Endotoxin Augments Myeloid Dendritic Cell Influx into the Airways in Patients with Allergic Asthma

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Rationale: Epidemiologic studies have shown that exacerbation of asthma is modulated by environmental endotoxin. High levels of endotoxin are associated with asthma symptoms and the current use of asthma medication. However, the underlying mechanisms by which endotoxin modulates asthma are not completely understood. Objectives: The aim of the study was to test whether endotoxin enhances the response of individuals with allergic asthma to allergen, and to determine if this interaction is associated with increased numbers of antigen-presenting cells in the airways. Methods: Seventeen subjects with mild allergic asthma underwent segmental challenge with allergen, endotoxin, and the combination of both in three different lung segments via bronchoscopy. The cellular influx including monocytes, myeloid dendritic cells (mDCs), and plasmacytoid dendritic cells (pDCs), as well as the level of cytokines, were assessed in bronchoalveolar lavage fluid obtained 24 hours after segmental challenge. Monocytes, mDCs, and pDCs were isolated and their capacity to induce T cell proliferation was determined. Measurements and Main Results: Endotoxin enhanced the cellular response to allergen. The combination of allergen and endotoxin resulted in increased numbers of total cells, lymphocytes, neutrophils, eosinophils, monocytes, and mDCs, as well as increased levels of lipopolysaccharide-binding protein, IL-1α, IL-6, and tumor necrosis factor-α in the bronchoalveolar lavage fluid compared with allergen alone. Isolated mDCs but not pDCs induced a strong T cell proliferation in vitro. Conclusions: Endotoxin augments the allergic inflammation in the lungs of individuals with asthma, and induces an enhanced influx of monocytes and functionally active antigen-presenting mDCs into the respiratory tract.

Keywords: dendritic cells; monocytes; bronchoalveolar lavage; endobronchial allergen challenge; mixed lymphocyte culture test

Endotoxin is a pro-inflammatory lipopolysaccharide-protein complex (LPS) from the outer membrane of gram-negative bacteria, which is ubiquitous in the environment. Significant amounts of endotoxin are detectable in ambient aerosols (2), and it is an important component of environmental tobacco smoke (3, 4). The exposure to endotoxin showed heterogeneous effects in asthma and allergy (5). While it is well established that early life exposure to endotoxin protects against the development of allergies (6), it has recently been shown that household endotoxin exposure is a significant risk factor for increased asthma prevalence in adults (7). Higher levels of endotoxin were significantly associated with the diagnosis of asthma, asthma symptoms, and the current use of asthma medication. Michel and coworkers have shown that the severity of asthma is significantly related to the endotoxin level in the house environment, with no association to the mite allergen concentration (8). Furthermore, it is known that in patients with asthma inhalation of endotoxin causes a significant decrease in lung function with an enhanced airway hyperreactivity (AHR) (9, 10). Boehlecke and colleagues (11) confirmed this finding, demonstrating an increase in AHR to mite allergen when the subjects were exposed to low-level ambient air endotoxin in an exposure chamber before inhaled allergen challenge.

Despite the well-documented negative effects of endotoxin on prevalence and severity of asthma, the underlying mechanism is not completely established. Patients with asthma show an increase in the eosinophilic nasal inflammation after nasal exposure to allergen and endotoxin (12). In healthy subjects, the inhalation of endotoxin skewed airway inflammation in a TH2 direction (13) and increased the maturation of pulmonary antigen-presenting cells, including dendritic cells (DCs) (14). However, the degree to which allergic inflammation is modified in the lower airways of patients with asthma after concomitant exposure to endotoxin and allergen is not known.

The aim of this study was to further elucidate the mechanism underlying the pro-inflammatory effect of endotoxin in the airways of patients with allergic asthma. The well-established model of segmental allergen challenge was combined with the endobronchial instillation of endotoxin (15) in subjects with mild allergic asthma.

We put the main focus of this study on allergen-presenting cells like monocytes and DCs. In particular, DCs link the innate and adaptive immune responses (16) and are thus modulators...
METHODS

Study Design

Subjects with mild asthma who are allergic to house dust mite underwent segmental challenges with allergen, endotoxin, and the combination of both during a first bronchoscopy. During a second bronchoscopy 24 hours later, bronchoalveolar lavages of the challenged segments were performed. The cellular influx including monocytes, mDCs, and pDCs, and the cytokine concentration in the BAL fluid, was determined. For functional tests, a second segmental challenge with allergen and endotoxin was done in a subgroup at least 8 weeks after the preceding challenge. BALs were performed 24 hours later, and monocytes, mDCs, and pDCs were isolated by a fluorescence-activated cell sorter (FACS). After a co-culture with autologous T cells, the T cell proliferation was determined.

Study Subjects

The study population consisted of 17 nonsmoking subjects (nine women and eight men; mean age 28 ± 5.2 yr) with mild persistent allergic asthma (according to the asthma classification defined at the GINA Report 2006, revised). The subjects only used β2-agonists if required for relief of asthma symptoms. None of them was treated with corticosteroids, sodium cromoglycate, theophylline, or leukotriene modifiers within 4 weeks of the study procedures. Therefore, the antigen-presenting capacity of DCs and monocytes was determined in a co-culture with autologous T cells. Finally, the level of cytokines and chemokines that could be involved in allergic inflammation and LPS-triggered DC function was determined in the bronchoalveolar lavage (BAL) fluid.

Some of the results of this study have been previously reported at the ATS Conference 2006 in the form of an abstract (1).

Dosage and Preparation of Challenge Substances

To determine the allergen dose for the segmental instillation, a skin-prick test with a dilution series of the allergen extract in saline was done as previously described (26). The allergen concentration that elicited at least a 3-mm-diameter skin weal response was selected for instillation (100–100,000 SQE diluted in 10 ml saline). Endotoxin challenge was done with 2 ng/kg endotoxin according to the protocol of O’Grady and coworkers (15). Endotoxin was prepared using lyophilized Clinical Center Reference Endotoxin (CCRE; Escherichia coli strain O:113) in a final volume of 10 ml sterile saline. The doses of allergen and endotoxin instilled in the combination segment were identical to those in the single challenge segments. They were dissolved in a final volume of 10 ml sterile saline.

To exclude endotoxin contamination of the allergen extract and to verify the batch status of the CCRE, vials were reconstituted in sterile saline and endotoxin activity was determined by an accredited reference laboratory using a quantitative kinetic analysis according to European and United States Pharmacopoeia (Ph. Eur./USP) guidelines. The allergen extract showed very low endotoxin levels of 0.059 EU/ml for the highest allergen dose of 100,000 SQE. The endotoxin activity of the CCRE was in high accordance with the labeled specification.

Bronchoscopy

After premedication (inhalation of 200 μg salbutamol, atropine 0.5 mg subcutaneously, and midazolam 0.05–0.1 mg/kg intravenously) and local anesthesia with topical lidocaine, bronchoscopy was conducted according to a standard protocol (27), which is consistent with international recommendations for investigative bronchoscopies (28). During the first bronchoscopy, the bronchoscope (BF 160 P; Olympus Optical, Tokyo, Japan) was placed into one segment of the left lower lobe in wedge position for a baseline BAL with 6 ± 20 ml saline solution (37°C). The first aliquot was aspirated separately and discarded. Next, 10 ml saline solution was instilled into a segment of the left upper lobe bronchus as a control challenge. Allergen solution was instilled in a segment of the lingular bronchus. The bronchoscope was then passed into the right upper lobe and endotoxin solution was segmentally instilled. Finally, a combination of allergen and endotoxin was instilled in a segment of the right middle lobe. A new microcatheter (Vygon, Aachen, Germany) was used for each instillation. After 24 hours, a second bronchoscopy was performed under identical premedication. During this bronchoscopy, a BAL as described for baseline was done in each challenged segment. In five subjects, a third bronchoscopy was performed with the instillation of allergen and endotoxin into a segment of the right middle lobe under identical conditions at least 8 weeks after the second bronchoscopy. During a fourth bronchoscopy 24 hours later, a BAL was performed in the challenged segment.

Processing and Staining of BAL Cells

BAL fluid samples were processed as previously described (27). Briefly, BAL fluid was filtered through a 100-μm filter and centrifuged. The supernatant was stored at −80°C. The total nucleated cell count was determined using a Neubauer hemocytometer. Differential cell counts were done using Diff-Quick staining (Dade Behring Inc., Marburg, Germany) of cytospin slides and by counting 400 cells per slide.

For immunofluorescence labeling, 5 × 105 BAL cells were used for each test. Cells were incubated for 30 minutes at 4°C with monoclonal antibodies or isotype controls according to the manufacturer’s recommendations (see below). The samples were lysed, fixed (Versa Lyse Lysing solution; Beckman Coulter, Krefeld, Germany), washed, resuspended in PBS, and kept on ice until flow cytometric analysis. Using a Cytomics FC 500 cytometer (Beckman Coulter), the data of 105 cells were recorded and the surface marker expression analyzed using Cytomics RXP software (Beckman Coulter).

Identification of Alveolar Monocytes and DC Subsets

Alveolar monocytes were differentiated from other BAL cells (alveolar macrophages, neutrophils, and eosinophils) by high expression of CD14 and specific properties in size, granularity, and autofluorescence as previously described (27). The identification of alveolar DC subsets was based on two different three-color-arrays (IOTest “myeloid subset” [CD14/16-FITC, CD85k-PE, CD33-PCS] and IOTest “plasmacytoid subset” [CD14/16-FITC, CD85k-PE, CD123-PCS]; Immunotech, Marseille, France). DCs were differentiated from monocytes and
macrophages based on the expression of ILT3 (CD85k) (20, 29), which is selectively expressed on monocytes, macrophages, and DCs, and the co-expression of the interleukin-3 receptor (CD123) or CD33 as well as a weak expression of CD14/CD16. pDCs were defined as cells with co-expression of ILT3 and CD123, whereas mDCs were defined by cells co-expressing ILT3 and CD33. A FACS plot is shown in Figure 1A to demonstrate the gating strategy. According to the manufacturer’s information, mDCs and pDCs identified with this methodology have shown an excellent correlation ($r^2 > 0.95$) to the classical definition markers of DC subsets (lineage-negative/HLA-DR$^+$/CD11c$^+$ for mDCs and lineage-negative/HLA-DR$^+$/CD123$^+$ for pDCs, respectively). For the detection of co-stimulatory molecules on DCs, BAL cells were additionally incubated with biotinylated antibodies against HLA-DR (BD Biosciences, Heidelberg, Germany), CD80 (Serotec, Düsseldorf, Germany), and CD209 (Natutec, Frankfurt, Germany) according to the manufacturer’s recommendations. In a second step, the cells were incubated with PE-CY7–labeled Streptavidin (Caltag, Hamburg, Germany).

**Isolation of Alveolar Monocytes and DC Subsets by Activated Cell Sorting**

In a subsequent part of the study, monocytes and DC subsets obtained during the fourth bronchoscopy were isolated using a FACSAria cell sorter (BD Biosciences). Since the sorting experiments were done at a time point when additional human pulmonary DC markers had been described by Demedts and colleagues (19), a combination of the above-described identification strategy with additional DC markers (BDCA-1; BDCA-2) was used. The DC subsets were identified by a six-color staining (BDCA-1-FITC, BDCA-2-PE, CD123-APC [Miltenyi Biotec, Bergisch Gladbach, Germany], CD33-PerCPCy5.5, CD14-APC-Cy7 [BD Biosciences], CD16-PE-Cy7 [Immunotech Beckman Coulter]), where pDCs were defined as cells with co-expression of BDCA-2 and CD123, and mDCs as cells co-expressing BDCA-1 and CD33. Both subpopulations of DCs had to show a dim expression of CD14/CD16 because it has been demonstrated that CD14/CD16 are expressed on DC precursors (30, 31). This strategy is in accordance with the identification of DC subsets in the human lavage by Bratke and coworkers (32). Monocytes were isolated by their strong co-expression of CD14 and CD33. The different populations were simultaneously sorted directly into continuously cooled (4°C) non-adherent tubes filled with culture medium.

**Mixed Lymphocyte Cultures**

Autologous T cells were isolated from subjects using anticoagulated peripheral blood, Lymphoprep (Axis Shield PoC, Oslo, Norway) and a T cell negative isolation kit with supraparamagnetic beads (Dynal Biotech ASA, Oslo, Norway). Nonactivated T cells were isolated by depleting non–T cells with a monoclonal antibody mix (CD14, CD16, HLA-DR, CD56, and CD235a). For mixed lymphocyte cultures, $3 \times 10^4$ sorted alveolar cells (monocytes, mDCs, and pDCs) were cocultured with $1 \times 10^5$ autologous T cells for 5 to 7 days at 37°C in round-bottom 96-well plates (duplicate or triplicate) in 200 µl RPMI 1640 (with 10% FCS, penicillin, and streptomycin; Cambrex, Taufkirchen, Germany). Cells were pulsed with 3.7 beq3H-thymidine for 16 h before harvest. Cell proliferation was assessed by scintillation using a Topcount liquid scintillation counter (PerkinElmer, Rodgau, Germany).

**Biochemical Analysis of BAL Fluid**

The concentration of LPS-binding protein (LBP) in the BAL fluid was determined by enzyme-linked immunosorbent assay (ELISA) (HBT, Uden, The Netherlands). Furthermore, the concentrations of 30 cytokines...
and chemokines were determined simultaneously with a multiplex assay kit (Lincoplex; Linco Research, Inc., St. Charles, MO) with premixed antibody-coated microsphere beads using a Luminex100 (Luminex Corporation, Austin, TX) according to the manufacturer’s recommendations.

Statistical Analysis
An overparameterized model as described in General Linear Models (33) was used for the statistical analysis. The model itself was a mixed model combining the fixed treatment effect and the random effect of the patients (34). In the main effect analysis, all data samples (control, allergen, endotoxin, and allergen+endotoxin) were used for the analysis. For each endpoint the paired *post hoc* analysis was performed using a Tukey-Kramer HSD test to adjust for multiplicity (35). In all analyses values of *P* < 0.05 were considered to be significant for both tests. The statistical analysis was performed using STATISTICA for Windows Version 7.1 (StatSoft, Inc., Hamburg, Germany).

RESULTS

Safety of the Procedures
After the incremental allergen inhalation, most of the subject showed a late phase reaction 4 to 6 hours after the allergen inhalation. Five subjects required treatment with inhaled steroids and two subjects with oral prednisolone. The broncho-scopies with the segmental challenges were well tolerated by most of the patients. One subject was excluded from the study after a complete atrioventricular block for 20 seconds after administration of atropine (0.5 mg, subcutaneously) before the first bronchoscopy. Another subject with a high AHR required intravenous treatment with 125 mg prednisolone after the second bronchoscopy. Furthermore, one subject was excluded from the rest of the study due to an elevated number of lymphocytes in the baseline lavage.

Cellular Inflammatory Response to Allergen and Endotoxin in the BAL
To investigate the effect of endotoxin on the allergic inflammation, we determined the absolute cell numbers of different cell populations in the BAL fluid after challenges with saline, allergen, endotoxin, and the combination of both (shown in Figure 2). When all challenges were compared for the analysis of the main effects, allergen had a significant effect on total BAL cells and the numbers of lymphocytes, eosinophils, pDCs, and mDCs, whereas endotoxin had an effect on total cells and the numbers of lymphocytes, neutrophils, macrophages, monocytes, pDCs, and mDCs. When the cellular response to allergen was compared in the paired *post hoc* analysis to the response to the combination of allergen with endotoxin, there was an increase in the number of total cells, lymphocytes, neutrophils, eosinophils, monocytes, and mDCs (*post hoc* analysis).

When surface marker expression on DC subsets was analyzed, there was a higher expression of HLA-DR and the co-stimulatory molecules CD86 and CD209 on mDCs compared with pDCs (see Figure 1B).

Capacity of DCs and Alveolar Monocytes to Induce T Cell Proliferation
For functional characterization of the newly immigrated DCs, alveolar monocytes and DC subsets (mDCs, pDCs) were isolated after segmental provocation with allergen and endotoxin from the BAL fluid and co-cultured with autologous T cells for 5 to 7 days. The capacity of mDCs to induce T cell proliferation was significantly higher compared with monocytes and pDCs and comparable to the mitogen control (Figure 3). The presence of house dust mite allergen during the co-culture did not change the T cell proliferation (data not shown).

Cytokine and Chemokine Levels in BAL Fluid in Response to Allergen and Endotoxin
The concentration of LBP in the BAL fluid after the different challenges determined by ELISA is shown in Figure 4. The results of cytokines and chemokines determined with a multiplex assay kit are shown in Table E1 in the online supplement. When all challenges were combined for the analysis of the main effects, allergen had an effect on IL-1α, IL-5, IL-6, IL-8, IL-13, macrophage inflammatory protein (MIP)-1α, sCD40L, and tumor necrosis factor (TNF)-α and endotoxin had an effect on IL-1α, IL-1α, IL-6, IL-10, MIP-1β, sCD40L, granulocyte colony-stimulating factor, and TNF-α. When the challenge with allergen was compared in the paired *post hoc* analysis to the combined challenge, there was a significant increase in the concentration of LBP, IL-1α, IL-6, and TNF-α in the BAL fluid. The concentration of IL-1β, IL-4, IL-12p40, IL-12p70, IL-15, IL-17, vascular endothelial growth factor, epidermal growth factor, MIP-1α, interferon-γ, and transforming growth factor-α was under the detection limit and is not listed.

DISCUSSION
It is well established from epidemiologic studies that the prevalence and the severity of asthma are dependent on the environmental concentration of endotoxin (7, 8). Furthermore, experimental studies have demonstrated a deterioration in lung function and an increase in AHR when patients with asthma are exposed to endotoxin (9, 10, 14, 36). Despite this well-described endotoxin effect on asthma, there is hardly any information on the underlying immunologic mechanism in the lung. Therefore, the aim of this study was to elucidate the effect a controlled endotoxin challenge had on the allergen-induced airway inflammation in patients with asthma.

When the cellular response to allergen alone was compared with the response to the combination of allergen with endotoxin, there was an increase in the number of lymphocytes, neutrophils, and eosinophils. Furthermore, the number of monocytes and mDCs, but not the number of pDCs, increased in the airways. When alveolar monocytes and DCs were isolated after challenge with allergen plus endotoxin, mDCs demonstrated the strongest T cell proliferation-inducing capacity. While the enhancing effect of endotoxin on the eosinophilic response in individuals with allergic asthma has already been described in a preliminary report in sputum (37), our data demonstrate for the first time that endotoxin augments the influx of functionally highly active mDCs into the airways of patients with asthma.

Several studies suggest that DCs play a crucial role in allergic inflammation (38, 39). Animal studies have shown an increased number of mDCs in the airways of sensitized and challenged mice during the acute phase (40). The depletion of mDCs at the time of allergen challenge eliminated airway inflammation and AHR (40). The intratracheal instillation of mDCs restored the reduced AHR and airway eosinophilia (23). While this suggests that mDCs play a key role in inducing and maintaining an adaptive immune response to allergens in the lung, pDCs have been identified as important cells that prevent asthmatic reactions to inhaled antigen by the induction of tolerance (17, 24, 25).

In human asthma, a rapid DC recruitment to the bronchial mucosa has been observed in response to local allergen challenge (41). However, there is uncertainty whether the concept of proallergic mDCs and antiallergic pDCs seen in animals is also
true in humans. In the peripheral blood of asthma patients a significantly higher number of pDCs in relation to mDCs was found compared with normal subjects (42). Consistent with our findings, Bratke and coworkers (32) recently showed that both mDCs and pDCs accumulate in BAL fluid in patients with allergic asthma after segmental allergen challenge. However, there are no data on functional properties of these cells available in asthma.

We investigated the additional effect of endotoxin on the DC accumulation in the lung and demonstrated that only increased numbers of mDCs, but not pDCs, enter the airways. Therefore, mDCs seem to play a prominent role at least in the enhancement of the allergic inflammation due to endotoxin. We also showed that functional properties of DC subsets are largely different and that mDCs but not pDCs induce T cell proliferation. This is in accordance with data from Demedts and colleagues (19), who showed in human tissue samples from patients with lung cancer that pulmonary mDCs are much stronger inducers of T cell proliferation than pDCs (43). Although it would be expected that the addition of HDM to the mDC/T cell co-cultures would increase T cell proliferation, we did not observe this effect. A possible explanation could be that the DCs were activated in vivo and, thus, could not present additional allergen effectively in vitro. The inability of pDCs to induce proliferation might be explained by their lack of co-stimulatory molecules. We have determined exemplarily the expression of activation markers on alveolar DCs and found a lower expression of HLA-DR and CD86 on pDCs compared with mDCs. This is in accordance with data from Demedts and colleagues (19) and Masten and colleagues (21). Furthermore, the co-stimulatory molecule DC-SIGN (CD209), which plays an important role in DC antigen presentation to T cells (44), is highly expressed on mDCs but not on pDCs.

Figure 2. Numbers of (A) total cells, (B) lymphocytes, (C) neutrophils, (D) eosinophils, (E) macrophages, (F) monocytes, (G) pDCs, and (H) mDCs in bronchoalveolar lavage fluid 24 hours after instillation with saline, allergen, endotoxin, and the combination of allergen+endotoxin (— Mean; *P < 0.05; **P < 0.01; ***P < 0.005).
In the presence of so-called danger signals like endotoxin, DCs undergo changes toward maturation and activation with a high expression of co-stimulatory molecules (45). Toll-like receptors (TLRs), which recognize these “pathogen-associated molecular patterns,” have been described on human DCs: TLR-2 and TLR-4 are expressed on mDCs, whereas pDCs express TLR-7 (21, 43). Therefore, endotoxin can bind and activate mDCs via TLR-4, resulting in an increased influx of mDCs with a high ability to induce T cell proliferation into the airways.

The role of monocytes in asthma, and particularly in endotoxin-enhanced asthma, is poorly understood. In individuals with asthma the recruitment of monocytes into the airways has been described after repeated allergen challenges (46, 47). In our study we found an even higher number of CD14+ monocytes in the lavage fluid when LPS was given in addition to allergen. Therefore, LPS enhances the monocyte recruitment into the lung in allergic asthma. LBP levels were investigated in BAL fluid because LBP enhances the binding of LPS to the endotoxin receptor CD14 on monocytes and LBP is one of the critical molecules regulating the acute airway response to LPS (48). While others have already shown increased levels of LBP after allergen challenge (49), we demonstrate here an additional effect after LPS exposure. Therefore, the presence of LPS further enhances the binding of LPS to CD14-expressing cells.

We saw increased levels of typical monocyte/DC-derived cytokines like IL-1α, IL-6, and TNF-α in the BAL fluid after allergen plus endotoxin challenge compared with allergen challenge alone. Interestingly, van Haarst and coworkers (50) described low autofluorescent monocytes in BAL fluid with a low T cell stimulatory capability which produced high levels of IL-1, IL-6, and TNF-α when they were isolated and stimulated with endotoxin. Therefore, monocytes recruited to the lung might be important sources of pro-inflammatory cytokines, which play a significant role in the allergic inflammation.

In conclusion, we have shown in patients with bronchial asthma that endotoxin induces an enhanced allergic inflammatory response in the lung with an enhanced influx of monocytes and highly potent antigen-presenting mDCs into the alveolar space. These data might provide a mechanism by which endotoxin increases the prevalence and severity of asthma shown in epidemiologic and clinical studies.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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