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ORIGINAL PAPER **Basic Mechanisms in Allergic Disease**

Allergen specific responses in cord and adult blood are differentially modulated in the presence of endotoxins

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Summary

Background Endotoxins are common contaminants in allergen preparations and affect antigen-specific cellular responses. Distinct effects of endotoxin on cells in human umbilical cord and adult blood are poorly defined.

Objectives To examine the effect of endotoxins in allergen preparations on cellular responses in human cord and peripheral blood (PB).

Methods The endotoxin content in β lactoglobulin (BLG), the peanut allergen Ara h 1 and the major birch pollen allergen Bet v 1 was assessed. Proliferation and cytokine response of mononuclear cells towards contaminated and lipopolysaccharide (LPS)-free allergens were evaluated at different time-points. Fractions of contaminated BLG were generated and assayed on their immuno-stimulatory capacity. The involvement of toll-like receptor (TLR) 2 and 4 was investigated by blocking antibodies and TLR-transfected human embryonic kidney cells.

Results The proliferative response of cord blood (CB)-derived mononuclear cells towards allergen-preparations at day 3 was related to the level of LPS contamination. At day 7, proliferation was also detected in the absence of endotoxin. Cytokine production in CB was strongly affected by the content of endotoxin, TLR-4 dependent and not related to the allergen content. Allergen- and endotoxin-induced proliferative responses were generally significantly higher in CB than in adult blood.

Conclusion Endotoxins in allergen preparations confound allergen-specific cellular responses. The impact of these contaminations varies with the blood source (CB vs. PB), the type of allergen and is time- and dose-dependent.

Keywords cord blood, endotoxin contamination, food allergens, T-cells, toll like receptors
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Introduction

Endotoxins in antigen preparations are a potential confounder in *in vitro* cellular assays [1, 2]. Lipopolysaccharide (LPS) contaminations play a role in daily laboratory practice. The milk-derived whey protein β lactoglobulin (BLG) is a prominent food antigen often used as a model allergen. High endotoxin contamination of commercially available formulations has been described [3]. The efficacy of removal procedures is limited. Residual endotoxin often remains present in amounts that might affect lymphoproliferation and cytokine production. LPS in allergen preparations has been shown to modulate cellular responses in animal studies [3, 4]. In these experimental models, the relevance of endotoxin-contamination seemed to vary with the time-point of exposure (prenatal, postnatal, adult animal). In humans, and in particular in cord blood (CB), the effects of endotoxin-contamination on allergen-specific cellular proliferation and cytokine production are poorly defined.

Immunostimulatory effects of LPS-contaminated allergen preparations on cord blood mononuclear cells (CBMCs) are particularly relevant, because large efforts have been made to predict the development of allergy on the basis of *in vitro* allergen-specific proliferation of CBMC [5, 6].

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Numerous reports indicate the presence of fetal T cells responsive to food and inhalant allergens in CB [5–17]. The priming of CB cells has been linked to prenatal antigen transfer [7, 9–12, 14–24]. It seems, however, that CB proliferative responses do not correlate with the amount of maternal allergen exposure [7]. In addition, up to 90% of CB preparations showed proliferation to BLG *in vitro*, which is higher than proliferative responses towards other allergens [11, 14, 19]. Thus, the physiological relevance of CB immune responses and their underlying mechanisms are under debate [25, 26]. It is, however, possible, that LPS in allergen preparations contribute to proliferative responses in CB and confound allergen-specific effects.

The aim of the present study was to examine the impact of endotoxins in commercially available allergen preparations on allergen-specific cellular responses. In particular, we aimed to investigate the differential confounding actions of endotoxins in a 'naïve' (CB) and a mature system (peripheral blood (PB) of adult individuals).

Here we demonstrate that endotoxin contamination in commercially available BLG preparations largely, but not exclusively, causes cellular proliferation and cytokine production in human CB cells. Effects of LPS were toll-like receptor (TLR)-4 dependent. Allergen- and endotoxin-induced proliferative responses were generally significantly higher in CB than in adult blood.

Material and methods

Allergen preparations

Endotoxin levels of the antigen preparations used in this study were quantified by limulus amoeboid lysate assay (Cambrex Bio Science, Walkersville, MD, USA; limit of detection 0.035 EU/mL) according to the manufacturer's instructions.

The following BLG preparations were used: BLG 3908 and L-0130 [BLG (NP), Sigma Sciences, St Louis, MO, USA] were contaminated with endotoxins (515 and 118 EU/mL, Table S1, online repository, >90% BLG). LPS-free BLG [BLG (LF), PSDI-2400, produced from bovine whey protein isolate, Arla Food, Arhus, Denmark, >95% BLG] contained 0.96 EU (Table S1) [3]. Fractions of BLG L-0130 [BLG (NP)] were obtained by size exclusion chromatography (SEC) as described by Brix et al. [3]. Briefly, BLG [NP] was dissolved in ammonium acetate (0.15 M, pH 6.0 at 20 mg/mL) and 5 mL were applied to a Superdex 75 column (Hiload 26/60, AP biotech, Uppsala, Sweden). Elution was carried out using the same buffer at 2.5 mL/min. Fractions of 10 mL were collected and frozen. Before inclusion in cell cultures, 2×1 mL from each fraction was vacuum dried, and twice evaporated with distilled water to remove any residuals of eluting buffer. The effects of all SEC-purified BLG fractions on murine leucocytes have previously been described by Brix et al. [3].

Purified, endotoxin-free Ara h 1, was provided by Dr E. N. Mills, (IFR Norwich, UK, <0.035 EU/mL). Recombinant *Betula verrucosa* allergen, Bet v 1, was purchased (Biomay, Vienna, Austria), and contained 1.96 EU/mL. Tetanus toxoid (tt) was purchased from Calbiochem (San Diego, CA, USA; 0.55 EU/mL).

Cord blood and peripheral blood mononuclear cells

The study protocol was approved by the local Ethics Committee of the Medical University of Vienna. Human umbilical CB from randomly chosen full-term healthy infants (>37 weeks of gestation) was obtained by venopuncture of the umbilical vein immediately after delivery and placed in sterile sodium heparin tubes. Adult volunteers with no history of food allergy and no sensitizations (specific IgE<0,35 kU/L, skin prick test negative) were used as controls. Heparinized blood was drawn over Ficoll-Paque (Pharmacia, Uppsala, Sweden). Mononuclear cells were isolated by density-gradient centrifugation as described [19].

Proliferation assays

Proliferation of CBMCs and peripheral blood mononuclear cells (PBMCs) towards allergens and LPS was determines by ^{3}[H]thymidine incorporation assay. According to the literature, an SI>2 was considered positive [15].

Cytokine measurement

CBMCs were co-cultured with the respective antigen at 5×10^{5}/mL ultra culture complete ultra culture medium (UCC, Biowhittaker, Walkersville, MD, USA) supplemented with 2 mM L-glutamine and 170 mg/L gentamycin sulphate (Sigma Sciences) in 96-well round-bottom plates in triplicates.

Unstimulated cells served as negative control. Supernatants were harvested at days 3 and 7 and frozen at −70 °C in cryotubes. IL-1β, IL-5, IL-10, TNF-α and INF-γ levels were measured by cytometric bead array (Flex Kit, Becton Dickinson, San José, CA, USA) using a FACS Canto (Becton Dickinson) according to the instructions provided by the manufacturer.

Toll-like receptor 2 and toll-like receptor 4 transfected human embryonic kidney cells (HEK293)

HEK293, stably transfected with human CD14 (293-hCD14), CD14 and TLR2 (293-hCD14/TLR2), or CD14 and TLR4 (293-hCD14/TLR4) were kindly provided by Prof. M. Yazdanbakhsh (Leiden University Medical Center, the Netherlands). Cells were cultured in standard Dulbecco's

modified Eaglés medium (DMEM; PAA laboratories GmbH, Pasching, Austria) containing 10% fetal bovine serum, gentamycine sulphate (0.1 μg/mL; Sigma Sciences) puromycin (5 μg/mL, Sigma Sciences) for 24 h. For stimulation experiments, 4×10^4 cells/well were placed in 96-well flat-bottom plates (Costar) in complete medium and co-incubated with the indicated antigens at 37 °C in 5% CO_2 for 4 days. Thereafter cell-free supernatants for cytokine measurements were collected. Untreated cells served as control. For stimulation of 293-hCD14/TLR4 cells, MD2 supernatant (a kind gift of Prof. M. Yazdanbakhsh, Leiden University Medical Center, the Netherlands) was added to the culture medium. The concentration of IL-8 in the supernatant was measured by ELISA (Thermo scientific, Rockford, IL, USA; limit of detection 25.6 pg/mL) according to the manufacturer's instruction.

Toll-like receptor-inhibition assays

Cord blood-derived monocytes were isolated via adherence in 48-well flat-bottom well plates. Cells were pretreated with 1 μg/mL blocking anti-TLR-4 monoclonal mouse anti human antibody or isotype control (IgG2a, all from eBioscience, San Diego, CA, USA) for 1 h, before allergen preparations or LPS (positive control) were added for 6 h. Cell-free supernatant was obtained and stored at −70 °C. TNF-α was measured by ELISA (Bender MedSystems, Vienna, Austria, limit of detection 7.8 pg/mL).

Statistics

Data were analyzed using SPSS 11.0.1 for Windows (SPSS Inc., Chicago, IL, USA). Non-parametric Wilcoxon's signed rank test was applied for matched pairs and Mann–Whitney *U*-test was used for independent samples. When Gaussian distribution was given, the Student *t*-test for independent samples or matched pairs *t*-test were performed. *P*-values of <0.05 were considered significant.

Results

Unpurified antigen preparations of β lactoglobulin confound proliferative and cytokine responses of cord and adult blood-derived mononuclear cells

Using limulus amoeboid lysate assay, we confirmed previous findings of high amounts of endotoxins present in commercially available BLG preparations [3] (online repository, Table S1). Purification via polymyxin B columns substantially reduced the endotoxin content of these preparations, but considerable amounts of endotoxin remained detectable (online repository, Table S1). The major peanut allergen Ara h 1 was endotoxin free. In initial experiments, we determined times of maximum CBMC-proliferation to endotoxin and allergen, respectively, as well as cut-off levels for LPS responses. We found that day 3 reflected endotoxin-related proliferation whereas day 7 most reliably was confined to allergen-specific responses (data not shown). Dose titration experiments with LPS revealed a cut-off level of around 1 ng LPS (∼10 EU) inducing a positive stimulation index >2 and 0.07 ng (∼0.7 EU) inducing TNF-α production above the limit of detection at day 3, and above baseline TNF-α production at day 7 in CB (online repository, Fig. S1).

We next performed dose allergen titration experiments from 0.1 to 50 μg/mL and the purified allergen spiked with the corresponding endotoxin doses to define the differential impact of allergen and endotoxin. At day 3, CBMCs proliferated in response to unpurified, but not to purified BLG [BLG (LF), ($P<0.05$; Wilcoxon's signed rank test, Fig. 1]. At day 7 though, the purified allergen [BLG (LF)] also induced proliferation (Fig. 1). The amount of BLG [NP] proliferation was significantly enhanced at a concentration of 0.1–5 μg/mL and remained borderline significant at a concentration of 10–50 μg/mL ($P=0.063$). Addition of LPS at levels similar to those present in the unpurified BLG [BLG (NP)] to the uncontaminated allergen preparation [BLG (LF)] restored the proliferative response (Fig. 1). Lymphoproliferation towards the endotoxin-free Ara h 1 could equally be spiked by addition of endotoxin (Fig. 1).

The impact of lipopolysaccharide on tetanus toxoid- and Bet v 1-specific proliferation

To control for naturally occurring proliferative responses in vaccinated adult individuals the recall antigen TT was tested. The TT preparation used herein contained also endotoxin, but below the limit of relevance (online repository, Table S1). A dose of 1 μg/mL TT was spiked with increasing levels of endotoxin corresponding to the amount of contamination measured in unpurified BLG. Doing so, a significantly increased proliferative response of PBMCs at day 7 was induced as compared with unspiked TT alone (Fig. 2, red lines).

In order to evaluate the relevance of endotoxin contamination in response to an inhalant allergen, the recombinant major birch pollen allergen Bet v 1 was chosen.

CBMCs and PBMCs of non-allergic donors were stimulated with titrated concentrations of rBet v 1 in the range of 0.1–50 μg/mL. No proliferative responses were detected in CBMCs (Fig. 3a, black line) whereas rBet v 1 induced proliferation in PBMCs from 3/6 donors (Fig. 3a, red line).

However, when PBMCs and CBMCs were stimulated with 10 μg of rBet v 1 and increasing concentrations of LPS, already the presence of 200 pg LPS/mL significantly increased proliferation of CBMCs ($P<0.05$). In PBMCs in contrast, although a trend towards an impact of LPS was observed, added concentrations from 200 pg to 200 ng

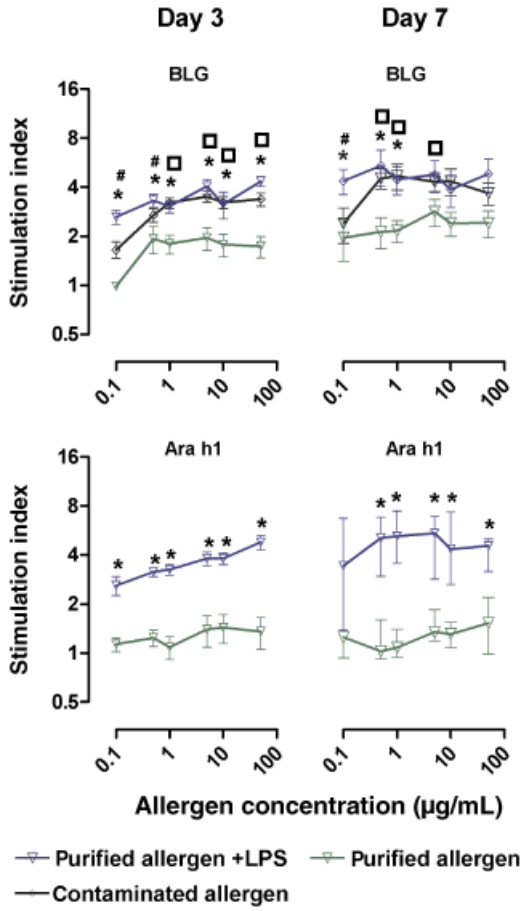

Fig. 1. Food allergen-specific proliferation of cord blood derived mononuclear cells. Cord blood-derived mononuclear cells were co-cultured with different food allergens (BLG, Ara h 1) either in its contaminated form BLG[NP], its purified form [BLG(LP), Ara h 1] or with LPS added according to the degree of contamination present in the respective concentration of the contaminated allergen [BLG(NP)]. (BLG: $n = 13$; mean±SEM; Ara h 1: $n = 7$ mean±SEM; $^{*}P < 0.05$ purified allergen+LPS vs. purified allergen; $^{\#}P < 0.05$ contaminated allergen vs. purified allergen+LPS; □, contaminated allergen vs. purified allergen). BLG, β lactoglobulin; LPS, lipopolysaccharide.

LPS did not cause significant increases in proliferation (Fig. 3b).

The proliferative response of cord blood cells to β lactoglobulin and β lactoglobulin plus endotoxins exceeds the corresponding response of adult blood cells

The proliferative response to the same amount of endotoxins and allergen was compared between cord and peripheral adult blood. In all experiments, CBMCs showed a significantly higher proliferative response to the purified allergen [BLG (LF)], as well as to allergen+LPS [applied in corresponding doses to the *per se* contaminated allergen BLG (NP) (Fig. 4)]. Again, the distinct influence of endotoxin on proliferation was detectable in cord and peripheral adult blood (Fig. 4).

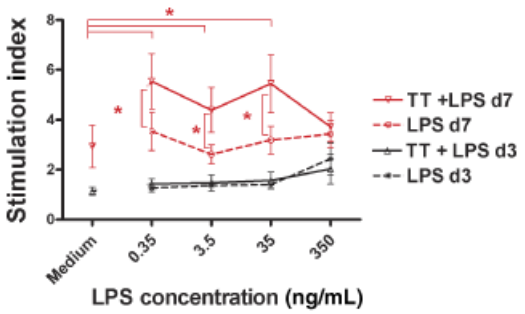

Fig. 2. Low level contaminants affect proliferative response to Th-1 type recall antigens at day seven in adult individuals. Tetanus toxoid was applied at a constant concentration of 1 µg/mL and LPS was added according to the amount present in contaminated BLG [NP]. While low-level contaminants (0.35–35 ng/mL) significantly increased proliferative responses to the antigen at day 7 (red lines) this was not detectable in the presence of higher endotoxin contamination (350 ng/mL). At day 3 (black lines) no effect on the readout parameters was measurable. Graph indicates the mean and the standard error of the mean ($n = 6$). LPS, lipopolysaccharide; Th-1, T-helper type 1; BLG, β lactoglobulin.

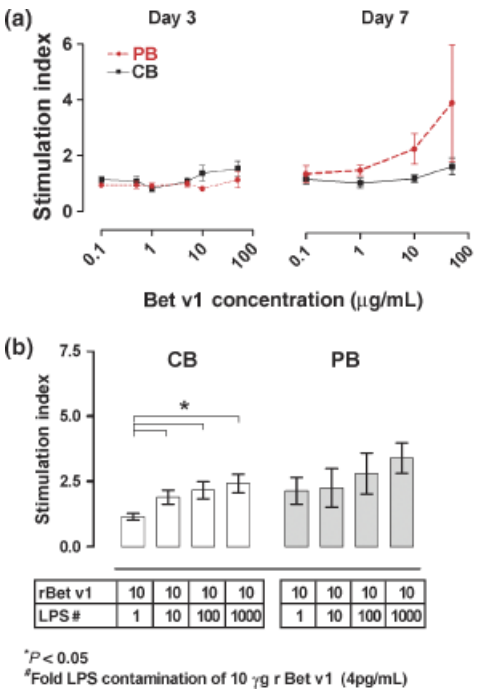

Fig. 3. Allergen-specific proliferation of adult and cord blood derived mononuclear cells to the major birch pollen allergen Bet v 1. Cord blood (CB) and peripheral blood (PB)-derived mononuclear cells were co-cultured for 3 and 7 days with the recombinant major birch pollen allergen Bet v 1 (20 pg endotoxin/10 µg rBet v 1) (a). Three out of six PBMC samples proliferated in the presence of the allergen at a concentration of 10 µg/mL. None of the CB-derived samples did so (0/6). Contamination at the relevant concentration of 10 µg/mL did not affect proliferation, neither in CB nor in adult blood (b). ($n = 6$ independent experiments CB and PB; results are expressed as mean±SEM). PBMCs, peripheral blood mononuclear cells.

Immunomodulatory properties of unpurified β lactoglobulin relate to high molecular weight fractions and act via a toll-like receptor 4 dependent mechanism

To determine the size of immuno-reactive components within the contaminated BLG samples [BLG (NP)], SEC was performed and 18 sub-fractions were sampled. At day 3, only the high molecular weight fraction, likely to represent the endotoxin-fraction (F1, F2), induced a positive proliferative response (Fig. 5a, upper panel), whereas at day 7 the BLG-corresponding fraction (F5 and F6, putatively representing BLG dimers) also induced clonal expansion (Fig. 5a, lower panel). This finding is in accordance with our previous experiments. Extremely low amounts of LPS-induced positive proliferative responses (Fig. 5b). Assuming a BLG-content of more than 90%, an at least ~100-fold higher potency can be attributed to the high molecular weight, endotoxin-contaminated fraction.

Purified BLG (LF) and the BLG-corresponding fractions (F5, F6) did not induce substantial cytokine production (online repository, Fig. S1). In contrast, the contaminated BLG-stimulated cytokine production at very low doses suggesting an innate immunity driven response corresponding to the LPS-titration results.

To prove the involvement of the most prominent receptors of endotoxins and to delineate whether LPS is mainly responsible for the described effects, TLR2 and TLR4 transfected HEK-cells were generated. Contaminated BLG initiated a robust IL-8 response via a TLR4 dependent mechanism (Fig. 6a) but had no effect in TLR2 transfected cells (Fig. 6b). To further investigate the relevance of TLR-4 in an *in vitro* system with all innate immunity receptors co-expressed, inhibition assays with anti-TLR4 antibodies were performed. Ultra pure LPS (S. minnesota; stem

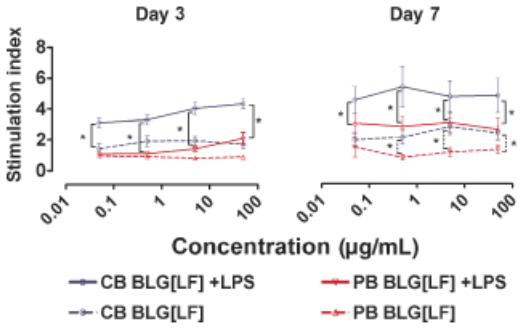

Fig. 4. Proliferative responses to allergen and allergen plus endotoxin are significantly higher in cord blood (CB) than in peripheral blood (PB) from adult donors. The impact of endotoxin contamination is significantly stronger in CB as compared with PB from adult donors (CB: $n = 17$; PB: $n = 10$). Graph indicates the mean and the standard error of the mean. BLG, β lactoglobulin;LPS, lipopolysaccharide.

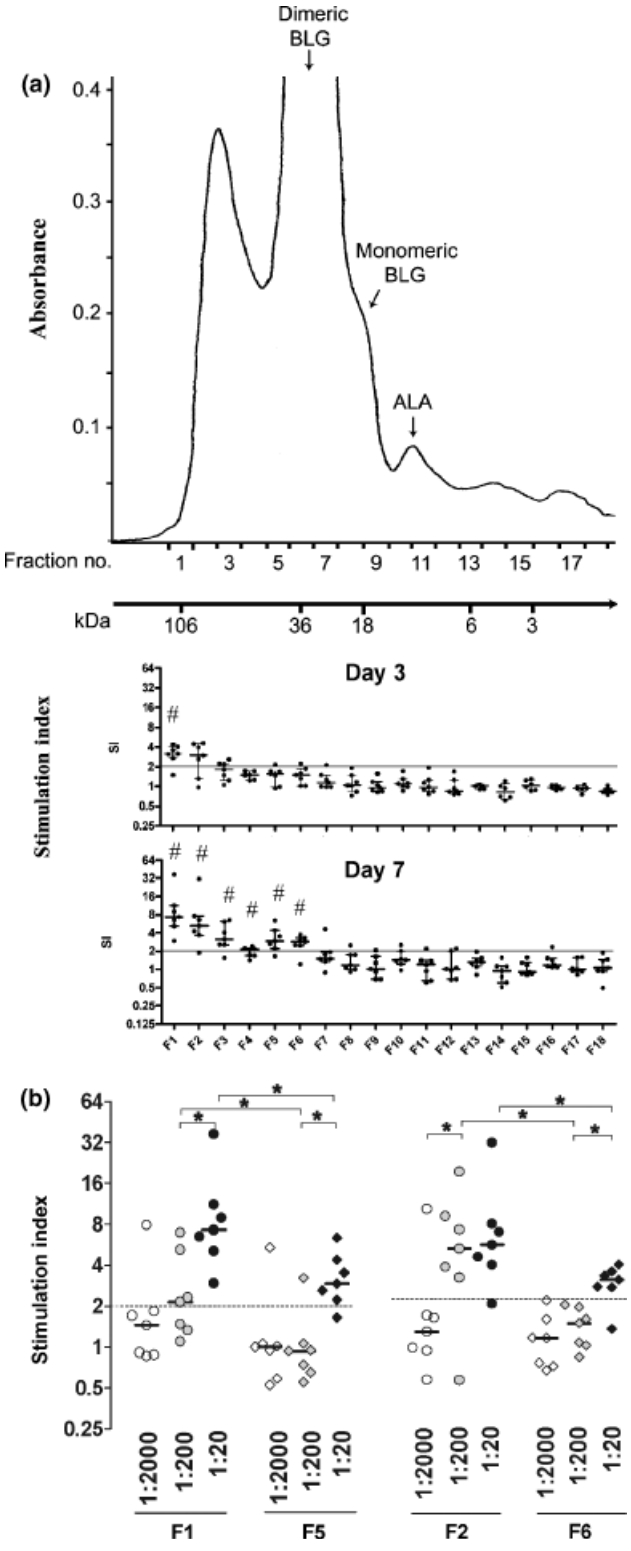

Fig. 5. High molecular weight fragments from unpurified BLG led to BLG independent proliferation. High molecular weight fragments isolated by size exclusion chromatography led to a BLG-independent proliferative response of CBMCs (a; $^{\#}P < 0.05$; Wilcoxon's signed rank test). Dose response experiments of high molecular contaminants and dimeric BLG revealed, despite of >90% purity of the BLG preparation, a more than 10-times higher potency of the contaminants (b). Dots represent data from seven independent experiments ($^{*}P < 0.05$; Wilcoxon's signed rank test). BLG, β lactoglobulin; CBMCs, cord blood mononuclear cells.

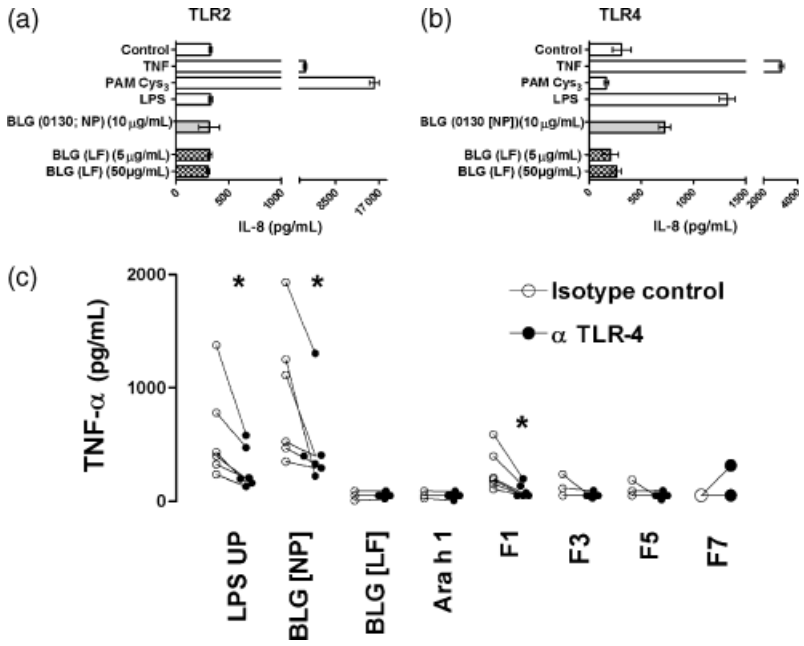

Fig. 6. BLG related release of pro-inflammatory cytokines is induced by high molecular contaminants and TLR 4 dependent. Human embryonic kidney cells (HEK293), stably transfected with an expression vector for human CD14 and TLR4 (293-hCD14/TLR4) and CD14 and TLR2 (293-hCD14/TLR2), were stimulated with the indicated preparations. The concentration of IL-8 in the supernatant was used as the read-out parameter and measured by ELISA (Endogene Thermo Scientific, IL, USA) according to the manufacturer's instructions ($n = 4$, two independent experiments; a and b). The relevance of this TLR 4 mediated effect in an *in vitro* cord blood system with all innate immunity receptors co-expressed was supported by inhibition assays with anti-TLR4 antibodies. Initially TLR 4- antibody or the respective isotype control [IgG2a, (1 μg/mL)] was added for 1 h. Thereafter allergens (BLG [LF], Ara h1, BLG [NP] and fractions from the size exclusion experiments, see Fig. 5) and the positive control [LPS ultra pure, Salmonella minnesota R 595 (0.1 μg/mL)] were added. TNF-α production was measured in the supernatant. Dots represent data from six independent experiments. Sub-fractions generated via size exclusion chromatography were applied. The high molecular weight fraction (F1; c) acted via TLR4 while the putative BLG-dimer (F5) did not induce TNF-α production (c). BLG, β lactoglobulin; HEK, human embryonic kidney; LPS, lipopolysaccharide; TLR, toll-like receptor.

R595), BLG[NP], BLG[LF], and BLG sub-fractions generated via SEC were tested. Ultra-pure LPS-induced TNF-α production was substantially inhibited by TLR4 blockade (Fig. 6c). No TNF-α production was induced by BLG [LF]. The high molecular weight fraction (F1; Fig. 6c) acted via TLR4 while the putative BLG-dimer (F5) did not induce TNF-α production (Fig. 6c).

Discussion

This study demonstrates that endotoxin in commercially available antigen preparations strongly affects *in vitro* cellular parameters. Endotoxins significantly increased proliferative responses and cytokine production of mononuclear cells towards BLG and the major birch pollen allergen Bet v 1. Comparable effects were detected when LPS was added to the more T-helper type 1-type recall antigen tt. LPS-contamination of the model allergen BLG led to TLR4 engagement. We further observed that CB-derived mononuclear cells were more sensitive than mononuclear cells from adults in terms of proliferative responses to both, purified allergens and allergen plus endotoxins.

Brix et al. proved in mice that the endotoxin fraction of contaminated BLG is more important in inducing proliferation of naïve T cells than the allergen fraction itself [3]. Human CB mainly harbours naïve T cells and therefore represents a perfect model system to assess the impact of endotoxins in BLG on naïve human cells. Its high frequency of recent thymic emigrants has been linked to this observation. It has been hypothesized that this might play a role in the immune defense of the neonate. However, human mononuclear cells are known to be less sensitive to endotoxin. Therefore, in human *in vitro* models, the relevance of endotoxin as a confounder could be less significant than in mice.

There are numerous potential confounders that could be responsible for the measured effects. LPS was an attractive candidate for a microbial contaminant, because high amounts were detected in the BLG fraction by limulus assay. In addition, we could prove its ability to induce proliferation and cytokine production by spiking allergen preparations with LPS. LPS is also known to be one of the naturally occurring ligands of TLR-4, and we could show that the contaminating fraction (Fig. 6b) binds to TLR-4 and that exposure to the contaminated BLG is of relevance also in the presence of different pathogen recognition receptors via the application of a TLR-4 blocking antibody (Fig. 6c). Nevertheless, the presence of additional biasing factors, such as endotoxins from Gram-positive bacteria

or subtle differences in the allergen preparations that are not LPS-related cannot be excluded. However, in our setting, LPS was the main contaminant within the BLG-preparations. This has been confirmed by the spiking experiments and by the TLR-4 inhibition experiments.

Importantly, purified BLG was capable of inducing proliferation in CB cells and led to a high percentage of positive responses comparable to previously published data [14, 15, 25, 27]. This strengthens the hypothesis that the measured proliferation in CB is also induced by the allergen itself, thus reflecting allergen specificity.

None of the investigated CB samples responded to rBet v 1. This strongly argues for a tolerable degree of contamination in case of Bet v 1 because the CB related responses to LPS are easier to induce. Regarding allergen specific response to inhalant allergens in CB a possible explanation could be that intra-uterine priming rather occurs with food allergens than with inhalant allergens. Food allergens are present at high concentrations in maternal PB and can cross the placenta [20]. The amount of inhaled birch pollen allergens by contrast is considered rather low and makes intrauterine priming less likely. This also helps to understand why the time-point of birth (within or outside the pollen season) does not correlate with the rate of proliferative response in CB [7]. On the other hand, recent studies have clearly pointed out that allergen-specific proliferation to inhalant allergen does also occur in CB but at lower frequencies [25, 27]. The sample size used in our study could have been too small to detect birch pollen specific proliferation.

In PBMCs from adult non-allergic donors in contrast, little or no proliferation in response to the purified BLG was measurable. Endotoxin-related effects occurred at a later time-point and were less prominent. However this impact is also considerable in case of recall responses as demonstrated for the spiking experiment with the tt.

Recombinant allergens and antigens display an important feature of immunological research. Although endotoxin-free conditions are very difficult to achieve due to the expression system in *Escherichia coli*, preparations with low levels of LPS are available. This is the case for rBet v 1 used in the present publication. Our data suggest that contaminations up to 20 pg/mL as measured in purified recombinant allergen preparations are acceptable because they did not affect proliferative responses significantly. While in our study no proliferative response to rBet v 1 was observed in CB, three out of six non-allergic adults responded to rBet v 1. This observation, however, is in line with data of Ebner et al. [28] demonstrating allergen-specific proliferation of PBMC also from non-allergic individuals .

Our data highlight the difficulty of differentiating between allergen-specific and unspecific responses in *in vitro* cellular assays. The effects highly depend on the cellular system (cord vs. adult blood cells) and the read out assay (proliferation, cytokine production). While CB-derived mononuclear cells readily respond at day 3 to both contamination and antigen at very low levels, this is not the case in PBMCs from adult donors. Consequently, proliferation data should be interpreted with caution, especially concerning positive responses of CMBC to low antigen concentrations.

In conclusion, we were able to demonstrate that endotoxins in commercially available allergen preparations act as a confounder on cellular responses via a TLR4-dependent mechanism. The impact of these contaminants on antigen-specific proliferation are dose- and time-dependent, as well as dependent on the type of allergen used and the regulatory properties related to the maturity of the donor system.

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Supporting Information

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

Table S1. Endotoxin contamination of used materials.
Fig. S1. Dose response in cord blood.
Fig. S2. Endotoxin in BLG preparations induces upregulation of the cytokines TNF-α, IL-1β, INF-γ, IL-10 and IL-5.

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