocytosis (IgG-opsonized zymosan particles) and cell-surface phenotypes were analyzed by flow cytometry of induced sputum cells obtained before and 6 hours after Clinical Center Reference Endotoxin (CCRE; 20,000 EU) inhalation in 9 healthy volunteers. **Results:** Neutrophils were elevated in the airways after CCRE inhalation ($67\% \pm 6\%$ vs $37\% \pm 6\%$; $P < .05$). Phagocytosis (monocytes, macrophages) was blunted (73% , 46% ; $P < .05$) and negatively correlated with PMN influx ($R = -0.73$; $P < .05$) after CCRE inhalation. GM-CSF and IL-1 β , potent DC maturation agents, were elevated after versus before CCRE inhalation ($217 \text{ pg/mL} \pm 103 \text{ pg/mL}$ vs $722 \text{ pg/mL} \pm 202 \text{ pg/mL}$; $83 \text{ pg/mL} \pm 24 \text{ pg/mL}$ vs $148 \text{ pg/mL} \pm 37 \text{ pg/mL}$, respectively; $P < .05$). Markers of DC maturation (CD80, CD86, HLA-DR) were upregulated on monocytes and macrophages ($P < .05$), and discrete populations of mature DC were observed ($P < .05$) after CCRE inhalation. **Conclusion:** Inhaled LPS, directly through TLR4 stimulation of immature DC and/or indirectly through stimulation of GM-CSF and IL-1 β , induces pulmonary DC maturation *in vivo*.

Inhaled LPS may enhance allergic airways responses to air pollution through its ability to induce DC maturation. (J Allergy Clin Immunol 2005;115:345-50.)

Key words: Inhaled LPS, phagocytosis, dendritic cell maturation

Normal individuals and those with asthma are often exposed to environmental endotoxin in doses that affect the immune system.¹ Indeed, the innate immune response to inhaled endotoxin (LPS) is thought to play a major pathogenic role in environmental exacerbations of asthma, and possibly in its initiation. We have been studying the immune response to inhaled LPS and have found that the phagocytic capacity of airway monocytes and macrophages in individuals with allergy and healthy volunteers is blunted after inhaled LPS challenge.²⁻⁵ Sputum macrophages obtained from subjects with asthma with elevated granulocytic inflammation also have decreased phagocytic capacity and downregulated surface expression of the complement-mediated phagocytosis receptor CD11b/complement receptor 3 (CR3) compared with subjects with asthma with minimal airways inflammation.⁶

In vitro studies on blood monocytes show that phagocytic monocytes can differentiate into immature dendritic cells (iDCs), then further into mature dendritic cells (mDCs)^{7,8} in the presence of potent dendritic cell (DC) maturation agents like GM-CSF, IL-4, LPS, and IL-1 β .⁹⁻¹¹ Blood monocytes enter tissues such as the lung, where they are highly phagocytic as iDCs. After encountering DC maturation agents that are usually associated with inflammation, the iDCs become mDCs with downregulated phagocytosis and upregulated surface expression of costimulatory molecules (CD80, CD86), MHC class II molecules (HLA-DR),¹²⁻¹⁴ and Fc γ Rs (Fc γ RI/CD64, Fc γ RIII/CD16).¹⁵ DC maturation also induces changes in chemokine receptor expression such that mDCs leave the tissue via afferent lymphatic channels and enter the lymph nodes, where they are effectively able to present antigen to and stimulate lymphocytes.¹⁰ Of note, the airways inflammatory response after inhaled LPS is marked by elevated neutrophils.^{2,16} Neutrophils play an important role in instructing recruitment and activation of DCs during microbial infection.¹⁷

In this study, we tested the hypothesis that inhaled LPS induces DC maturation *in vivo* in human beings, serving to bridge innate with adaptive airways immune responses.

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Abbreviations used

CBC: Complete blood count
CCRE: Clinical Center Reference Endotoxin
DC: Dendritic cell
iDC: Immature dendritic cell
mDC: Mature dendritic cell
MFI: Mean fluorescence intensity

We challenged healthy volunteers with inhaled LPS and examined phagocytosis of IgG-opsonized zymosan particles by airway monocytes and macrophages. We further examined the expression of MHC class II molecules (HLA-DR), costimulatory molecules, and Fc receptors on airway monocytes and macrophages, and measured levels of cytokines in the airways known to enhance DC maturation. We show that LPS inhalational challenge induces the generation of mature lung DCs *in vivo*.

METHODS**Healthy subjects undergoing LPS inhalation challenge**

Nine healthy subjects (3 male, 6 female) between the ages of 18 and 50 years underwent LPS inhalation challenge. All subjects were nonsmoking volunteers with no history of lung disease (asthma, chronic obstructive pulmonary disease, fibrosis) and had been free of upper and lower respiratory tract infection for at least 4 weeks before beginning the study. All women provided a urine sample for pregnancy testing, and a positive pregnancy test resulted in exclusion from the study. All subjects had a FEV₁ of greater than 80% of predicted values for a population of similar height, weight, sex, age, and race.

This study was approved by the Committee on the Protection of the Rights of Human Subjects, the School of Medicine, the University of North Carolina at Chapel Hill, the General Clinical Research Center Ethics Review Board at the University of North Carolina at Chapel Hill, and the Food and Drug Administration under IND BB9998. Subjects received a medical examination on a separate screening day before beginning the study, at which informed written consent was obtained.

LPS inhalation challenge protocol

The study protocol consisted of 3 consecutive days. The first day of each study period was for baseline measurements of spirometry, venesection for complete blood count (CBC) analysis, vital signs, symptom score, and induced sputum endpoints. On the second day, baseline spirometry, vital signs, and symptom scores were recorded immediately before inhalation challenge. Endotoxin (20,000 EU) was provided by the National Institutes of Health Clinical Center and is referred to as Clinical Center Reference Endotoxin (CCRE). Spirometry was measured 1 time each hour for 6 hours after the inhalation challenge. Venesection for CBC analysis and induced sputum was performed 6 hours after the inhalation challenges. After the completion of the induced sputum procedure, vital signs and symptom scores were recorded, and the subjects were observed overnight in the clinical unit of the GCRC at the University of North Carolina Hospitals. Follow-up measurements of spirometry, vital signs, and symptom scores were recorded on the following morning (third day).

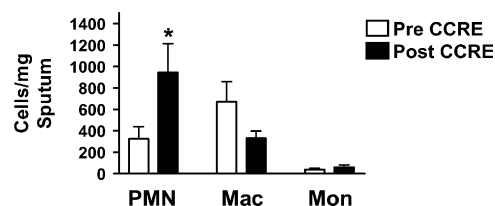


FIG 1. The mean $\pm$ SEM number of polymorphonuclear neutrophils (PMN), macrophages (Mac), and monocytes (Mon) (cells/mg sputum) before and 6 hours after CCRE inhalation recovered from induced sputum in 9 subjects (* $P < .05$).

Induced sputum and fluid phase analysis

Sputum induction and processing were performed according to previously published methods.^{2,18} Cytokines (GM-CSF, IL-1 β , TNF- α , IFN- γ , IL-12, soluble CD14, IL-13, IL-6, IL-8) were analyzed by using commercially available ELISA kits (R&D Systems, Minneapolis, Minn).

Flow cytometry and immunofluorescent staining

Flow cytometry was performed by using a FACSORT (Becton Dickinson, Miami, Fla) as previously described.^{2,18} Macrophages, monocytes, neutrophils, and lymphocytes in sputum were identified on the basis of light scatter properties and positive expression for CD45 (pan leukocyte marker), HLA-DR/CD14 (macrophages/monocytes), CD16 (neutrophils), and CD3 (lymphocytes). DCs were further analyzed by examining surface expression of CD80 and CD86. Analysis was performed by using Cell Quest software (Becton Dickinson).

Phagocytosis assay

Phagocytosis of fluorescein isothiocyanate-labeled IgG-opsonized *Saccharomyces cerevisiae* zymosan A BioParticles (Molecular Probes, Inc; Eugene, Ore) was performed as previously described.^{2,18} All samples were analyzed by flow cytometry within 24 hours of fixation. Particle uptake was identified as a rightward shift in fluorescence into the particle (zymosan)-exposed region (M3) from the control (nonparticle-exposed) region (M1). Phagocytosis was defined as the mean fluorescence intensity (MFI) of the cells in the zymosan-exposed region less the MFI of the cells in the control region. A uniform shift in all sputum phagocyte populations was routinely observed when cells were exposed to particles, such that at least 80% of the cells shifted (with particle exposure) into the exposed region compared with the unexposed region.

Data analysis

Differences in neutrophil levels, phagocytosis, cell-surface marker expression, and cytokine levels between before and after CCRE inhalation challenge were measured by using nonparametric paired *t* tests (Wilcoxon sign-rank) with the overall α level set at 0.05. Data are presented as means $\pm$ SEMs unless otherwise noted. Correlation analysis was performed by using the Spearman rank correlation coefficient (*R*) with the overall α level set at 0.05.

RESULTS**Lung function, symptoms, vital signs, CBC analysis**

Subjects did not develop systemic symptoms such as chills or cough, and no significant changes in vital signs (fever, blood pressure, heart rate, breath sounds) or lung

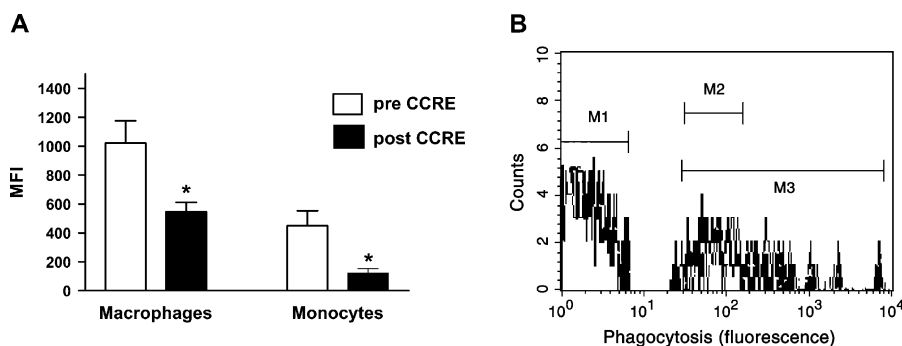


FIG 2. A, Mean $\pm$ SEM (N = 9) phagocytosis (MFI) of IgG-opsonized *S cerevisiae* zymosan A BioParticles in sputum derived macrophages and monocytes before (open bar) and 6 hours after (solid bar) inhaled challenge with CCRE (* P < .05). **B**, A typical histogram overlay demonstrating the changes in phagocytosis.

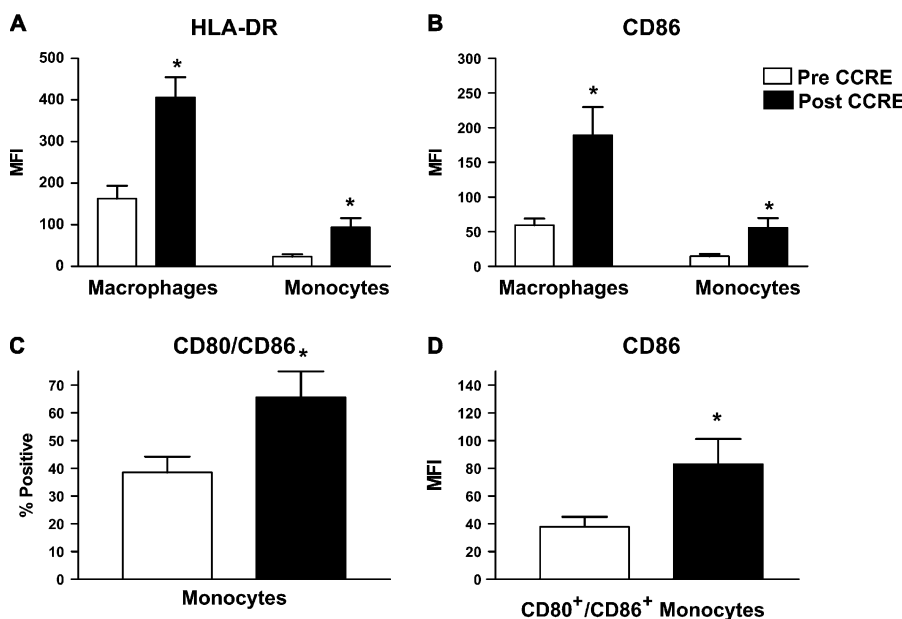


FIG 3. Mean $\pm$ SEM expression (MFI) of DC maturation markers (MHC class II/HLA-DR (**A**), CD86/B7-2 (**B**) CD80/CD86) on sputum macrophages and monocytes 6 hours after CCRE challenge (solid bar) compared with prechallenge (open bar) levels (* P < .05). The percentage of monocytes coexpressing CD80/CD86 (% positive cells) (**C**) and the level of CD86 expression on CD80⁺/CD86⁺ monocytes (**D**) were significantly (* P < .05) elevated 6 hours after CCRE exposure compared with pre-exposure values.

function (% predicted forced vital capacity [FVC] and forced expiratory volume in 1 second [FEV₁]) were observed at 1 hour, 6 hours, or 24 hours after CCRE (20,000 EU) challenge compared with before CCRE challenge. Peripheral blood CBC analysis showed no significant changes compared with pre-exposure baseline.

Neutrophil response

To examine whether CCRE challenge alters the leukocyte milieu in the airways, we measured leukocyte subsets in sputum before and after CCRE challenge by microscopic analysis and flow cytometry. Fig 1 shows the

relative changes in the number of neutrophils, macrophages, and monocytes before and after CCRE. The number of neutrophils was markedly increased after CCRE challenge. Likewise, the percentage of neutrophils increased by 1.8-fold (P < .05) after CCRE challenge.

Phagocytosis

Phagocytosis of IgG-opsonized zymosan (*S cerevisiae*) particles was blunted in airway monocytes (73% decrease; P < .05) and macrophages (46% decrease; P < .05) after inhaled challenge with CCRE compared with prechallenge values (Fig 2, A). A typical histogram overlay

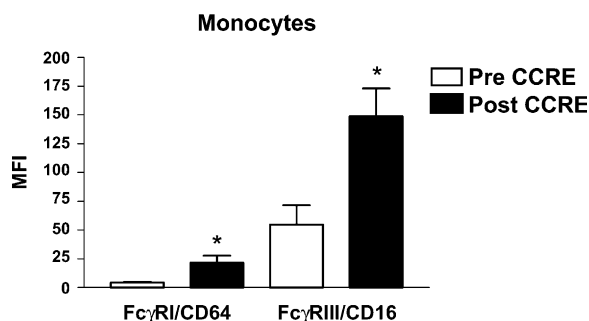


FIG 4. Mean $\pm$ SEM expression (MFI) of Fc γ RI (CD64) and Fc γ RIII (CD16) on sputum monocytes before versus after CCRE inhalation. Both CD64 and CD16 were significantly (* $P < .05$) elevated after versus before CCRE inhalation.

demonstrating the changes in phagocytosis is shown in Fig 2, B. Uptake of zymosan particles occurred both before and after CCRE exposure as measured by a rightward shift in MFI from the control region (M1) into the total zymosan-exposed region (M3). Greater zymosan uptake was observed pre-CCRE exposure (black) compared with post-CCRE exposure (white, M2 region) and is shown as a greater rightward shift in MFI. A uniform shift in cells from the control region (M1) into the zymosan-exposed region (M3) was observed before and after CCRE exposure in most subjects. The mDC population (by forward scatter/side scatter) was included in the phagocytosis analysis.

To determine whether the influx of neutrophils after CCRE was related to the decreased phagocytosis response, we examined the association between neutrophil levels and macrophage phagocytosis after CCRE. Phagocytosis was negatively correlated with PMN influx ($R = -0.73$; $P < .05$) after CCRE inhalation.

Cell surface phenotypes

To determine whether the observed change in phagocytosis was a result of maturation of monocytes and macrophages (immature DC, iDC) to mDC, we evaluated the expression of DC maturation markers (MHC class II/HLA-DR, CD80/B7-1, CD86/B7-2, CD80/CD86) on sputum macrophages and monocytes after CCRE challenge compared with prechallenge levels. Whereas CD11b (CR3) expression (MFI) on monocytes and macrophages was unaffected by CCRE exposure (16 ± 2 vs 17 ± 2 ; 23 ± 4 vs 25 ± 4 , respectively), the expression of HLA-DR, CD80, and CD86 all increased on a per cell and population basis (Fig 3). DCs express receptors for the Fc portion of IgG (Fc γ RI/CD64, Fc γ RIII/CD16), which mediate internalization of antigen-IgG complexes and promote efficient MHC class II-restricted antigen presentation. Both CD64/Fc γ RI and CD16/Fc γ RIII expression on monocytes were significantly ($P < .05$) increased after CCRE exposure compared with pre-exposure (Fig 4).

In 6 of 9 subjects, we identified a distinct population of mDCs after CCRE exposure. This distinct population is

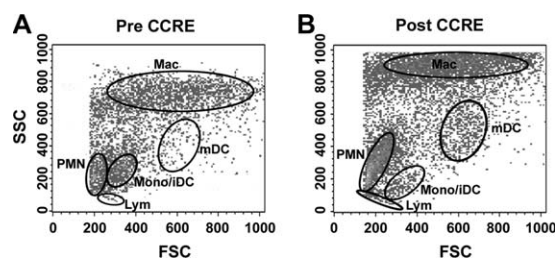


FIG 5. A dot plot of sputum leukocytes based on light scatter properties (x-axis: forward scatter, FSC; y-axis: side scatter, SSC) before (A) and 6 hours after (B) CCRE inhalation. Gated populations are identified as macrophages (Mac), neutrophils (PMN), lymphocytes (Lym), monocytes (Mono/iDC). In 6 subjects, a population of mDCs was observed.

shown in Fig 5, B. In Fig 5, A (pre-CCRE), the mDC population is less well organized and contains relatively fewer cells. Compared with the monocyte population, the mDC population from these subjects had a relatively higher expression (MFI) of HLA-DR (507 vs 128), CD80 (12 vs 7), and CD86 (122 vs 59).

Cytokine levels in sputum

LPS can stimulate DC maturation either directly or indirectly through cytokines. Cytokines that can induce DC maturation including GM-CSF, IL-1 β , IL-4, IL-12, and TNF- α were analyzed in sputum before and after CCRE challenge. We found that GM-CSF and IL-1 β levels were increased after CCRE exposure (Fig 6). CCRE exposure had no significant effect on any of the other cytokines measured (Table I).

DISCUSSION

In this study, we tested the hypothesis that stimulation of the lung innate immune system with inhaled LPS induces changes within cytokine and cellular milieu on the airways surface to generate and stimulate adaptive immune responses more effectively by promoting the maturation of lung DCs. We showed that inhaled clinical center reference endotoxin (CCRE, 20,000 EU) causes (1) a decrease in the phagocytic capacity of airways surface myeloid cells; (2) an increase in monocyte/macrophage expression of costimulatory molecules, MHC class II, and Fc receptors; (3) the appearance of a phenotypically distinct population of mDC in the airways; and (4) the induction of cytokines capable of inducing DC maturation. These changes are consistent with inhaled LPS causing DC maturation *in vivo*.

Downregulated phagocytosis is a feature of DC maturation. In previous studies, we showed in healthy subjects and subjects with asthma that airway cells (monocytes, macrophages, neutrophils) have reduced phagocytosis after inhaled LPS (5 μ g $\sim$ 30,000 EU) challenge.² In this study, by using a Food and Drug Administration–approved CCRE (20,000 EU), we confirm that in healthy

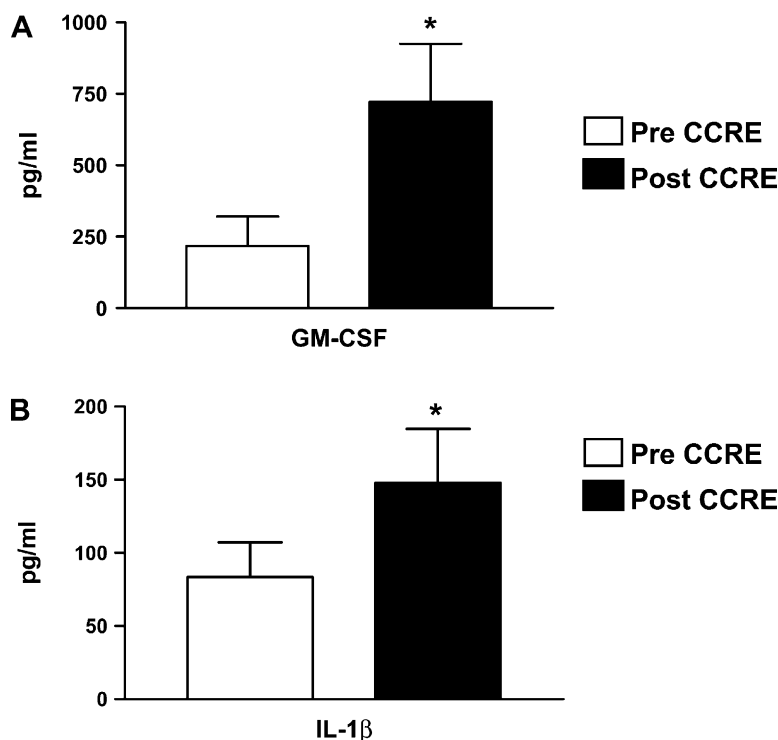


FIG 6. Mean $\pm$ SEM (N = 9) GM-CSF (pg/mL) (**A**) and IL-1 β (pg/mL) (**B**) levels in sputum supernatant before (open bar) versus after (solid bar) CCRE exposure (* $P < .05$).

TABLE I. Mean ($\pm$ SEM) cytokine levels in induced sputum before and 6 hours after CCRE challenge

Cytokine (pg/mL)	GM-CSF	IL-1 β	IL-13	IL-12	IFN- γ	IL-6	IL-8	IL-10	sCD14
Pre-CCRE	217 (103)	84 (24)	669 (142)	980 (373)	149 (76)	120 (24)	9312 (4013)	111 (82)	29,676 (5564)
Post-CCRE	722 (203)*	148 (36)*	931 (132)	467 (84)	200 (148)	144 (39)	5346 (1408)	218 (102)	35,636 (7086)

* $P < .05$.

volunteers, sputum macrophages and monocytes have reduced phagocytic capacity after acute challenge with inhaled LPS. Importantly, phagocytic capacity was modified when the dose of LPS was capable of inducing significant neutrophil influx into the airways. In a recent study in which we used a dose of inhaled LPS (10,000 EU) that was below the threshold for inducing a neutrophil influx, we observed enhanced rather than downregulated phagocytic capacity.¹⁹ Indeed, neutrophils stimulated by microorganisms release factors that induce upregulation of DC costimulatory molecules CD86 and CD40.¹⁷ Whether the decrease in phagocytosis is causally or incidentally related to airways neutrophilia remains to be understood. It may be that neutrophils have a regulatory role in DC function during microbial infection and that cross-talk between neutrophils and DCs is an important component of innate host defense responses.

Immature DCs can capture antigens and phagocytose pathogens, but compared with mDCs, they express lower levels of molecules required for antigen presentation and T-cell stimulation, such as MHC class II (HLA-DR) and costimulatory molecules CD80 and CD86. mDCs are

much less able to phagocytose pathogens than iDCs or phagocytic monocytes but have relatively higher expression of HLA-DR, CD80, CD86, and CD80/CD86, enabling them to activate antigen-specific T cells and induce primary immune responses.¹² In this study, LPS inhalation caused an upregulation of HLA-DR, CD80, and CD86, markers of DC maturation on airway monocytes and macrophages, 6 hours after exposure. Our findings of increased expression of Fc γ Rs (CD64, CD16) after CCRE exposure further support the notion of enhanced DC maturation. DCs can be activated and induced to mature and to upregulate MHC class II presentation by engagement of CD16 and CD64.^{15,20}

The mechanism by which inhaled LPS causes DC maturation *in vivo* is not entirely clear. It is possible that the effect is direct through activation of TLR4. The time course of activation (within 6 hours) is consistent with a direct effect. Our data in this respect are consistent with *in vivo* animal observations in mice in which systemic administration of LPS induced maturation of splenic DCs within 4 to 6 hours of exposure.²¹ Other investigators have also shown that markers of DC maturation are enhanced

within 24 hours of exposure to bacterial LPS.¹⁴ However, it is also possible that the effects are indirectly mediated as well. It has been well established from *in vitro* work that GM-CSF and IL-4 have the ability to induce maturation of blood monocytes into iDCs. These cytokines as well as LPS, TNF- α , and IL-1 β can promote the maturation of iDCs to mDCs.^{7,14,22,23} Our finding that inhaled CCRE induced GM-CSF and IL-1 β , 2 potent DC maturation factors, suggests that indirect mechanisms of DC maturation may also be initiated.

In summary, our findings suggest that inhaled LPS induces DC maturation in human airways *in vivo*. The biologic consequences of airway DC maturation after acute LPS inhalation may be to initiate or potentiate an allergic response, which in turn can initiate or exacerbate asthma. Indeed, inhaled LPS enhances airways hypersensitivity to inhaled allergen in atopic subjects with asthma.^{24,25}

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