

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Lipopolysaccharide amplifies eosinophilic inflammation after segmental challenge with house dust mite in asthmatics

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To cite this article: Berger M, de Boer JD, Bresser P, van der Poll T, Lutter R, Sterk PJ, van der Zee JS. Lipopolysaccharide amplifies eosinophilic inflammation after segmental challenge with house dust mite in asthmatics. *Allergy* 2015; **70**: 257–264.

Keywords

allergen; asthma; bronchodilators; endotoxin; inflammation.

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Accepted for publication 5 November 2014

DOI:10.1111/all.12544

Edited by: Hans-Uwe Simon

Abstract

Background: House dust contains mite allergens as well as bacterial products such as lipopolysaccharide (LPS). Asthma exacerbations are associated with the level of exposure to allergens and LPS. LPS can potentiate allergen effects in steroid-naïve patients. Long-acting β_2 -agonists (LABA) were shown to inhibit LPS-induced bronchial inflammation in healthy volunteers. The aim of this study was to assess the effect of LPS on the allergen-induced eosinophilic inflammation [primary endpoints: eosinophil counts and eosinophil cationic protein (ECP)] induced by bronchial instillation of house dust mite (HDM) in patients with asthma on maintenance treatment with inhaled corticosteroids (ICS).

Methods: Thirty-two nonsmoking asthmatics with HDM allergy were treated with run-in medication (fluticasone propionate 100 μ g bid) during 2 weeks before the study day. All patients underwent bronchial challenge with HDM, and half of them were randomized to receive additional LPS. Both groups were randomized to receive pretreatment with a single inhalation of 100 μ g salmeterol 30 min before bronchial segmental challenge. Six hours later, bronchoalveolar lavage (BAL) was collected for leukocyte cell count, differentials, and cellular activation markers.

Results: Challenge with HDM/LPS induced a significant increase in eosinophil cationic protein ($P = 0.036$) and a trend toward an increase in BALF eosinophils as compared to HDM challenge.

Conclusion: Lipopolysaccharide promotes eosinophilic airway inflammation in patients with asthma despite being on maintenance treatment with ICS.

Abbreviations

ANOVA, analysis of variance; ATS, American Thoracic Society; BALF, bronchoalveolar lavage fluid; ECP, eosinophil cationic protein; ERS, European Respiratory Society; FEV₁, forced expiratory volume in 1 second; GINA, Global Initiative for Asthma; HDM, house dust mite; ICS, inhaled corticosteroids; IL, interleukin; LABA, long-acting β_2 -agonists; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MIP-1 α , macrophage inflammatory protein-1 alpha; MIP-1 β , macrophage inflammatory protein-1 beta; MPO, myeloperoxidase; PC-20, provocative concentration (mg/ml) of methacholine inducing a decline in FEV₁ of 20% from baseline; RANTES, regulated and normal T cell expressed and secreted; RAST, radioallergosorbent test; TGF- β , transforming growth factor-beta; TNF- α , tumor necrosis factor-alpha.

Asthma is characterized by variable airflow limitation, airway hyper-responsiveness, and airway inflammation, accompanied with symptoms of shortness of breath, cough, wheezing, and chest tightness (1). It is thought to be caused and driven by a combination of genetic factors and environmental factors such as allergens, viruses, bacterial products, molds, and air pollution (1–3).

Exposure to house dust mites (HDMs) is associated with asthma exacerbations and eosinophilic inflammation in mite-sensitive patients (1). House dust is a complex mixture, containing mite allergens and microbial products such as lipopolysaccharide (LPS, endotoxin). LPS is known to induce a neutrophilic inflammation in healthy subjects (4–6).

Patients with asthma have an increased sensitivity toward inhaled LPS with respect to lung function with a more pronounced fall in FEV₁ (7). Also, exposure to LPS might be associated with occurrence of asthma symptoms (8).

While some investigators showed positive (4, 9) and others negative (8, 10) effects of LPS exposure on asthma occurrence, severity, and airway remodeling, little is known about the interaction and underlying mechanisms between allergen-driven activation via the adaptive immune system and LPS-induced activation of the innate immune system. Interestingly, de Boer et al. (11) showed that LPS inhibits the Th2 lung inflammation induced by HDM allergens in mice.

Asthma treatment is built on inhalation corticosteroids (ICS) and bronchodilators (1). Corticosteroids strongly inhibit allergen-induced eosinophilic inflammation (12), but are less effective in suppressing LPS-induced neutrophilic inflammation (13). Previous work from our group showed that the long-acting β_2 -agonist (LABA) salmeterol is capable of inhibiting LPS-induced inflammation in healthy volunteers (14). Moreover, in patients with asthma, salmeterol suppresses allergen-induced early release of interleukin (IL)-5, which is pivotal for eosinophilic inflammation and suggests an inhibitory effect on mast cell activation (15, 16). Therefore, LABAs may be effective in suppressing both allergen-induced and LPS-induced pulmonary inflammation in asthma and thereby reduce airway remodeling.

Using the clinically relevant model of bronchial segmental HDM provocation (5, 6, 17), we tested the hypothesis that addition of LPS to allergen, in a ratio similar to natural house dust, induces an increase in the eosinophilic inflammatory response in stable asthmatics on maintenance treatment with inhaled corticosteroids.

Furthermore, we tested whether pretreatment with a single dose of salmeterol has an inhibitory effect on allergen and allergen/LPS-induced inflammation in asthmatics while on maintenance treatment with ICS.

Methods

Subjects

Thirty-nine adult, nonsmoking and stable patients with intermittent-to-mild asthma (according to GINA) (1), with allergy to HDM, were recruited by advertising. Patients were included after screening, consisting of a questionnaire, physical examination, blood investigation, lung function (ATS) (18, 19), and allergy test (RAST). The institutional ethics committee approved the study, and subjects gave informed consent (Dutch Trial Register no. 1807). Additional information is provided in the online data supplement.

Study design

In this investigator-initiated, randomized, controlled, single-blinded study (Fig. 1), all patients used run-in medication [fluticasone propionate (GlaxoSmithKline, Zeist, the Netherlands)] 100 μ g bid during 2 weeks before the study day (20). Patients who used ICS before screening were asked to stop their regular medication and switch to the study run-in medication. After run-in, patients were randomized to receive bronchial segmental challenge with HDM or HDM/LPS in a lung subsegment. Sham challenge (saline) was performed in the contralateral lung. In addition, patients were randomized to pretreatment by inhalation of 100 μ g of salmeterol (GlaxoSmithKline) 30 min before the challenge or no pretreatment. After 6 h, bronchoalveolar lavage (BAL) was performed (ATS guidelines)(21, 22). Primary endpoints were markers of eosinophilic inflammation (eosinophil cell count and eosinophil cationic protein) in BAL fluid (BALF).

Materials

U.S. Standard Reference endotoxin (lipopolysaccharide *E. coli*, 10 000 EU/vial) was kindly supplied by Dr Suffredini from the National Institutes of Health, Bethesda, Maryland, USA.

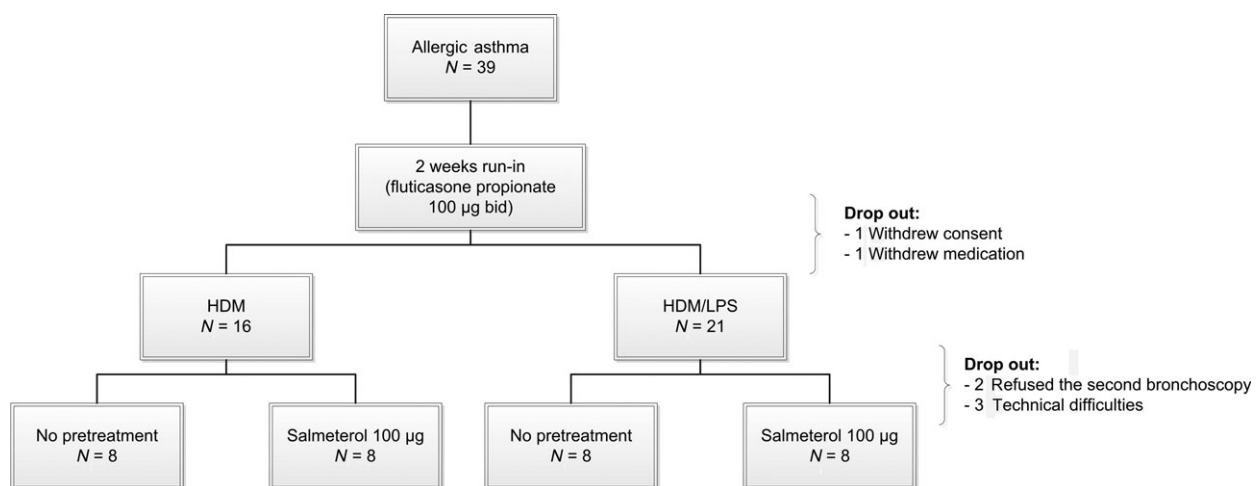


Figure 1 Study design flow diagram. HDM, house dust mite; LPS, lipopolysaccharide.

Standardized D. Pteronyssinus extract (10 000 BU/ml) was derived from ALK Abello, Nieuwegein, the Netherlands. All patients received 50 biological units in 5 ml saline, which contained 0.097 ng endotoxin.

Measurements

Cell differentials from BALF were performed on cytospins (Diff-Quick; Dade Behring AG, Düringen, Switzerland). Cell concentrations were calculated as (% cells $\times$ total cell count)/volume of BALF. When the percentage of a cell differential was zero, we assigned a quarter of the detection limit (0.1%) for statistical reasons (23). Inflammatory mediators in BALF and serum were determined by multiplex bead flow assays (Bio-Rad, Hercules, CA, USA) and read on a Bioplex 200 reader (Bio-Rad Laboratories, Inc.) or enzyme-linked immunosorbent assay (ELISA).

Statistical analysis

A sample size of 16 patients was estimated to have a power of 80% to detect a 50% change in BAL leukocytes (14). Differences were considered significant at *P*-values equal or <0.05 . Block randomization was performed by envelopes using fixed block sizes of four treatment options. The pulmonologist administering the intervention and the laboratory analysts performing the measurements on the material were completely blinded to the treatment. Baseline differences were analyzed by one-way ANOVA. To determine the effect of LPS on HDM-induced pulmonary inflammation, we performed a univariate analysis with LPS and salmeterol as fixed factors and baseline values (blood) or control segment values (BALF) as covariates. All analyses were performed with SPSS 20 for Windows (IBM, Armonk, NY, USA).

Results

Participants were investigated between August 2010 and August 2011. Subject characteristics were not significantly different between groups (*P* > 0.05, Table 1). Two of 39 screened patients withdrew during the run-in period. After

the run-in period, we randomized 37 patients on the study day. During the study day, two patients refused to undergo the second bronchoscopy, and in three cases, the procedures were not completed due to medical reasons (Fig. 1). We were able to collect blood samples before and 6 h after provocation from 36 patients. Per-protocol analysis of BALF was performed in 32 patients. Nine of these 32 patients used ICS maintenance treatment with a mean fluticasone equivalent of 300 μ g/day before the start of run-in. We observed no significant differences in FEV₁ during the study day between different groups.

We started with an LPS dose of 4 ng/kg, which was based on studies in healthy volunteers. We lowered the LPS dosage to 1 ng/kg because of adverse respiratory effects in the first two patients who received HDM plus 4 ng/kg LPS (no pretreatment with salmeterol). For the standard provocation dose of 4 ng/kg, it is reported in the literature (6) that it does not induce any eosinophilic inflammation. We composed an additional control group of six asthmatics to verify that the lower dose of 1 ng/kg LPS also did not cause an increase in baseline eosinophilic inflammation but did induce a neutrophilic inflammation. In this group, we did not find an effect on any of the parameters for eosinophilic inflammation. Increase in BALF IL-6 (*P* = 0.029) and CXCL10 (*P* = 0.013) and increase in peripheral blood leukocytes (*P* = 0.023), neutrophils (*P* = 0.029), and monocytes (*P* = 0.021) 6 h after low-dose LPS challenge did confirm proinflammatory effects of the low-dose LPS.

To study the effect of LPS on HDM-induced eosinophilic inflammation, the results of the two patients with 4 ng/kg LPS were included in the analysis. To study the effect of salmeterol pretreatment, results of these two patients were omitted as both patients did not receive salmeterol pretreatment.

Effect of LPS on HDM-induced eosinophilic inflammation (*n* = 32)

Primary endpoints

Table 2 shows the primary and secondary endpoints in BALF of subjects challenged with HDM and HDM/LPS.

Table 1 Baseline characteristics

	HDM <i>N</i> = 8	HDM/LPS <i>N</i> = 8	HDM (S) <i>N</i> = 8	HDM/LPS (S) <i>N</i> = 8	<i>P</i> -value
Age*	26.5 (20–30)	28.0 (19–42)	21.0 (19–26)	22.3 (19–25)	>0.05
Male gender (%)	37.5	25.0	25.0	50.0	>0.05
ICS use before run-in (%)	25.0	62.5	12.5	12.5	>0.05
Prebronchodilator FEV ₁ in liter†	4.2 (0.8)	3.8 (0.7)	3.6 (0.6)	4.0 (0.5)	>0.05
Prebronchodilator FEV ₁ % of predicted†	106.0 (11.3)	101.4 (14.0)	97.5 (14.0)	103.4 (15.0)	>0.05
PC-20‡ in mg/ml	9.9 (3.6)	6.7 (4.7)	7.5 (5.3)	2.8 (4.6)	>0.05
Total IgE‡ in kU/l	305.1 (2.6)	383.5 (3.2)	168.7 (4.0)	306.8 (2.9)	>0.05
IgE HDM‡ in kU/l	25.4 (3.5)	24.2 (5.2)	7.6 (5.4)	32.2 (2.9)	>0.05

HDM, house dust mite; LPS, lipopolysaccharide; (S), salmeterol; ICS, inhalation corticosteroids; FEV₁, forced expiratory volume in 1 second; PC-20, provocative concentration (mg/ml) of methacholine inducing a decline in FEV₁ of 20% from baseline.

Values are expressed as *median (range), †mean (standard deviation), ‡geometric mean (geometric standard deviation) and analyzed by one-way ANOVA.

Addition of LPS to HDM induced a significant increase in BALF eosinophil cationic protein (ECP) ($P = 0.04$) and a nonsignificant increase in eosinophils ($P = 0.09$) (Fig. 2 and Table 2). The interaction term of LPS and salmeterol was not significant in the univariate analysis, indicating that the effect of LPS was independent of the presence or absence of pretreatment with salmeterol.

Secondary endpoints

Addition of LPS to HDM induced a significant increase in leukocytes, neutrophils (Fig. 3), lymphocytes, monocytes, basophils, plasma cells, IL-1 β , IL-6, IL-8, myeloperoxidase (MPO) (Fig. 3), MCP-1, MIP-1 α , MIP-1 β , chymase, tryptase, CXCL-10, and TGF- β (Fig. 3) in BALF. Measurements of IL-4, IL-5, IL-10, IL-13, IL-17, and stem cell factor in BALF were below the detection limit.

In peripheral blood, addition of LPS to HDM caused an increase in leukocytes ($P = 0.001$), neutrophils ($P < 0.001$), and IL-10 ($P = 0.02$) and a decrease in blood eosinophils ($P = 0.04$) (Table S1). These results did not

depend on the presence or absence of salmeterol pretreatment.

Effect of salmeterol in HDM and HDM/LPS-challenged patients ($n = 30$)

Primary endpoints

The slight inhibition of the HDM-induced increase in eosinophils and ECP in BALF by pretreatment with salmeterol did not reach statistical significance (Table 3). This result was not affected by the presence or absence of LPS challenge.

Secondary endpoints

After pretreatment with salmeterol, we measured a significant inhibition of the increase in lymphocytes ($P = 0.02$), monocytes ($P = 0.03$), IL-1 β ($P = 0.03$), and CXCL10 ($P = 0.003$) in BALF after challenge.

In peripheral blood, salmeterol significantly augmented the decrease in lymphocytes ($P = 0.002$) and eosinophils ($P = 0.001$) and an increase in IL-8 ($P = 0.021$).

Table 2 Effect of provocation with HDM, HDM/LPS, or saline (control) in BALF

	Control $n = 32$	HDM $n = 16$	HDM/LPS $n = 16$	P -value
Primary outcomes				
Eosinophils, cells $\times 10^4/\text{ml}$	0.07	0.47 $\dagger$	1.70 $\dagger$	0.09
Eosinophil cationic protein, ng/ml	0.84	2.74*	8.26 $\dagger$	0.04
Secondary outcomes				
Leukocytes, cells $\times 10^4/\text{ml}$	11.89	12.79	29.58*	0.02
Neutrophils, cells $\times 10^4/\text{ml}$	1.10	1.23	10.19*	0.001
Lymphocytes, cells $\times 10^4/\text{ml}$	0.87	0.79	1.31	0.03
Monocytes, cells $\times 10^4/\text{ml}$	0.01	0.01	0.02*	0.04
Basophils, cells $\times 10^4/\text{ml}$	0.01	0.01	0.02*	0.006
Macrophages, cells $\times 10^4/\text{ml}$	7.68	7.94	10.72	0.30
Mast cells, cells $\times 10^4/\text{ml}$	0.01	0.01	0.03*	0.05
Plasma cells, cells $\times 10^4/\text{ml}$	0.01	0.01	0.02*	0.02
IL-1 β , pg/ml	0.02	0.01	0.13*	0.002
IL-6, pg/ml	1.33	1.06	1188.5 $\dagger$	<0.001
IL-8, pg/ml	25.86	19.54	785.2 $\dagger$	<0.001
IL-9, pg/ml	0.57	0.38	0.39	0.97
IL-18, pg/ml	0.04	0.04	0.05	0.44
GRO- α , ng/ml	0.41	0.64	0.95*	0.21
Myeloperoxidase, ng/ml	12.08	12.33	37.67 $\dagger$	0.002
MCP-1, pg/ml	5.95	4.70	42.56*	0.01
MIP-1 α , pg/ml	0.03	0.04	9.10 $\dagger$	<0.001
MIP-1 β , ng/ml	0.09	0.03	2.30 $\dagger$	<0.001
CXCL-10, ng/ml	1.04	0.74	13.80 $\dagger$	<0.001
RANTES, pg/ml	0.08	0.16	0.48*	0.17
TGF- β , pg/ml	7.12	7.23	15.38*	<0.004
Chymase, pg/ml	5.10	5.32	21.33 $\dagger$	<0.001
Tryptase, kU/l	0.72	0.87	1.88 $\dagger$	<0.009
Eotaxin, pg/ml	1.80	1.54	1.92	0.69

HDM, house dust mite; LPS, lipopolysaccharide.

Geometric means for saline challenge and estimated marginal means for HDM and HDM/LPS challenge site (means adjusted for saline challenge values). P -values for the difference between HDM and HDM/LPS as found in the univariate analysis with LPS and salmeterol as fixed factors and saline values as covariate.

* $P < 0.05$ vs saline, $\dagger P \leq 0.001$ vs saline.

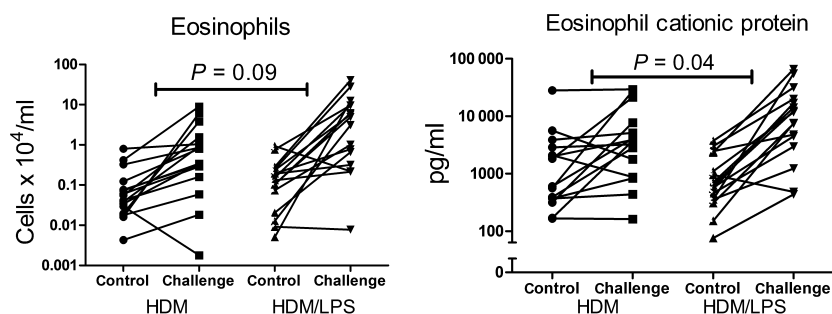


Figure 2 Additional effect of LPS to HDM on primary outcomes in BALF. Paired differences in eosinophil cell count and concentration of eosinophil cationic protein in bronchoalveolar lavage fluid (BALF), 6 h after challenge with saline and HDM, or saline and HDM/LPS.

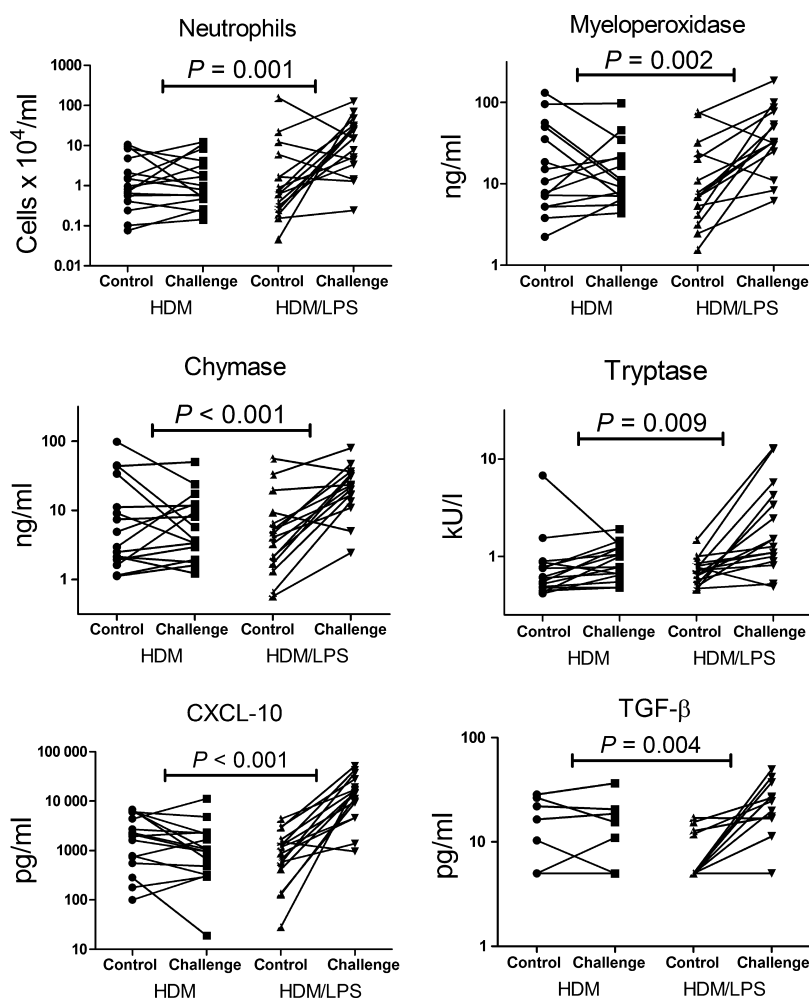


Figure 3 Additional effect of LPS to HDM on secondary outcomes in BALF. Paired differences in neutrophil cell count and concentrations of myeloperoxidase, chymase, tryptase, CXCL-10, and TGF- β

in bronchoalveolar lavage supernatant, 6 h after challenge with saline and HDM, or saline and HDM/LPS.

Discussion

This study shows a potentiating effect of LPS on allergen-induced eosinophilic inflammation in patients with mild asthma on maintenance treatment with ICS. We observed a significant

increase in BALF eosinophil cationic protein and a trend toward an increase in BALF eosinophils after HDM/LPS challenge as compared to HDM challenge only. As expected, addition of LPS to HDM challenge induced significant increases in BALF neutrophils and myeloperoxidase. Moreover, there was

Table 3 Effect of salmeterol on outcomes in BALF after provocation with HDM $\pm$ LPS

Primary outcomes	Salmeterol – (N = 14)	Salmeterol + (N = 16)	P-value
Primary outcomes			
Eosinophils, cells $\times 10^4$ /ml	1.50	0.41	0.11
Eosinophil cationic protein, ng/ml	5.22	3.63	0.56
Secondary outcomes			
Leukocytes, cells $\times 10^4$ /ml	20.98	16.25	0.25
Neutrophils, cells $\times 10^4$ /ml	3.84	2.70	0.34
Lymphocytes, cells $\times 10^4$ /ml	1.33	0.87	0.02
Monocytes, cells $\times 10^4$ /ml	0.02	0.01	0.03
Basophils, cells $\times 10^4$ /ml	0.01	0.01	0.51
Macrophages, cells $\times 10^4$ /ml	10.65	7.78	0.14
Mast cells, cells $\times 10^4$ /ml	0.02	0.01	0.08
Plasma cells, cells $\times 10^4$ /ml	0.01	0.01	0.14
IL-1 β , pg/ml	0.07	0.02	0.03
IL-6, pg/ml	29.51	22.49	0.43
IL-8, pg/ml	142.82	83.87	0.86
IL-9, pg/ml	0.40	0.41	0.70
IL-18, pg/ml	0.04	0.04	0.66
Myeloperoxidase, ng/ml	17.84	21.10	0.67
GRO- α , ng/ml	0.77	0.94	0.75
MCP-1, pg/ml	15.97	13.38	0.79
MIP-1 α , pg/ml	1.45	0.15	0.08
MIP-1 β , ng/ml	0.26	0.21	0.87
CXCL-10, ng/ml	3.90	2.05	0.003
RANTES, pg/ml	0.38	0.16	0.28
TGF- β , pg/ml	8.40	11.40	0.44
Chymase, pg/ml	9.70	10.24	0.95
Tryptase, kU/l	1.09	1.24	0.96
Eotaxin, pg/ml	2.11	1.19	0.32

Estimated marginal means (means adjusted for saline challenge) and *P*-values for the difference between salmeterol/HDM $\pm$ LPS and HDM $\pm$ LPS as found in the univariate analysis with LPS and salmeterol as fixed factors and saline challenge values as covariate.

also an increase in chymase, tryptase, TGF- β , and CXCL-10 among others. Additional treatment with single-dose salmeterol did not significantly inhibit any of the primary endpoints. However, there was significant inhibition of the increase in BALF lymphocytes, monocytes, IL-1 β , and CXCL10.

To our knowledge, this is the first study investigating the additional effect of LPS on HDM-induced bronchoalveolar inflammation in allergic asthmatics on maintenance treatment with ICS, using the safe, clinical relevant, and well-tolerated research model of bronchial segmental provocation.

The ratio LPS: HDM in the current study was 1 : 1.41 (based on weight), which resembles the composition of floor dust in regular, temperate climate homes (24).

This study has a few limitations.

It is known that there is a large interindividual variability in sensitivity to endotoxin (25), and its effects are time and dose dependent, complicating every study investigating the effect of LPS exposure. Because we intended to limit the burden to the patients, we did not perform a cross-over study to correct for this effect.

We observed a relatively high neutrophil count in the control segments of some patients, which may be explained by more pronounced collateral inflammation of asthmatics as

described by Braunstahl et al. (26). Alternatively, as the control segment was always challenged first, contamination with endotoxins from the upper airways during insertion of the bronchoscope could cause this high neutrophil count. However, regardless of the origin of the phenomenon, it may have masked differences rather than being responsible for the differences, as we corrected the effect of LPS and salmeterol for the values in the control segments.

Some investigators report maximal eosinophil influx in BALF of asthmatics at 42 h after allergen provocation (27), while others report eosinophil influx in BAL as early as 4 h after allergen challenge (28). The design of our study, with a time frame of 6 h, covers the early phase of the inflammatory response after allergen challenge. We may have missed possible effects of LPS and salmeterol on inflammation later on.

In our small groups, there seems to be some heterogeneity in baseline characteristics (Table 1) concerning PC20 and allergic parameters. This might be a result of differences in ICS usage prior to recruitment. However, based on additional information available, we believe that these differences did not influence the final results.

First, all patients had mild persistent or intermittent asthma. The groups were homogeneous in this respect. Sec-

ond, all patients, including the ones on ICS treatment before recruitment, used the same run-in medication of flutide 200 µg/day for 2 weeks prior to the study day.

Baseline values of the primary endpoints did not differ significantly for patients who used and did not use ICS before run-in and were not dependent on baseline PC20 methacholine or levels of HDM-specific IgE. Moreover, we performed an analysis with the baseline variables (blood) and saline values (BALF) as covariates minimizing the effect of differences. Introducing the use of ICS before run-in as a fixed factor in the analysis did not show any indication for an independent contribution of this factor.

In line with the results of Schaumann et al. (17) in steroid-naïve patients with asthma, we demonstrate a potentiating effect of LPS on allergen-induced local pulmonary inflammation as shown by a definitive increase in ECP and a possible increase in eosinophil numbers.

Maris et al. (14) previously observed a significant inhibition of neutrophil recruitment and reduced production of MPO and TNF- α after a single dose of 100 µg salmeterol previous to inhalation challenge with LPS in healthy males. In the current study, we did not investigate the effect of salmeterol on challenge with LPS only, but salmeterol did not appear to inhibit neutrophil influx by LPS in combination with HDM. The simultaneous challenge with house dust mite may have modified the effect of salmeterol on neutrophil recruitment, and it cannot be ruled out that the effect of salmeterol on LPS-induced neutrophil recruitment is modified by the maintenance treatment with inhaled corticosteroids. Furthermore, there were additional differences between the study by Maris et al. and our current study in the type of challenge (inhalation vs bronchial segmental challenge) and the total dose of LPS dosage (100 µg by inhalation vs 1 ng/kg with a mean dose of 0.07 µg by local endobronchial deposition). It should be tested whether the effect of salmeterol on LPS-induced neutrophil recruitment in asthmatics differs from the effect as found in healthy volunteers.

We were not able to demonstrate alterations in Th2 proteins, such as IL-4, IL-5, and IL-13, in BALF. In general, protein levels of these cytokines are very low in BAL due to dilution by the lavage procedure. Unfortunately, we were not able to perform PCR for these parameters. Previous studies showed efficacy of combination therapy with ICS and LABA on mast cells (16, 29) and smooth muscle cells (30). Interestingly, a single dose of salmeterol, next to ICS maintenance treatment, significantly inhibited the increase in lymphocytes, monocytes, IL-1 β , and CXCL10 and had a borderline effect on mast cells after HDM or HDM/LPS exposure. This is consistent with the results of Wallin et al. (29) who demonstrated a significant decrease in mast cells in BALF, after 12 weeks of treatment with fluticasone propionate and salmeterol. Interestingly, we also observed an increase in chymase, tryptase, and CXCL10 after addition of LPS to HDM, which suggests an effect on mast cell recruitment and/or activation (31, 32). These mediators affect the airway smooth muscle layer, which is thought to play an important role in airway remodeling in asthma (32). Likewise, TGF- β ,

which is also potentiated by LPS, is associated with airway remodeling (33) and asthma severity (34).

Based on our findings and others, we could speculate that exposure to LPS in combination with mite allergen potentiates mechanisms associated with airway remodeling. Possibly, salmeterol due to its effect on CXCL10 might interfere with these mechanisms, at least during acute administration.

In conclusion, we showed that low-dose LPS significantly promotes eosinophilic airway inflammation, in particular as indicated by ECP levels, after allergen exposure in patients with asthma. Most interestingly, while they were using maintenance treatment with ICS. In addition to its effect on ECP, LPS appears to promote parameters associated with mast cell recruitment and activation, and potentiates TGF- β . These pathways are connected with airway remodeling. Secondly, salmeterol may inhibit some aspects of HDM/LPS-induced bronchial inflammation, but did not inhibit the main parameters for eosinophilic and neutrophilic inflammation.

Acknowledgments

The authors gratefully acknowledge Mrs. B.S. Smids-Dierdorp, Mrs. T. Dekker, Ms. A. Dijkhuis, and Ms. S. Chowdhury for their excellent laboratory assistance; Dr K.F. van der Sluijs, Dr M.A. van de Pol, Drs C.J. Majoor, and Drs L.N. Venekamp for their professional assistance in performing the bronchoscopies; and Dr E.M. Kemper and Ms. E.S. Brans for their assistance during preparation of the HDM solution and LPS solution.

Author contributions

P.B., T.P., R.L., P.J.S., and J.S.Z. were involved in conception and design. M.B., J.D.B., P.B., and J.S.Z. participated in data collection. M.B., J.D.B., R.L., P.J.S., and J.S.Z. performed analysis and interpretation. All authors contributed to the writing of this manuscript.

Funding

This study was financially supported by an unrestricted grant from GlaxoSmith Kline, the Netherlands.

Conflicts of interest

All authors state that they have no conflict of interests to declare.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Effect of provocation with HDM or HDM/LPS in peripheral blood.

Table S2. Effect of provocation with LPS or HDM/LPS in BALF.

References

- From the Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA) 2012. Available from: <http://www.ginasthma.org/>. Last Accessed 18 December 2014
- Dulek DE, Peebles RS Jr. Viruses and asthma. *Biochim Biophys Acta* 2011;**11**:1080–1090.
- Vieira SE, Stein RT, Ferraro AA, Pastro LD, Pedro SS, Lemos M et al. Urban air pollutants are significant risk factors for asthma and pneumonia in children: the influence of location on the measurement of pollutants. *Arch Bronconeumol* 2012;**48**:389–395.
- Hernandez ML, Herbst M, Lay JC, Alexis NE, Brickey WJ, Ting JP et al. Atopic asthmatic patients have reduced airway inflammatory cell recruitment after inhaled endotoxin challenge compared with healthy volunteers. *J Allergy Clin Immunol* 2012;**130**:869–876.
- Hoogerwerf JJ, de Vos AF, Bresser P, Van der Zee JS, Pater JM, de Boer A et al. Lung inflammation induced by lipoteichoic acid or lipopolysaccharide in humans. *Am J Respir Crit Care Med* 2008;**178**:34–41.
- O'Grady NP, Preas HL, Pugin J, Fiuza C, Tropea M, Reda D et al. Local inflammatory responses following bronchial endotoxin instillation in humans. *Am J Respir Crit Care Med* 2001;**163**:1591–1598.
- Lawson JA, Dosman JA, Rennie DC, Beach J, Newman SC, Senthilselvan A. The association between endotoxin and lung function among children and adolescents living in a rural area. *Can Respir J* 2011;**18**:e89–94.
- Heederik D, von Mutius E. Does diversity of environmental microbial exposure matter for the occurrence of allergy and asthma? *J Allergy Clin Immunol* 2012;**130**:44–50.
- Gehring U, Strikwold M, Schram-Bijkerk D, Weinmayr G, Genuneit J, Nagel G et al. Asthma and allergic symptoms in relation to house dust endotoxin: Phase Two of the International Study on Asthma and Allergies in Childhood (ISAAC II). *Clin Exp Allergy* 2008;**38**:1911–1920.
- Michel O. Role of house-dust endotoxin exposure in aetiology of allergy and asthma. *Mediators Inflamm* 2001;**10**:301–304.
- de Boer JD, Roelofs JJ, de Vos AF, de Beer R, Schouten M, Hommes TJ et al. Lipopolysaccharide inhibits Th2 lung inflammation induced by house dust mite allergens in mice. *Am J Respir Cell Mol Biol* 2013;**48**:382–389.
- Gauvreau GM, Boulet LP, Postma DS, Kawayama T, Watson RM, Duong M et al. Effect of low-dose ciclesonide on allergen-induced responses in subjects with mild allergic asthma. *J Allergy Clin Immunol* 2005;**116**:285–291.
- Green RH, Brightling CE, McKenna S, Hargadon B, Parker D, Bradding P et al. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet* 2002;**360**:1715–1721.
- Maris NA, de Vos AF, Dessing MC, Spek CA, Lutter R, Jansen HM et al. Antiinflammatory effects of salmeterol after inhalation of lipopolysaccharide by healthy volunteers. *Am J Respir Crit Care Med* 2005;**172**:878–884.
- Hoshino M, Handa H, Miyazawa T. Effects of salmeterol and fluticasone propionate combination versus fluticasone propionate on airway function and eosinophilic inflammation in mild asthma. *Allergol Int* 2009;**58**:357–363.
- Koopmans JG, Lutter R, Jansen HM, Van der Zee JS. Adding salmeterol to an inhaled corticosteroid reduces allergen-induced serum IL-5 and peripheral blood eosinophils. *J Allergy Clin Immunol* 2005;**116**:1007–1013.
- Schaumann F, Muller M, Braun A, Luetzig B, Peden DB, Hohlfeld JM et al. Endotoxin augments myeloid dendritic cell influx into the airways in patients with allergic asthma. *Am J Respir Crit Care Med* 2008;**177**:1307–1313.
- Crapo RO, Casaburi R, Coates AL, Enright PL, Hankinson JL, Irvin CG et al. Guidelines for methacholine and exercise challenge testing-1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. *Am J Respir Crit Care Med* 2000;**161**:309–329.
- Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A et al. Standardisation of spirometry. *Eur Respir J* 2005;**26**:319–338.
- Baraket M, Oliver BG, Burgess JK, Lim S, King GG, Black JL. Is low dose inhaled corticosteroid therapy as effective for inflammation and remodeling in asthma? A randomized, parallel group study. *Respir Res* 2012;**13**:11.
- Du Rand IA, Barber PV, Goldring J, Lewis RA, Mandal S, MUnavvar M et al. Summary of the British Thoracic Society guidelines for advanced diagnostic and therapeutic flexible bronchoscopy in adults. *Thorax* 2011;**66**:1014–1015.
- Meyer KC, Raghu G, Baughman RP, Brown KK, Costabel U, du Bois RM et al. An official American Thoracic Society clinical practice guideline: the clinical utility of bronchoalveolar lavage cellular analysis in interstitial lung disease. *Am J Respir Crit Care Med* 2012;**185**:1004–1014.
- Shah VP, Midha KK, Findlay JW, Hill HM, Hulse JD, McGilveray IJ et al. Bioanalytical method validation—a revisit with a decade of progress. *Pharm Res* 2000;**17**:1551–1557.
- Michel O, Kips J, Duchateau J, Vertongen F, Robert L, Collet H et al. Severity of asthma is related to endotoxin in house dust. *Am J Respir Crit Care Med* 1996;**154**:1641–1646.
- Michel O, Dentener M, Corazza F, Buurman W, Rylander R. Healthy subjects express differences in clinical responses to inhaled lipopolysaccharide that are related with inflammation and with atopy. *J Allergy Clin Immunol* 2001;**107**:797–804.
- Braunstaal GJ, Kleinjan A, Overbeek SE, Prins JB, Hoogsteden HC, Fokkens WJ. Segmental bronchial provocation induces nasal inflammation in allergic rhinitis patients. *Am J Respir Crit Care Med* 2000;**161**:2051–2057.
- Lommatzsch M, Julius P, Kuepper M, Garn H, Bratke K, Irmischer S et al. The course of allergen-induced leukocyte infiltration in human and experimental asthma. *J Allergy Clin Immunol* 2006;**118**:91–97.
- Brown JR, Kleimberg J, Marini M, Sun G, Bellini A, Mattoli S. Kinetics of eotaxin expression and its relationship to eosinophil accumulation and activation in bronchial biopsies and bronchoalveolar lavage (BAL) of asthmatic patients after allergen inhalation. *Clin Exp Immunol* 1998;**114**:137–146.
- Wallin A, Sue-Chu M, Bjerrmer L, Ward J, Sandstrom T, Lindberg A et al. Effect of inhaled fluticasone with and without salmeterol on airway inflammation in asthma. *J Allergy Clin Immunol* 2003;**112**:72–78.
- Kaur M, Chivers JE, Gienbycz MA, Newton R. Long-acting beta2-adrenoceptor agonists synergistically enhance glucocorticoid-dependent transcription in human airway epithelial and smooth muscle cells. *Mol Pharmacol* 2008;**73**:203–214.
- Brightling CE, Ammit AJ, Kaur D, Black JL, Wardlaw AJ, Hughes JM et al. The CXCL10/CXCR3 axis mediates human lung mast cell migration to asthmatic airway smooth muscle. *Am J Respir Crit Care Med* 2005;**171**:1103–1108.
- Tan X, Alrashdan YA, Alkhouri H, Oliver BG, Armour CL, Hughes JM. Airway smooth muscle CXCR3 ligand production: regulation by JAK-STAT1 and intracellular Ca(2+). *Am J Physiol Lung Cell Mol Physiol* 2013;**304**:L790–L802.
- Savov JD, Gavett SH, Brass DM, Costa DL, Schwartz DA. Neutrophils play a critical role in development of LPS-induced airway disease. *Am J Physiol Lung Cell Mol Physiol* 2002;**283**:L952–L962.
- Ierodiakonou D, Postma DS, Koppelman GH, Gerritsen J, ten Hacken NH, Timens W et al. TGF-beta1 polymorphisms and asthma severity, airway inflammation, and remodeling. *J Allergy Clin Immunol* 2013;**131**:582–585.

Supplement Table I: Effect of provocation with HDM or HDM/LPS in peripheral blood

Parameters	Baseline	HDM	HDM/LPS	P-value
	n = 36	n = 16	n = 20	
Leukocytes, cells x 10 ⁹ /L	5.50	7.26	9.53	0.001
Neutrophils, cells x 10 ⁹ /L	2.81	4.48	6.87	<0.001
Eosinophils, cells x 10 ⁹ /L	0.17	0.10	0.08	0.04
Lymphocytes, cells x 10 ⁹ /L	1.81	1.90	1.80	0.28
Monocytes, cells x 10 ⁹ /L	0.44	0.53	0.61	0.08
Basophils, cells x 10 ⁹ /L	0.03	0.03	0.03	0.67
IL-5, pg/ml	1.90	2.22	2.40	0.53
IL-6, pg/ml	0.43	0.60	1.24	0.12
IL-8, pg/ml	0.96	1.25	1.36	0.85
IL-10, pg/ml	1.08	0.58	1.40	0.02
GRO-α, ng/ml	18.43	14.03	11.91	0.70
MCP-1, pg/ml	0.92	0.67	0.70	0.37
MIP-1α, pg/ml	0.90	0.89	0.69	0.25
MIP-1β, ng/ml	28.76	24.49	29.72	0.12
TGF-α, pg/ml	0.31	0.29	0.25	0.71

Footnote: Geometric mean data for baseline values and estimated marginal means (adjusted for baseline values) for HDM and HDM/LPS. P-values for the difference between HDM and HDM/LPS as found in the univariate analysis with LPS and salmeterol as fixed factors and baseline values as covariate.

Definition of abbreviations: HDM; House Dust Mite, LPS; Lipopolysaccharide. Cell differentials, cytokines and chemokines were measured in peripheral blood before and 6 hours after bronchial segmental challenge with HDM or HDM/LPS.

Supplement Table II: Effect of provocation with LPS or HDM/LPS in BALF

Primary outcomes	LPS n = 6	HDM/LPS n = 14	P-value
Eosinophils, cells x 10 ⁴ /ml	0.29	1.82	0.07
Eosinophil cationic protein, ng/ml	0.78	5.81	0.06
Secondary outcomes			
Leukocytes, cells x 10 ⁴ /ml	33.04	29.44	0.76
Neutrophils, cells x 10 ⁴ /ml	14.29	9.95	0.97
Lymphocytes, cells x 10 ⁴ /ml	1.53	1.46	0.49
Monocytes, cells x 10 ⁴ /ml	0.02	0.02	0.53
Basophils, cells x 10 ⁴ /ml	0.02	0.02	0.59
Macrophages, cells x 10 ⁴ /ml	15.28	11.38	0.95
Mast cells, cells x 10 ⁴ /ml	0.02	0.03	0.19
Plasma cells, cells x 10 ⁴ /ml	0.02	0.02	0.89
IL-1 β , pg/ml	0.09	0.14	0.27
IL-6, ng/ml	0.59	0.99	0.21
IL-8, pg/ml	660.69	659.17	0.64
IL-9, pg/ml	0.34	0.40	0.95
IL-18, pg/ml	0.04	0.04	0.87
Myeloperoxidase, ng/ml	22.96	31.48	0.52
GRO- α , ng/ml	0.93	1.13	1.00
MCP-1, pg/ml	82.41	49.89	0.68
MIP-1 α , pg/ml	1.19	8.39	0.33
MIP-1 β , ng/ml	1.66	1.73	0.51
CXCL-10, ng/ml	15.92	10.62	0.99
RANTES, pg/ml	0.04	0.49	0.008
TGF- β , pg/mL	8.87	12.91	0.74
Chymase, ng/ml	14.96	19.59	0.52
Tryptase, kU/L	0.71	1.54	0.16

Eotaxin, pg/ml	1.69	1.49	0.89
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Footnote: Estimated marginal means (means adjusted for saline challenge) and p-values for the difference between LPS and HDM/LPS as found in the univariate analysis with HDM and salmeterol as fixed factors and saline challenge values as covariate.

Definition of abbreviations: HDM; House Dust Mite, LPS; Lipopolysaccharide. Cell differentials, cytokines and chemokines were measured in bronchoalveolar lavage fluid, 6 hours after instillation of LPS or HDM/LPS in a lung subsegment of allergic asthmatics.