

Low Levels of Endotoxin Enhance Allergen-Stimulated Proliferation and Reduce the Threshold for Activation in Human Peripheral Blood Cells

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Key Words

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T cell proliferation

Abstract

Background: Endotoxins, comprised of bacterial cell wall lipopolysaccharides (LPS), have been reported to have both protective and exacerbating effects on the development and maintenance of allergic disease in humans and on markers of allergic inflammation in animal models of allergy. In this study, we investigated the effect of low concentrations of LPS on human peripheral blood mononuclear cells (PBMC) stimulated with the major cat allergen Fel d 1. **Methods:** Extensive purification of recombinant (r) Fel d 1 yielded essentially endotoxin-free rFel d 1 (0.2 ng LPS/mg protein). PBMCs prepared from 15 subjects having IgE to cat (>0.7 kU_A/l) and 8 subjects IgE negative to cat were stimulated with 2, 10 or 25 µg/ml of rFel d 1 in the presence or absence of 50 pg/ml LPS. Proliferation was measured after 7 days of culture and supernatants were analyzed for IFN γ , IL-5 and IL-10. **Results:** LPS (50 pg/ml) increased rFel d 1-stimulated proliferation of PBMCs both from subjects IgE-positive and subjects negative to cat allergens. PBMCs from 13 of the subjects did not proliferate in response to stimulation with 2 and 10 µg/ml

rFel d 1 alone but did so in the presence of LPS. Moreover, LPS increased the levels of rFel d 1-stimulated IFN γ in cultures from cat-negative subjects, IL-5 from cat-positive subjects and IL-10 from both groups. **Conclusion:** Very low doses of LPS enhance proliferation and decrease the apparent threshold level for cell activation, prompting careful evaluation of allergen stimulated T cell activation in vitro.

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Introduction

Numerous signals activating innate and adaptive immunity interact in order to raise an appropriate immune response to infectious agents. Stimulation of innate immune responses is also critical for the outcome of an adaptive immune response to common noninfectious antigens, including allergens. A potent stimulus of the innate immune system is the bacterial outer cell wall component endotoxin, which is composed of lipopolysaccharides (LPS). LPS stimulates the innate immune system by interaction with Toll-like receptor 4 (TLR-4), preferentially expressed on professional antigen-presenting cells such as dendritic cells and macrophages, but also on monocytes and neutrophils [1, 2]. Effective TLR-4 activa-

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tion by LPS requires the interaction of LPS with the coreceptor CD14, the adapter molecule MD-2 and LPS-binding protein, LBP [2, 3]. LPS signaling induces T lymphocyte proliferation and cytokine production but exactly how T cell activation is triggered is not fully understood. Proposed mechanisms are through blocking of suppression by regulatory T cells (Treg) [4] and through direct cell-to-cell contact with monocytes [5].

Endotoxins are ubiquitous in our environment and contaminate many common allergen sources like mites and dander from furred animal, as well as experimental allergens such as chicken ovalbumin (OVA) [6]. The role of LPS in the mounting and maintenance of allergic diseases is contradictory. In farm environments endotoxins have been proposed as a protective factor, decreasing the risk of developing allergic disease [7, 8]. In other settings LPS may have the opposite effect. Asthma severity has for example been shown to correlate more with endotoxin levels in house dust than with mite allergen concentration [9–11].

In different experimental models LPS exhibits variable effects on parameters measuring allergic immune responses, inflammation and bronchial hyperresponsiveness. In some animal models for asthma endotoxins have been reported to inhibit the allergic response [12–14], while in others LPS enhanced the allergic immune response and inflammation [15, 16]. An LPS effect on the allergic response is also supported by findings from mice deficient for TLR4 or the TLR-signaling molecule MyD88, demonstrating that allergic inflammation and induction of pulmonary T helper (Th) 2 responses depends on these activation pathways [17–20]. Thus, endotoxins may lead to opposing effects on the allergic response depending on, for example, the LPS dose, the time-course of exposure and the route of allergen and LPS challenge.

The dose of LPS present during allergen sensitization is critical for the outcome of the immune response. In a mouse model for OVA sensitization low levels of inhaled LPS together with allergen induced a Th2 type of response in contrast to high levels of LPS, which led to a Th1 response to the allergen [18]. The allergic inflammation resulting from LPS exposure during allergen challenge in already sensitized animals was also demonstrated to be differentially modulated depending on the level of endotoxin [13, 15, 17]. The effect of endotoxin exposure on human allergic disease is even more complex, since the levels of endotoxin vary considerably in different environmental settings. Low doses of endotoxin that are subthreshold for inducing airway neutrophilia seem to result in a Th2 skewed airway inflammatory response in

humans [21]. However, a recent experimental study addressing the question how inhalation of low-dose LPS affects the immediate- and late-phase bronchial and inflammatory response in cat allergic asthmatic patients failed to detect any significant effect of exposure to LPS prior to allergen challenge [22].

Here we sought to investigate the effect of adding very low concentrations of LPS to human peripheral blood mononuclear cell (PBMC) cultures stimulated with a highly purified recombinant preparation of the major cat allergen Fel d 1. We show that LPS is able to enhance the Fel d 1-stimulated proliferative response of PBMCs. Furthermore, we demonstrate that the Fel d 1-induced cytokine production increases when LPS is present in the culture. Thus, low levels of LPS have a direct effect on allergen-induced cell responses. Our results suggest that these low concentrations of LPS decrease the apparent threshold level for in vitro allergen-induced cell activation.

Materials and Methods

Preparation and Purification of rFel d 1

Recombinant Fel d 1 (rFel d 1) was expressed in *Escherichia coli* and purified using Ni²⁺ affinity and size exclusion chromatographies [23]. Additional to the previously described purification procedure, rFel d 1 was subjected to in vitro refolding in 6 M guanidine hydrochloride and 25 mM β -mercaptoethanol. The refolding process proceeded over night by dialysis against 20 mM Tris, pH 7.6. The protein was purified by ion-exchange chromatography on a Mono Q column (Amersham Bioscience, Uppsala, Sweden). Binding to the resin was achieved in a starting buffer, 20 mM Tris pH 7.6, followed by a 10 column volumes gradient to 1 M NaCl in 20 mM Tris, pH 7.6. Fractions containing rFel d 1 were dialyzed against the starting buffer and the ion-exchange chromatography was repeated using the same conditions. In parallel, refolded rFel d 1 was purified by polymyxin B bead affinity chromatography (Affi-Prep®, BioRad, Hercules, Calif., USA), according to manufacturer's instructions. Purity was assessed by SDS-PAGE in 15% homogeneous gels. The purified rFel d 1 was stored at -80°C . Protein concentration was determined by BCA assay (Pierce, Rockford, Ill., USA) with bovine serum albumin as standard.

Endotoxin activity was determined in a limulus amoebocyte lysate endochrome assay (Charles River Endosafe, Charleston, S.C., USA) according to the manufacturer's instructions. Natural cat allergen was prepared from cat dander (Allergen AB, Ängelholm, Sweden) by aqueous extraction.

Subjects

Peripheral blood was obtained from 23 subjects in total. Subjects that had >0.7 kU_A/l IgE to cat dander (median 3.2, range 1–66; Pharmacia CAP System, Phadia AB, Uppsala, Sweden) were defined as positive to cat ($n = 15$) and subjects that lacked IgE to cat (≤ 0.35 kU_A/l) as negative to cat ($n = 8$). These two groups will hereafter be referred to as 'cat-positive' and 'cat-negative'. For

fractionation of CD4+CD25+ and CD4+CD25- cells, blood was drawn from 10 individuals CAP-negative to cat dander (≤ 0.35 kU_A/l) and for isolation of monocytes from 2 subjects CAP positive to cat (5.3 and 18 kU_A/l). The study was approved by the local ethics committee.

PBMC Cultures

PBMCs isolated by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) gradient centrifugation were cultured at a cell density of 1×10^6 cells/ml in 96-well plates in complete RPMI (cRPMI: RPMI 1640 supplemented with 25 µg/ml gentamicin, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (Gibco, Invitrogen Corporation, UK), 50 µM 2-mercapto-ethanol (Sigma Aldrich, Steinheim, Germany) and 10% (v/v) heat-inactivated bovine growth serum (Hyclone, Logan, USA) at 37°C in a humidified atmosphere with 6% CO₂. PBMCs were stimulated with rFel d 1 (2, 10 and 25 µg/ml) alone or with 50 pg/ml of LPS (from *E. coli* O26:B6, Sigma Aldrich). After 6 days supernatants were collected for determination of cytokines (IL-5, IL-10 and IFN-γ) using the Th1/Th2 cytometric bead array kit (BD Biosciences, San Jose, Calif., USA) according to the manufacturer's instruction (detection limit 2.5 pg/ml). 1 µCi/well [³H]thymidine (Amersham Biosciences) was added for the final 16 h of incubation and the measured proliferation was expressed as stimulation indices (SI), i.e. mean of triplicate counts per minute (cpm) obtained from stimulated cells divided by cpm values from unstimulated wells. Mean cpm (for all subjects) in unstimulated cultures was 1,480 cpm, in cultures stimulated with 2 µg/ml rFel d 1 2,190 cpm, 10 µg/ml rFel d 1 2,810 cpm, 25 µg/ml rFel d 1 5,630 cpm, 2 µg rFel d 1 + 50 pg/ml LPS 4,680, 10 µg rFel d 1 + 50 pg/ml LPS 7,920, 25 µg rFel d 1 + 50 pg/ml LPS 9,840 and in cultures stimulated with LPS alone 4,840.

Polymyxin Blocking

PBMCs were cultured and stimulated as described above, in the presence or absence of 1 µg/ml of polymyxin B (Sigma Aldrich). The stimulation index was calculated as the ratio of cpm obtained in rFel d 1- and/or LPS-stimulated cultures divided with cpm in the appropriate unstimulated control culture (with or without polymyxin). Three independent experiments were performed on PBMC cultures from three cat-positive subjects.

Isolation and Stimulation of Monocytes

CD14+ monocytes were isolated by MACS microbead separation (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions from PBMCs obtained from two cat-positive subjects. Purity of the isolated monocyte fractions from the two subjects were assessed to 92 and 98% CD14+ cells on a FACSCalibur flow cytometer (BD Biosciences) after staining with anti-CD14-FITC and isotype-control IgG2a-FITC (BD Pharmingen, San Jose, Calif., USA). Isolated monocytes were stimulated (10^6 cells/ml cRPMI, 100,000 cells/well) with LPS at 10, 50, 100 pg/ml, 1, 10 and 50 ng/ml or with Fel d 1 (10 and 25 µg/ml) alone or together with 50 pg/ml LPS.

Isolation and Co-Cultures of CD4+CD25- and CD4+CD25+ Cells

CD4+CD25+ cells were isolated from PBMCs using activated magnetic bead sorting according to the manufacturer's protocol (CD4+CD25+ Treg isolation kit, Miltenyi Biotec). Mean purity of

the CD4+ cells was 96% (95–98%) and for CD4+CD25+ cells 83% (66–94%) as determined by staining with anti-CD25-PE (Miltenyi Biotec), anti-CD4-FITC, IgG1-PE and IgG1-FITC (BD Pharmingen) and analysis on a FACSCalibur flow cytometer (BD Biosciences). A detailed description of the characterization of isolated Treg and Teffector cell fractions has been published elsewhere [24]. Cells in the CD4- fraction were irradiated (30 Gray) and used as antigen-presenting cells. A total of 2×10^4 CD4+ cells (100 µl/well) were cultured in cRPMI in the presence of antigen and 8×10^4 autologous irradiated CD4- cells per well for 6 days. The cultures containing different ratios of CD4+CD25+ and CD4+CD25- cells (1:0, 3:1, 1:1, 1:3 and 0:1) were stimulated with 10 µg/ml rFel d 1 in the presence or absence of 50 pg/ml of LPS. To evaluate the suppressive function of the isolated CD4+CD25+ cells, co-cultures were stimulated with 1 µg/ml plate-bound anti-CD3 (OKT3).

Statistical Analysis

The Wilcoxon matched pairs test was used for within group comparisons and the Mann-Whitney U test for between-groups comparisons. The STATISTICA software, version 7.1 (StatSoft Scandinavia AB, Sweden), was used for statistical calculations. $p < 0.05$ was considered significant (* < 0.05 , ** < 0.01 , *** < 0.001).

Results

Purification of rFel d 1 Yielded Highly Pure and Almost Endotoxin-Free Protein

To be able to study the effect of LPS on allergen-stimulated cell cultures it was essential to obtain a very pure preparation of rFel d 1. In addition to previously described purification steps [23], the recombinant protein was further purified by repeated ion exchange chromatography. The purity of rFel d 1 was analyzed on SDS-PAGE displaying a single band with an apparent molecular mass of 19 kDa (not shown). The endotoxin content of the purified rFel d 1 was measured to 2 EU/mg corresponding to 0.2 ng/mg protein (giving 5 pg/ml cell culture at the highest rFel d 1 stimulation concentration). In comparison, rFel d 1 purified by polymyxin affinity chromatography instead of ion exchange contained 1.86 ng endotoxin/mg protein and an aqueous extract of cat dander 320 ng/mg protein.

LPS Enhances rFel d 1-Stimulated Proliferation of PBMCs

In order to assess the impact of LPS on allergen-stimulated cell proliferation, PBMCs from all subjects were stimulated with 2, 10 and 25 µg/ml rFel d 1 or were left unstimulated in the presence or absence of 50 pg LPS. As shown in figure 1a, LPS stimulates proliferation at 50 pg/ml, without any other stimuli added to the cultures. In

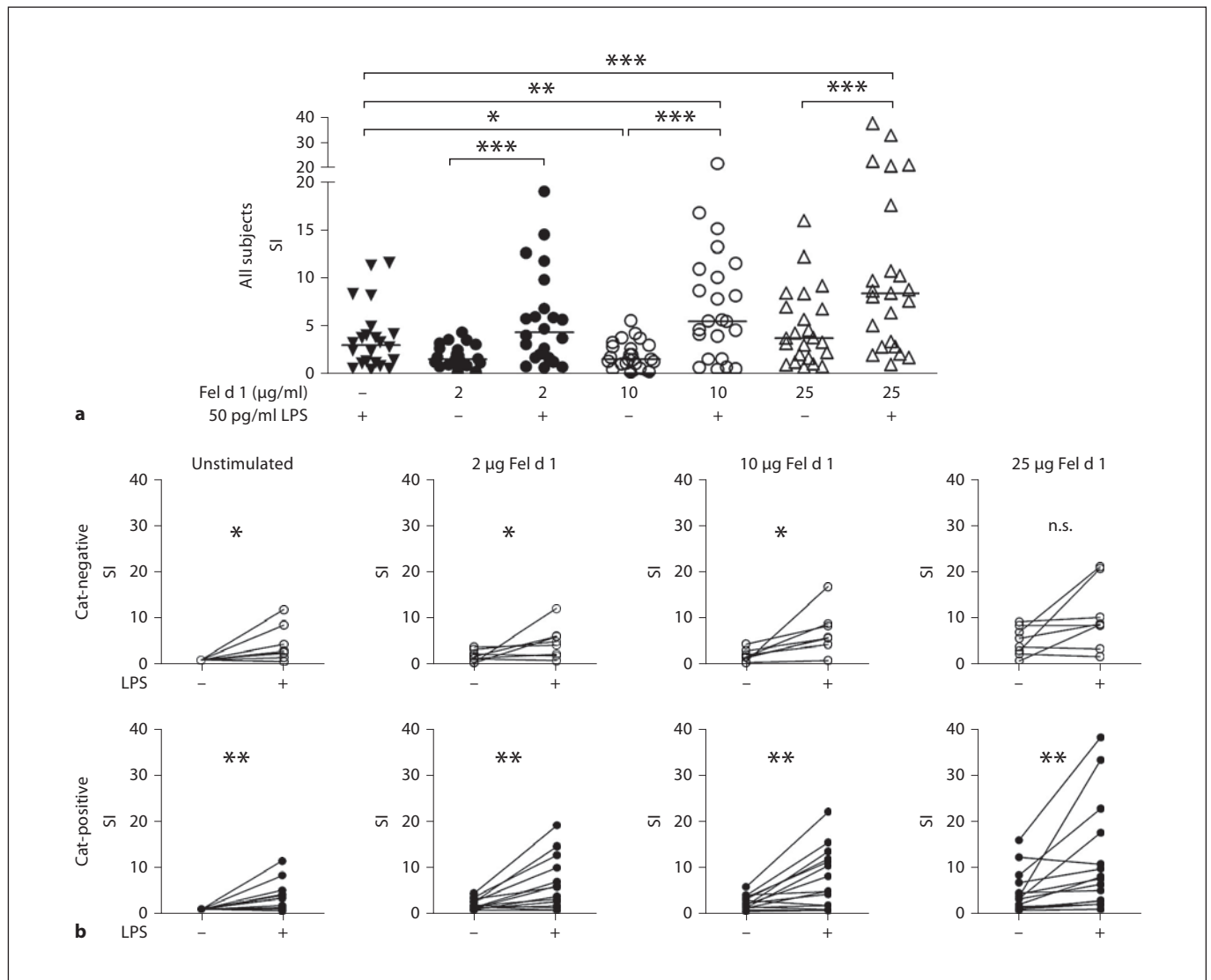


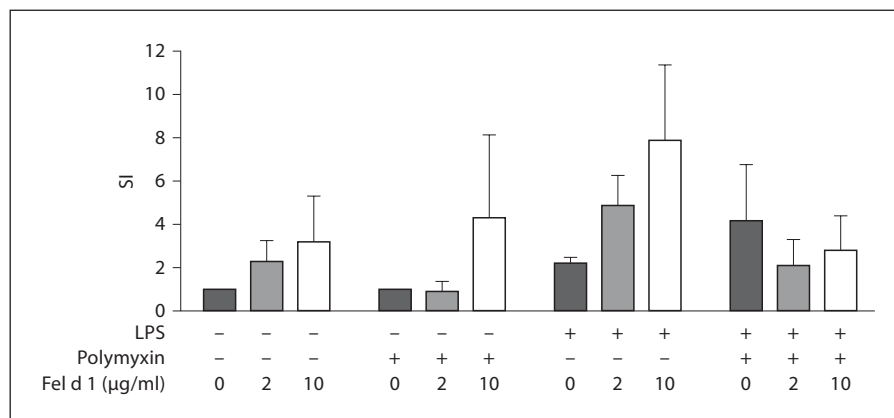
Fig. 1. LPS increases the Fel d 1-stimulated proliferation of PBMC from cat-positive and cat-negative subjects. **a** Proliferation data from PBMC cultures, expressed as stimulation indices (SI), from all subjects. SI values were obtained from cultures stimulated with 2 μg/ml (filled circles), 10 μg/ml (open circles) and 25 μg/ml (open triangles) in the absence (-) or presence (+) of 50 pg/ml LPS, as well as from cultures stimulated with LPS alone (filled triangles). Median values are indicated. * $p \leq 0.05$, ** $p \leq 0.01$,

*** $p \leq 0.001$. **b** Proliferation data from PBMC cultures, expressed as SI, from subjects IgE-positive and IgE-negative to cat. The data for unstimulated cells (in cRPMI only) and cells stimulated with 2, 10 and 25 μg/ml rFel d 1 are shown for cat-positive subjects ($n = 15, 14$ at 2 μg/ml rFel d 1; filled symbols) and cat-negative subjects ($n = 8$; open symbols). * $p \leq 0.05$, ** $p \leq 0.01$; n.s. = not significant.

fact LPS alone stimulates higher proliferation than 10 μg/ml of highly purified rFel d 1. LPS in combination with rFel d 1 at 2, 10 or 25 μg/ml stimulates higher proliferation than rFel d 1 alone at all three allergen concentrations ($p < 0.001$). Moreover, the proliferation stimulated by 10 and 25 μg/ml of rFel d 1 in combination with 50

pg/ml LPS is significantly higher than the proliferation stimulated by LPS alone (fig. 1a). There was no significant difference between SI values obtained from cultures stimulated with rFel d 1 together with 50 pg/ml LPS compared to the corresponding sum of SI values from cultures stimulated with LPS alone and rFel d 1 alone. Thus,

Fig. 2. Proliferative responses in PBMC cultures stimulated by 2 and 10 $\mu\text{g/ml}$ rFel d 1 together with 50 pg/ml are decreased by polymyxin. Mean SI values $\pm$ SEM are shown for unstimulated PBMC cultures (dark grey) and PBMC cultures stimulated with 2 $\mu\text{g/ml}$ rFel d 1 (grey) or 10 $\mu\text{g/ml}$ rFel d 1 (white). At each rFel d 1 stimulation concentration 50 pg/ml LPS was added to indicated cultures and all stimulations were done both in the presence (+) or absence (-) of polymyxin. The data are from three independent experiments on PBMCs obtained from 3 cat-positive subjects.



the increase in proliferation appears to be mainly additive, although a clear synergistic effect was detected in rFel d 1 + LPS-stimulated cultures for some individuals.

The highly purified rFel d 1 was not able to stimulate proliferation in PBMC cultures from several subjects, especially at the lower stimulation concentrations. Both at 2 and at 10 $\mu\text{g/ml}$ rFel d 1 13 subjects responded with a negative proliferation stimulation index, i.e. $\text{SI} < 2$, when stimulated in absence of added LPS. At 25 $\mu\text{g/ml}$ rFel d 1 6 subjects did not reach a positive SI value. In PBMC cultures from these individuals, 9/13 exhibited positive SI values in response to 2 $\mu\text{g/ml}$ rFel d 1, 8/13 to 10 $\mu\text{g/ml}$ rFel d 1 and 5/6 to 25 $\mu\text{g/ml}$ rFel d 1 when 50 pg/ml LPS was added to the cultures. For the cultures with negative SI values, the mean SI for 2, 10 and 25 $\mu\text{g/ml}$ rFel d 1 was, respectively, 1.2, 1.2 and 1.2, compared to 4.1, 6.0 and 3.4 in the presence of 50 pg/ml LPS. The corresponding SI values to LPS alone were 3.7, 4.1 and 2.7. Thus, addition of LPS led to positive SI values in PBMC cultures not responding to rFel d 1 alone, reducing the apparent threshold level for cell activation of PBMCs.

We further wanted to investigate if IgE positivity to cat affected the proliferation of PBMCs in response to rFel d 1 and LPS. The proliferation increased significantly when 50 pg LPS was added to all stimulating concentrations of rFel d 1 and to medium only, except for the cat-negative group at 25 $\mu\text{g/ml}$ (fig. 1b). The LPS-induced increase of proliferation was most pronounced for the cat-positive group, particularly at the highest rFel d 1 concentration (25 $\mu\text{g/ml}$). However, there was no significant difference in proliferation levels between the cat-positive group and the cat-negative group at any stimulation condition.

LPS-Dependent Amplification of Proliferation Is Inhibited by Polymyxin

We next wanted to ensure that the observed enhancement of proliferation was mediated by added LPS. In three independent experiments we evaluated the effect on proliferation of adding the endotoxin-neutralizing agent polymyxin B to PBMC cultures stimulated with 2 or 10 $\mu\text{g/ml}$ rFel d 1, as well as unstimulated cultures, in the presence or absence of 50 pg/ml LPS. The results reveal that polymyxin reduces the enhanced rFel d 1-stimulated proliferation observed in cultures with added LPS to levels comparable to those stimulated by rFel d 1 alone (fig. 2). Based on these experiments, we conclude that the amplification effect on PBMC proliferation is mediated mainly by LPS and not by other possible contaminants in the commercial LPS preparation, such as bacterial lipopeptides [25, 26].

LPS Does Not Stimulate Proliferation of Monocytes

Since monocytes express receptors for LPS, a control experiment was performed to assess the ability of a PBMC fraction enriched for monocytes to proliferate after stimulation with a range of different LPS concentrations (10 pg/ml to 50 ng/ml). The isolated monocytes did not proliferate to any of the tested LPS concentrations (< 500 cpm, data not shown). Neither did the monocytes proliferate in response to Fel d 1 stimulation (10 and 25 $\mu\text{g/ml}$), nor to Fel d 1 with 50 pg/ml added LPS.

Allergen Stimulated Cytokine Concentrations Are Elevated by the Addition of LPS

The concentrations of IFN- γ , IL-10 and IL-5 were measured in supernatants from the rFel d 1- and LPS-stimulated PBMC cultures. As shown in figure 3 signifi-

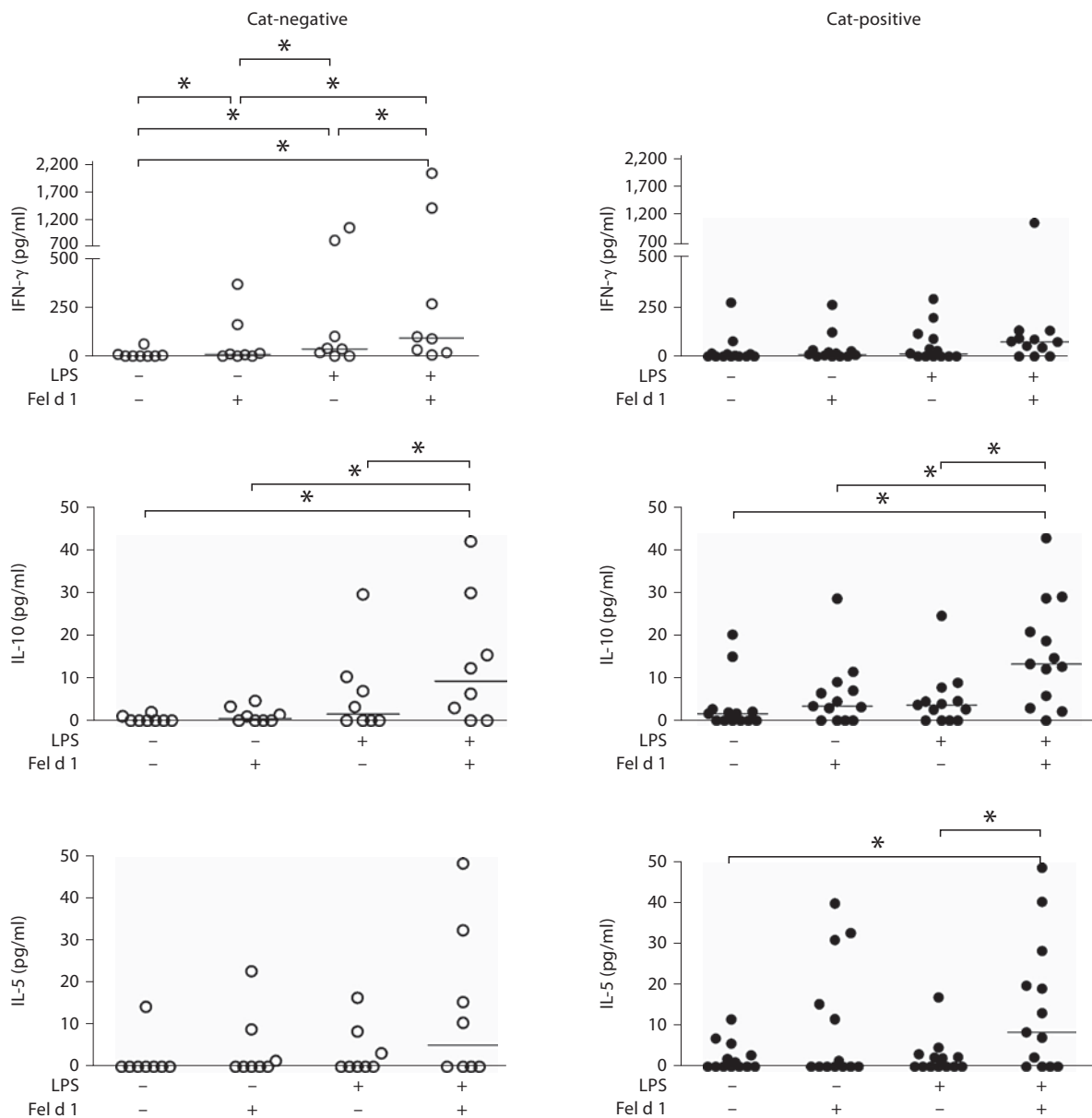


Fig. 3. Elevated levels of IFN- γ are stimulated by LPS in PBMC cultures from cat-negative subjects and of IL-5 in PBMC cultures from cat-positive subjects. Cytokine concentrations (IFN- γ , IL-10 and IL-5) measured by cytometric bead array in supernatants of PBMC cultures from cat-positive subjects ($n = 13$) and cat-nega-

tive subjects ($n = 8$) stimulated with 10 $\mu\text{g/ml}$ Fel d 1, 50 pg/ml LPS, 10 $\mu\text{g/ml}$ Fel d 1 + 50 pg/ml LPS or unstimulated. Concentrations are given as pg/ml. Median values are indicated * $p \leq 0.05$.

cant differences were detected for IFN γ concentrations between the different stimulation conditions (unstimulated and stimulated with 10 μ g/ml rFel d 1 with or without 50 pg/ml LPS) for the cat-negative subjects, in contrast to the cat-positive group where no difference in the IFN γ levels was observed in response to the different stimuli. Higher concentrations of IL-10 were obtained in cultures with rFel d 1 and LPS compared to the other stimulation conditions for both groups ($p < 0.05$). Finally, elevated levels of IL-5 were stimulated by rFel d 1 together with LPS compared to by LPS only or to unstimulated cells in culture supernatants from cat-positive ($p < 0.05$) but not from cat-negative subjects. When comparing the cat-positive and cat-negative groups no difference in the levels of any of the analyzed cytokines stimulated at any stimulation condition was observed.

LPS Affects the Suppression of Proliferation by CD4+CD25+ Treg Cells Only at a High Treg to Effector Cell Ratio

A proposed mechanism through which LPS activate T cells is by inhibition of suppressive function of Treg cells. Therefore, we investigated if suppression by Treg cells was affected by LPS. The ability of isolated CD4+CD25+ Treg cells to suppress proliferation of CD4+CD25- effector cells was first evaluated in Treg:effector cell co-cultures with polyclonal anti-CD3 stimulation. Isolated fractions of CD4+CD25+ cells from ten nonallergic healthy subjects were all able to suppress anti-CD3-stimulated autologous CD4+CD25- cells (not shown). Co-cultures with CD4+CD25+ Treg cells and CD4+CD25- effector cells were then stimulated with 10 μ g/ml rFel d 1 alone or rFel d 1 with 50 pg/ml LPS. Only at a 3:1 Treg:effector cell ratio, stimulation with rFel d 1 together with LPS resulted in a slightly higher proliferation than stimulation with rFel d 1 without added LPS ($p < 0.01$, fig. 4).

Discussion

Recombinant proteins obtained by expression in Gram-negative bacteria, such as *E. coli*, may contain high amounts of endotoxin, a constitutive component of bacterial cell walls. Thus, recombinant allergens produced in bacteria have to be extensively purified if intended for mechanistic studies of the allergen per se in biological in vivo or in vitro systems. A general protocol for endotoxin removal from proteins is not available. Different approaches include two-step extraction protocols, ion-exchange or affinity chromatography based on polymyxin

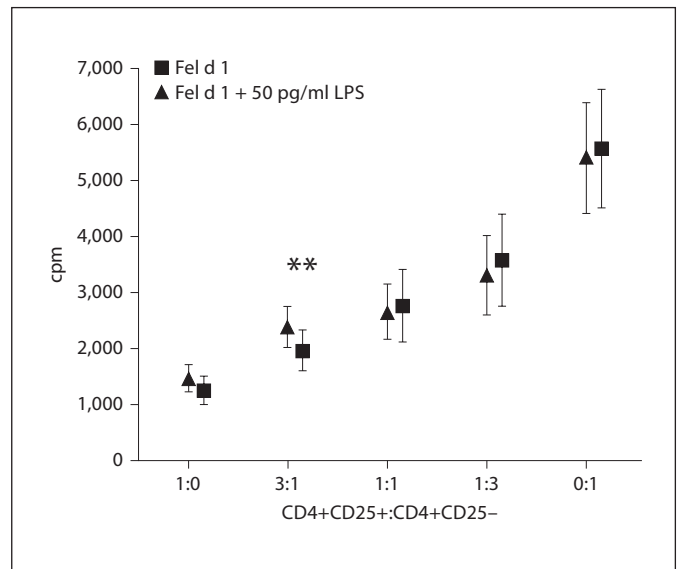


Fig. 4. Effect of LPS on Treg-mediated suppression of rFel d 1-stimulated proliferation in co-cultures of CD4+CD25+ Treg cells and CD4+CD25- effector T cells. A total of 2×10^4 CD4+ cells stimulated with 10 μ g rFel d 1 only (squares) or rFel d 1 + 50 pg/ml LPS (triangles) were cultured at different ratios of CD4+CD25+ and CD4+CD25- cells (1:0, i.e. CD25+ alone, 3:1, 1:1, 1:3 and 0:1 i.e. CD25- alone) in triplicates together with 8×10^4 irradiated antigen-presenting cells. The cells were isolated from 10 subjects IgE-negative to cat and the proliferation (mean $\pm$ SEM) at each CD25+:CD25- ratio was compared between rFel d 1 only and rFel d 1+LPS-stimulated cells. ** $p \leq 0.01$.

B and have been applied with varying success [27, 28]. In order to obtain highly purified rFel d 1, we included an in vitro refolding step to remove endotoxin contaminations and subjected the protein to two consecutive steps of ion-exchange purification. This approach gave a homogenous and essentially endotoxin free preparation of rFel d 1. The different purification strategies involved also ensured that the protein was highly purified from other possible contaminants. Such contaminants may activate T cells, as recently shown for flagellin contaminated recombinant heat shock protein 70 [29].

In our studies we added 50 pg/ml of LPS to PBMC cultures. This dose was chosen on the premises that the LPS contamination in rFel d 1 would not exceed 10% of added LPS in any experiment. Still, 50 pg/ml is a very low dose compared to the endotoxin level in the natural allergen source, cat dander, which contained 1,600-fold higher amounts of endotoxin than the purified rFel d 1. The range of LPS contamination reported for different aller-

gen preparations is quite broad [30]. Similarly, commercially available protein preparations, such as OVA [6] and β -lactoglobulin may contain different amounts of LPS, depending on the manufacturer and the way of preparation [31]. Compared to the varying LPS levels reported in different studies, the endotoxin content of our rFel d 1 preparation and the amount of LPS added to cell cultures in this study can be considered as low doses.

We could demonstrate that addition of LPS enhanced the proliferation in Fel d 1-stimulated PBMC cultures from all subjects, regardless if they had IgE to cat or not. The LPS-stimulated increase in proliferation was mainly additive, since LPS could stimulate proliferation on its own. Both for LPS and Fel d 1-stimulation the proliferation derived from dividing lymphocytes, as demonstrated by the inability of an isolated monocyte cell fraction to proliferate in response to LPS. We also observed that PBMCs from many of the subjects did not respond to stimulation with rFel d 1 alone, in particular at lower allergen concentrations (2 and 10 μ g/ml). In fact, at these lower allergen stimulation concentrations the proliferation was equal to, or even lower than, the proliferation stimulated by LPS alone. The rFel d 1 preparation used for stimulation contained exceptionally low levels of endotoxin, due to the extensive purification. Thus, LPS contamination in allergen preparations normally used for *in vitro* cell stimulation experiments may cause proliferation in cultures that is not a result of allergen stimulation. On the other hand, an additional stimulation by LPS or other TLR ligands may be critical for reaching a threshold for cell activation. In our experiments, LPS was able to decrease the apparent threshold concentration of Fel d 1 able to activate a positive proliferative response. Even though at least part of the proliferation stimulated by 2 and 10 μ g/ml of rFel d 1 in combination with LPS resulted from LPS stimulation, proliferation could be stimulated by 25 μ g/ml rFel d 1 alone in a majority of the PBMC cultures. A similar effect of low-dose LPS (100 pg/ml) on PBMC proliferation to tetanus toxoid-derived peptides has been described [32].

In the present study, the subjects were divided into cat IgE-positive and cat IgE-negative subjects. We could not detect any difference between the groups either in proliferation or cytokine levels in rFel d 1- and LPS-stimulated PBMC cultures. However, for cat-negative subjects both rFel d 1 and rFel d 1 together with LPS specifically stimulated increased amounts of IFN- γ , while IL-5 production was induced by rFel d 1 + LPS in PBMC cultures from cat-positive subjects. Thus, subjects having IgE to cat responded with enhancement of a Th2-cytokine response

and cat-negative subjects with elevated levels of a typical Th1 cytokine. It is likely that PBMCs from cat-positive subjects tend to have a Th2-skewed response and those from cat-negative subjects a more Th1-skewed response to Fel d 1 and our data indicate that the Th1/Th2 profile of the cytokine response is not affected by addition of low-dose LPS. It has been shown that LPS and LPS derivatives are able to induce Th1 responses and IFN γ [33–35]. In our study, LPS alone was able to stimulate production of IFN γ in PBMCs obtained from the cat-negative group. The reason why LPS did not promote IFN γ production in PBMCs from cat-positive subjects could possibly be that the doses of LPS used were too low to overcome a decreased propensity to respond with a Th1 type of response in this group.

Although endotoxin evidently interacts with and exerts many of its effects through cells responding to innate immunity signals, cells normally linked to adaptive immunity have also been proposed as targets for LPS and other TLR ligands. Activated memory T cells can express TLR-2 and TLR-2 ligands, such as bacterial lipoprotein, are able to amplify T cell responses [36]. CD4+CD25+ regulatory T cells have been shown to express TLRs, including TLR-4, and respond to LPS independently of antigen-presenting cells [37]. Therefore, we wanted to investigate if the observed LPS-stimulated enhancement of Fel d 1-specific cell activation was affected by increasing the ratio of a cell fraction enriched for CD4+CD25+ regulatory T cells over a CD4+CD25– cell fraction. We could only detect an effect of LPS at a 3:1 ratio of CD25+:CD25– cell fractions, where rFel d 1 together with LPS induced a slightly higher proliferation than rFel d 1 alone. This might be a result of an effect of LPS on regulatory T cell function. However, since a difference in proliferation could only be detected at a high Treg to effector cell ratio and the difference is probably too small to be biologically relevant, the observed effect of LPS on allergen-stimulated PBMC cultures cannot be explained by a reduced suppressive function of regulatory T cells. A more likely mechanism is that LPS acts on monocytes, as LPS-stimulated activation of T cells has been shown to require cell-to-cell contact with monocytes [5, 38].

In conclusion, we here demonstrate that the activation of allergen-specific T cells is affected by LPS, since LPS has the ability to increase the allergen-stimulated response. Consequently, even low levels of LPS contamination in allergen preparations may obstruct the *in vitro* measurement of cell activation and should be taken in account when evaluating allergen stimulated T cell activation.

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