

ORIGINAL ARTICLE

Children with atopic histories exhibit impaired lipopolysaccharide-induced Toll-like receptor-4 signalling in peripheral monocytes

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Summary

Background The hygiene hypothesis states that early exposure to bacterial products such as lipopolysaccharide (LPS) may be protective against the development of allergic diseases. Whether atopic disease affects the ability of immune cells to respond to LPS is unclear. Our laboratory has demonstrated previously that children express high levels of Toll-like receptor (TLR)-4 on CD4⁺ cells in nasal mucosa.

Objective To determine if children with a history of allergic disease have impaired responses to LPS on circulating CD4⁺ leucocytes.

Methods Peripheral blood mononuclear cells from children (aged 2–18) and adults with or without a history of atopic conditions were cultured with/without IL-4 or LPS for up to 24 h. Expression of surface TLR-4, CD14, CD4, CD3, as well as of intracellular phosphorylated p^{42/44}ERK and p38 mitogen-activated protein kinase (MAPK) were assessed by flow cytometry.

Results A history of atopy in children was associated with impaired LPS-induced TLR-4-dependent phosphorylation of p^{42/44}ERK and p38 MAPK by CD4⁺ monocytes. Decreased LPS signalling was reproduced by pre-incubation of control cells with recombinant IL-4. LPS stimulation also decreased TLR-4 expression on monocytes from children without atopic histories but not from atopic subjects. CD4⁺ T lymphocytes showed limited LPS responsiveness, regardless of atopic status. In contrast with non-atopic children, TLR-4 expression on monocytes of children with atopic histories decreased as a function of age. **Conclusions** This study provides evidence for defective LPS recognition on circulating CD4⁺ leucocytes of subjects with atopic histories compared with those from non-atopic children. CD4⁺TLR4⁺ monocytes from children with atopic histories failed to phosphorylate MAPKs. Our results suggest that a history of atopic disease is associated with impaired TLR-4-mediated innate immune function compared with non-atopic children.

Keywords CD4, lipopolysaccharide, lymphocytes, monocytes, pediatric atopy, signal transduction, Toll-like receptor-4

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Introduction

The hygiene hypothesis has been used to explain the increasing prevalence of allergic disorders. Extensive epidemiologic data from multiple European and North American studies suggest that exposure to microbial products modifies immune responses in children against developing allergic diseases [1]. Decreased atopy in rural areas has been correlated with exposure to farm animals [2, 3], unpasteurized milk products [4], multiple household pets [5] and bacterial endotoxin or lipopolysaccharide (LPS) [6]. How bacterial endotoxin modifies immune responses in childhood is an area of intense investigation.

Few studies have investigated how allergic diseases and Th2-type microenvironment affect immune cells' ability to respond to endotoxin.

Recognition of bacterial endotoxins is largely mediated via Toll-like receptors (TLR). TLR are hallmarks of the innate immune system, facilitating a rapid recognition of common infectious patterns encountered by higher organisms (via pattern-associated molecular patterns) [7]. TLR-4 recognizes LPS, and is part of a key signalling complex comprising CD14, MD-2 and intracellular components which initiate protective immune responses against bacterial invaders [8]. In mucosal tissues, LPS' targets are assumed to be mainly innate immune system

cells such as macrophages, dendritic cells and epithelial cells. TLRs are also expressed by structural cells, and to a lesser extent on cells of the adaptive immune system, including B- and T lymphocytes [9, 10]. In the periphery, monocytes are commonly used to study LPS effects as they constitutively express the TLR-4 complex [11]. Upon binding, LPS and TLR-4 complexes are rapidly internalized [12] and activate cells through a variety of signal transduction pathways including the nuclear factor of kappa B (NF- κ B), mitogen-activated protein kinase (MAPK) p38 and extracellular signal-regulated kinase (ERK) 1/2 ($p^{42/p44}$ ERK) [8, 13]. LPS was shown to sequentially induce the monocyte production of pro-inflammatory (TNF- α , IL-1 β , IL-6, IL-8, etc.) and anti-inflammatory mediators (IL-1Ra, IL-10, etc.) [14, 15]. While exposures to high concentrations of bacterial LPS induce detrimental levels of pro-inflammatory mediators, exposure to low-level LPS, especially early in life, may provide immunoregulatory effects [16].

Beside epidemiological evidence supporting the hygiene hypothesis, the role of LPS in the development of allergic diseases remains a matter of controversy. While exposure of newborn mice to low levels of LPS abolished Th2 responses and bronchial hyperresponsiveness to inhaled allergens in an asthma model [17], studies in mature mice yielded conflicting results [18–22]. Previous work demonstrated that LPS down-regulated allergen-induced Th2-type responses in explanted nasal mucosa from children, but not adults [23], reflecting that age affects LPS responsiveness [24]. Studies also reported differences in the LPS-induced cytokine production and signalling pathway activation by blood mononuclear cells based on maternal atopy [25–28], and by peripheral blood mononuclear cells (PBMC) from asthmatic and non-asthmatic adults [29, 30]. Although a difference in LPS-mediated responses may reflect a differential TLR-4 complex expression/function in atopy, few studies have investigated whether atopic status and/or Th2-type microenvironment affect the way LPS activates TLR-4-expressing peripheral leucocytes of children. In this study, we determined whether a history of atopic disease affects the ability of CD4-expressing PBMC, namely T 'helper' lymphocytes and monocytes [31] to respond to LPS.

Materials and methods

Study subjects

Subjects were recruited from the Pediatric Test Center of the Montreal Children's Hospital. After informed, written consent was obtained, each subject had 2–5 cm³ of whole blood procured in heparinized tubes in the context of other clinical testing. Each subject answered a short screening questionnaire to ascertain the presence of a personal or family history of allergic conditions [including asthma, eczema (atopic dermatitis), food allergies,

aeroallergen reactivity and rhinitis], based on International Study of Asthma and Allergic Disease in Childhood (ISAAC) questionnaires [32]. The study was approved by the Research Ethics Board of the Montreal Children's Hospital, McGill University Health Center. A total of 376 patients (ages 2–45) were recruited.

Peripheral blood mononuclear cells isolation and culture

PBMCs were isolated by Ficoll–Paque centrifugation (GE Healthcare, Baie D'Urfé, QC, Canada). Cells were maintained in RPMI medium: RPMI 1640 (Invitrogen, San Diego, CA, USA), supplemented with 10% v/v heat-inactivated foetal-bovine serum (FBS; HyClone, Logan, UT, USA), 4 mM L-glutamine, 50 U/mL penicillin G, 50 μ g/mL streptomycin, 1 mmol/L sodium pyruvate and 1.5% of 1 M HEPES buffer, incubated at 37 °C and 5% CO₂. Before flow cytometry staining and analysis, cells were incubated with or without recombinant human IL-4 (13.5 ng/mL; Mediatech, Montreal, QC, Canada) or LPS from *Salmonella minnesota* R595 (from 0.05 to 5 μ g/mL; Alexis Biochemicals, San Diego, CA, USA) for up to 24 h.

Flow cytometry cell staining of peripheral blood mononuclear cells

PBMC were stained for the presence of cell surface TLR-4 (using AlexaFluor 488-conjugated mouse anti-human IgG_{2a}, clone HTA125, Serotec, Cedarlane, Burlington, ON, Canada), CD14 (phycoerythrin-conjugated mouse IgG_{2a}, clone M5E2, BioLegend, San Diego, CA, USA), CD4 (allophycocyanin-conjugated mouse IgG₁, clone RPA-T4, BioLegend), as well as CD3 (FITC-conjugated mouse IgG₁, clone UCHT1, Becton Dickinson, Pharmingen, Toronto, ON, Canada) or corresponding isotype control antibodies. Before staining, PBMC were pre-incubated with intravenous immune globulin (20 μ g/mL) to diminish non-specific FC receptor binding. Ten thousand events were acquired by flow cytometry (FacsCalibur, BD-Biosciences, Toronto, ON, Canada) and analysed using CellQuestTM Pro software version 4.0.2 (BD-Biosciences). Cell viability uniformly remained above 90% in all experiments. Data are presented as percentages of stained cells within gated cell populations, and/or as median fluorescence intensity values. In all cases, the percentage of cells stained with isotype-control antibodies was subtracted from that of antigen-specific antibodies.

Detection of phospho- $p^{42/44}$ ERK and -p38 mitogen-activated protein kinase

PBMC from age-matched children with or without atopic histories were incubated for 1 h in FBS-depleted complete RPMI with or without IL-4 (13.5 ng/mL) or human TLR-4 (5 μ g/mL, mouse anti-human IgG_{2a}, clone HTA125, eBioscience, San Diego, CA, USA) or IL-4 neutralizing

antibody (68 ng/mL, mouse IgG₁, clone AVU02, R&D Systems, Minneapolis, MN, USA) or corresponding isotype control antibodies. Cells were then stimulated with LPS (0.25 µg/mL) for 1–60 min. Cells were stained for CD4 before fixation in 4% formaldehyde (Polysciences Inc., Warrington, PA, USA) and permeabilization in 100% cold methanol (Fisher Scientific, Montreal, QC, Canada). Cells were incubated with rabbit IgG antibodies against human phosphorylated ^{p42/44}ERK (Thr202/Tyr204) or phospho-p38 MAPK (Thr180/Tyr182) for 15 min at room temperature (Cell Signaling Technology, Boston, MA, USA). MAPK antibodies were detected using FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA). Data were acquired by flow cytometry.

Statistical analysis

Appropriate parametric or non-parametric one-way ANOVAS (with Bonferroni's or Kruskal–Wallis tests, respectively), followed by *post hoc* tests (Tukey's or Dunn's multiple comparison tests, respectively) were used to analyse all phenotyping data sets. For analysing LPS-induced signaling data, non-parametric one-way ANOVAS/Friedman tests followed by Dunn's tests were performed. Data are representative of the indicated number of subjects (*n*). Appropriate *t*-tests were used when comparing two groups. Spearman's rank tests were conducted for correlation analyses. Values are presented as means ± SEM. *P*-values < 0.05 were considered significant.

Results

Discriminating between CD4-expressing monocytes and T 'helper' lymphocytes

To study the LPS responsiveness of circulating CD4⁺ leucocytes, we studied a sample of children aged 2–18, and adult volunteers (18–45). The demographics and histories of atopic disease are presented in Table 1. Using flow cytometry, we discriminated different CD4-expressing cell populations present in PBMC. Among CD4-expressing cells, the CD4⁺Low FSC/SSC large population

incorporated CD14⁺ monocytes, but few CD3⁺ T lymphocytes (Figs 1a, b, d and e). In comparison, 40% to 55% of the FSC/SSC small lymphocytes (Fig. 1a) consisted of CD4⁺High-expressing T 'helper' lymphocytes as they co-expressed CD3 (Figs 1c and e). We also detected approximately 5% of the large FSC/SSC cells that co-expressed CD4⁺High (Fig. 1b) and CD3, which were analysed as T lymphocytes. CD14-expressing monocytes were uniformly TLR-4⁺ (≥ 85%) and predominantly (> 95%) detected in the CD4⁺Low subset (Figs 1d and f). In contrast, a small percentage of the CD3⁺CD4⁺High T 'helper' lymphocytes also co-expressed CD14 and TLR-4 whereas < 1% of CD4⁺ cells were CD14⁺ (data not shown).

A history of atopy impairs lipopolysaccharide-mediated signal transduction in CD4⁺ cells

We determined whether the TLR-4 complex on peripheral CD4⁺ cells was able to respond (*in vitro*) to LPS by phosphorylation of p38 MAPK (Fig. 2) and ^{p42/44}ERK (Fig. 3). Stimulation with LPS induced rapid and consistent increases in phosphorylated p38 MAPK in CD4⁺Low monocytes of children with no atopic history (Fig. 1a, left graph). Significantly elevated levels of phosphorylated p38 MAPK were observed within 5 min after LPS exposure. In contrast, limited or no phosphorylation was observed among the CD4⁺High T 'helper' cells (Fig. 2b, left graphs, respectively). Surprisingly, there was a clear difference in the LPS-induced p38 MAPK phosphorylation in cells from children with histories of atopic conditions compared with those from non-atopic children (Figs 2a and b, right vs. left graphs). In contrast with non-atopic controls, CD4⁺Low cells from individuals with atopic histories appeared relatively insensitive to the effects of LPS.

The difference in LPS-induced p38 MAPK phosphorylation between monocytes of non-atopic and atopic children was confirmed through an assessment of ^{p42/44}ERK phosphorylation (Fig. 3). CD4⁺Low monocytes from non-atopic children exhibited significantly increased intracellular phosphorylated ^{p42/44}ERK from 1–5 min after LPS exposure, compared with monocytes from individuals with atopic histories (Fig. 3a, left vs. right graph). LPS failed to increase the phosphorylation of ^{p42/44}ERK in CD4⁺High

Table 1. Characterization of the subject's atopic status and demographics

Atopic status	Atopic subjects				Non-atopic subjects			
	2–6	7–12	13–18	> 18	2–6	7–12	13–18	> 18
Age	2–6	7–12	13–18	> 18	2–6	7–12	13–18	> 18
Mean age (±SD)	4±1.4	10±1.7	15.1±1.4	24.9±10.3	4.1±1.6	10±1.8	15.3±1.3	24.2±9.2
Number of subjects	25	49	77	23	39	63	86	14
History of allergies (% of subjects with diagnosed allergic disease)								
Asthma	28	39	47	44	0	0	0	0
Eczema	28	16	25	26	8	16	14	0
Food allergies	52	33	26	35	0	0	0	0
Aeroallergen reactivity	28	55	60	70	0	0	0	0
Multiple allergies	28	41	34	52	0	0	0	0

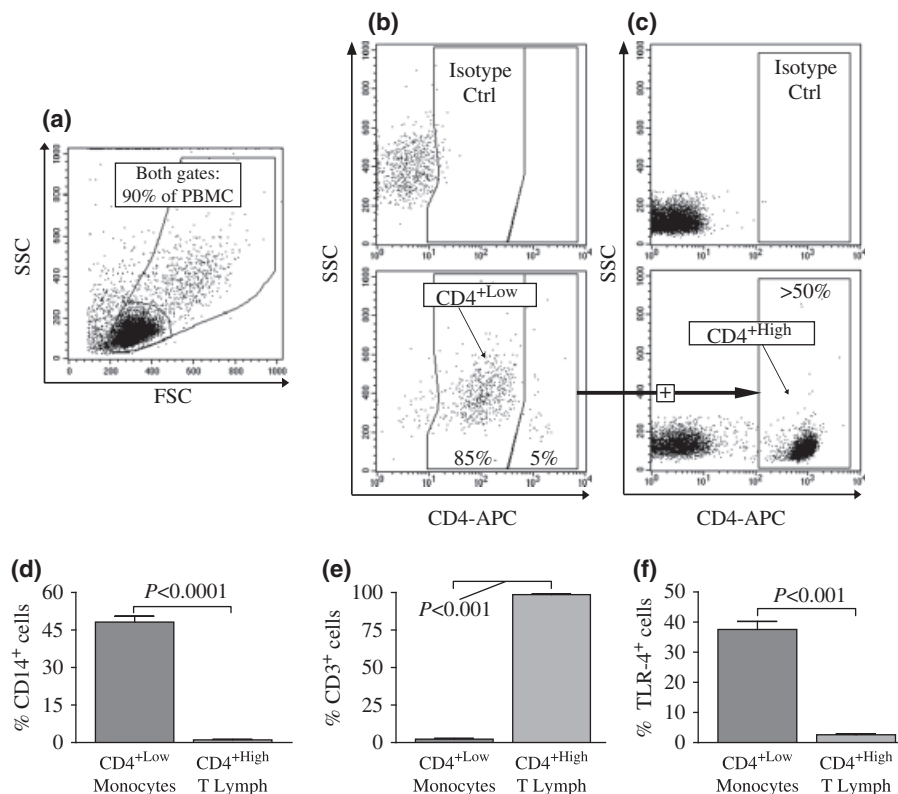


Fig. 1. Characterization of circulating TLR-4⁺CD4-expressing cell populations: monocytes and T 'helper' lymphocytes. Peripheral blood mononuclear cells (PBMC) were analysed by flow cytometry for the surface expression of CD4, CD14, CD3 and Toll-like receptor (TLR)-4. Representative FSC/SSC dot plot for PBMC, with gates around two populations (a). CD4 staining/SSC dot plot of monocytes (b) and lymphocytes (c) identified as CD4⁺Low and CD4⁺High cells, respectively. (d) Relative percentage of CD14⁺ cells ($n = 22$), (e) CD3⁺ ($n = 4$) and (f) TLR-4⁺ cells ($n = 61$) among the CD4⁺ cell populations.

T lymphocytes, regardless of status (Fig. 3b). Furthermore, culturing PBMC from non-atopic children for 1 h with IL-4-supplemented medium markedly attenuated LPS-induced $p^{42/44}$ ERK phosphorylation (Fig. 3c). This effect was specific because no reduction in $p^{42/44}$ ERK phosphorylation was observed when recombinant IL-4 was pre-incubated with IL-4 neutralizing antibody (data not shown). Culturing cells from subjects with atopic conditions with exogenous anti-IL-4 antibodies did not reverse the impaired LPS-induced phosphorylation (data not shown). The LPS-mediated effect on $p^{42/44}$ ERK phosphorylation in non-atopic monocytes was TLR-4-dependent because it was inhibited by pre-incubation with TLR-4 neutralizing antibody (Fig. 3d), not by an isotype antibody (data not shown). Taken together, these results demonstrate that LPS can activate at least two major signal transduction pathways in peripheral monocytes from non-atopic, but not in cells from children with a history of atopy.

A history of atopy is associated with impaired lipopolysaccharide-induced decreases in Toll-like receptor-4 expression

It was reported previously that LPS recognition and response induce rapid internalization of both LPS and

TLR-4 [12]. We therefore assessed whether LPS could differentially affect TLR-4 expression present at the surface of CD4⁺ leucocytes from children with and without a history of atopy. Interestingly, LPS induced a stable and consistent decrease in TLR-4 expression only on monocytes from non-atopic donors compared with monocytes from children with atopic histories (Figs 4a vs. b), regardless of the LPS dose. The LPS-mediated TLR-4 decrease was prolonged for 72 h without affecting cell viability, and was accompanied by a down-regulation of TLR-4 mRNA (data not shown). Peripheral CD4⁺Low monocytes from subjects without an atopic history displayed an increased TLR-4 expression and LPS-induced signalling compared with T lymphocytes (Figs 1f, 2a and b, respectively), and were also the only cells to show significantly reduced TLR-4 expression upon LPS exposure ($P < 0.05$; data not shown). These data support the notion that leucocytes from children with histories of atopic conditions display defective LPS responsiveness.

Lipopolysaccharide unresponsiveness is not due to variability in Toll-like receptor-4/CD14 expression

In an attempt to explain the differences seen in LPS responsiveness between monocytes from children with/

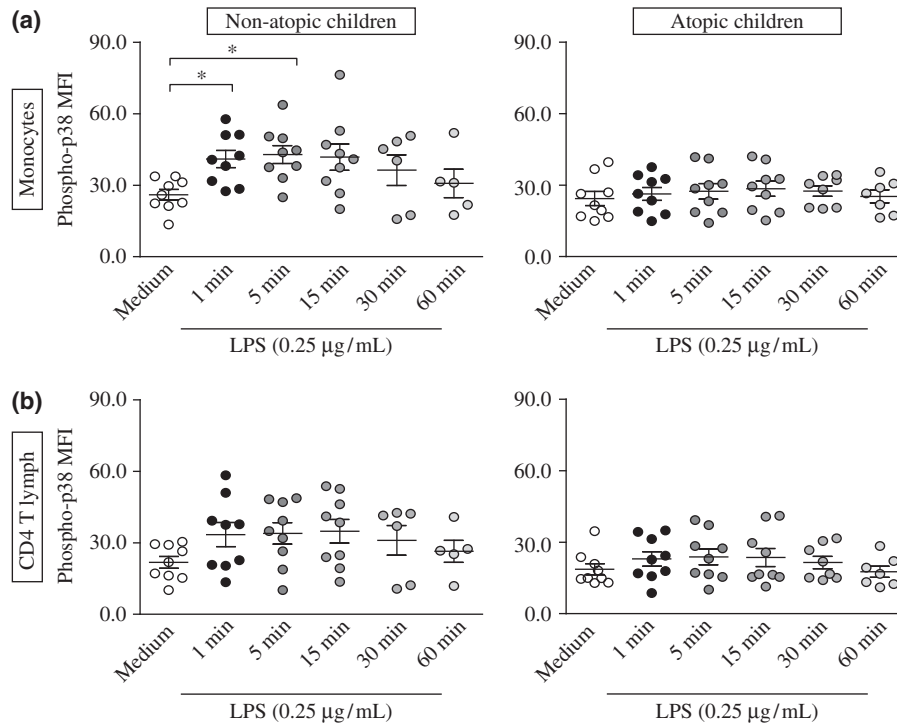


Fig. 2. Lipopolysaccharide (LPS)-induced signal transduction via p38 is impaired in CD4⁺ cells from children with atopic histories compared with those from non-atopic children. Cultured peripheral blood mononuclear cells were stimulated with 0.25 µg/mL LPS for 1–60 min, and then stained for CD4 and intracellular phospho-p38 mitogen-activated protein kinase and analyzed by flow cytometry. Data are presented as median fluorescence intensity (MFI) of phospho-p38 MAPK detected in (a) CD4⁺ monocytes and (b) T 'helper' lymphocytes from both non-atopic (left graphs; $n = 9$; age: 14.0 ± 5.5) and children with atopic histories (right graphs; $n = 9$; age: 13.9 ± 9.5) * $P < 0.05$.

without atopic histories, we surveyed our paediatric cohort for the expression of surface TLR-4 on CD4-expressing leucocytes and compared it with adults (e.g. ≥ 18 years old). To account for variations due to age, we stratified samples into three age groups (e.g. 2–6, 7–12 and 13–17 years old) as well as by atopic history. As depicted in Fig. 5, we did not observe significant variation in TLR-4 expression in either monocytes (Fig. 5a) or T 'helper' lymphocytes (Fig. 5b), regardless of age, atopic history or IL-4 stimulation, compared with adults. We then evaluated the association between TLR-4 expression in CD4⁺ leucocytes and the age of the subjects through correlation analyses in atopic and non-atopic children, separately. TLR-4 expression on monocytes was inversely correlated with age in subjects with atopic histories, whereas TLR-4 expression on monocytes from non-atopic children did not vary throughout childhood (Fig. S1). In contrast, TLR-4 expression on T 'helper' lymphocytes from children with an atopic history increased significantly with age, whereas no significant increase with age was observed in non-atopics. Finally, we looked at surface CD14 expression on CD4⁺ leucocytes, because CD14 is mandatory for proper TLR-4-mediated signal transduction. Similar to TLR-4, there was no difference between monocyte CD14 expression in children with atopic histories compared with non-atopic children, at baseline

(Fig. 6). However, adding IL-4 in the culture significantly down-regulated the CD14 expression on monocytes. Taken together, these data regarding expression of TLR-4 and CD14 in monocytes enabled us to conclude that impaired LPS-induced signalling in cells from children with a history of atopic disease is not primarily due to variability in TLR-4 complex expression, but may be due to the influence of IL-4 on CD14 expression.

Discussion

The role of LPS in the development and manifestation of allergic diseases remains controversial, and the focus of extensive research efforts. Numerous studies supportive of the hygiene hypothesis suggested exposure to bacterial endotoxin e.g. LPS can be protective against allergen sensitization and the development of the allergic phenotype, whereas other studies reported LPS worsens inflammation and causes exacerbations in asthmatic individuals. However, few studies have investigated whether a history of Th2-type disorders in infancy affect the ability of the developing immune system to sense bacterial LPS. In this study, we evaluated a cohort of children and demonstrated that atopic status is associated with reduced LPS responsiveness in monocytes, as determined by the impaired phosphorylation of MAPK pathways and the impaired decrease in TLR-4

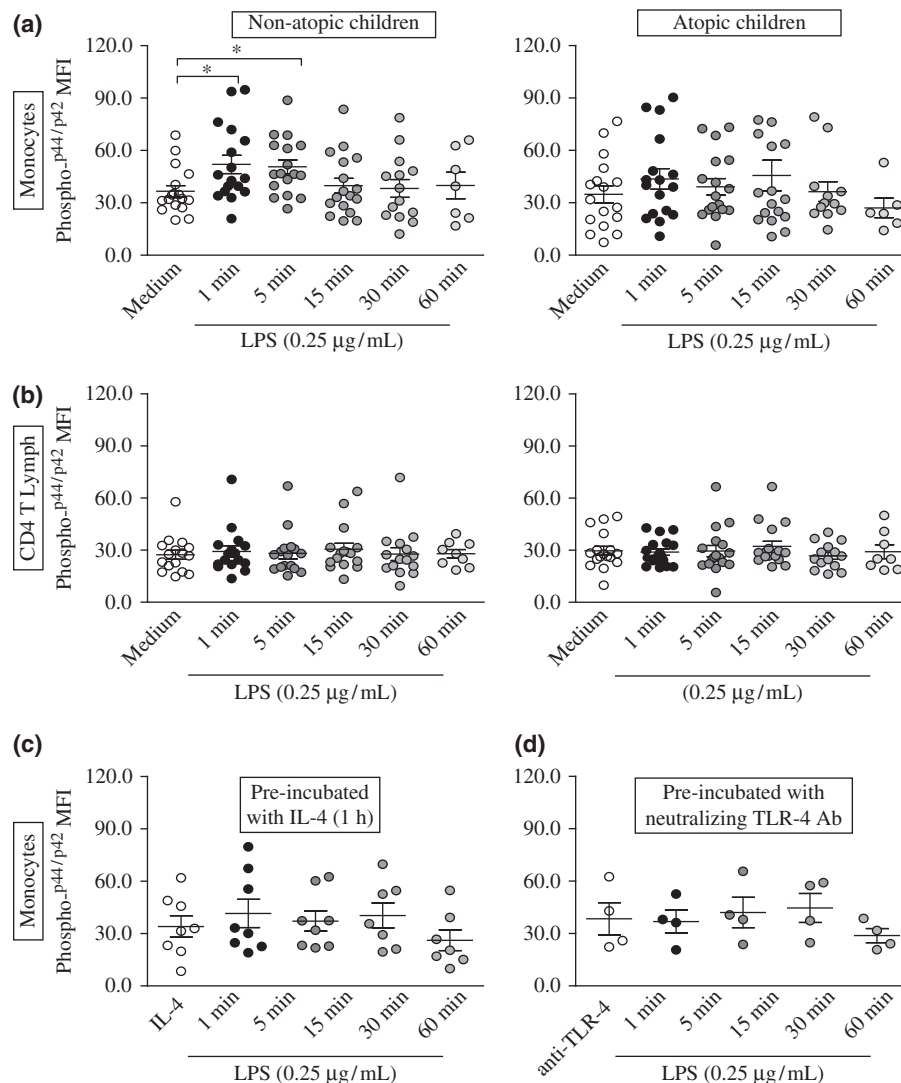


Fig. 3. Impaired lipopolysaccharide (LPS)-induced signal transduction via $p^{42/44}$ ERK in $CD4^{+}$ cells from children with atopic histories compared with non-atopic children. Peripheral blood mononuclear cells pre-incubated with/without IL-4 were stimulated with 0.25 µg/mL LPS for 1–60 min, and then stained for CD4 and intracellular phospho- $p^{42/44}$ ERK and analyzed as in Fig. 2. Data are presented as median fluorescence intensity (MFI) of phospho- $p^{42/44}$ ERK detected in (a) $CD4^{+Low}$ monocytes and (b) $CD4^{+High}$ T 'helper' lymphocytes from non-atopic (left graphs; $n = 16$; age: 12.1 ± 3.5) and children with a history of atopic conditions (right graphs; $n = 17$; age: 12.4 ± 5.4). (c) Monocytes from non-atopics pre-incubated with recombinant IL-4 ($n = 8$) or (d) Toll-like receptor (TLR)-4 neutralizing antibody for 1 h before LPS exposure. * $P < 0.05$.

surface expression upon LPS exposure. This report is also the first to use a large paediatric cohort to survey the expression of TLR-4 on CD4-expressing circulating leucocytes, including monocytes and T 'helper' lymphocytes.

One limitation of this study was that atopic status and history were determined using a questionnaire, as in the ISAAC studies [32]. Within the limitation of our protocol, we screened several hundred children undergoing laboratory testing for other purposes. We accepted children as 'atopic' primarily if they had history of allergic diseases and past skin prick testing. The consistency of the results appears to underline the accuracy of the screening process. However, our follow-up studies incorporate more rigorous proofs including IgE measurement.

Previous work from our laboratory [23] detected TLR-4 on $CD4^{+}$ cells in nasal mucosa in both atopic and non-atopic children. Similar to other studies [9], consistently low percentages of TLR-4⁺ cells were detected among peripheral blood $CD4^{+}$ T cells; TLR-4 was mainly expressed on the surface of activated $CD4^{+}$ T cells. Monocytes represented the most important TLR-4⁺ circulating leucocyte population expressing CD4 [31]. Monocytes from non-atopic children significantly responded to LPS exposure by increasing $p^{42/44}$ ERK and p38 MAPK phosphorylation [24]. In contrast, we did not detect significant LPS-induced ERK or p38 MAPK phosphorylation among $CD4^{+High}$ T 'helper' lymphocytes regardless of atopic status, which reflects the relative LPS unresponsiveness

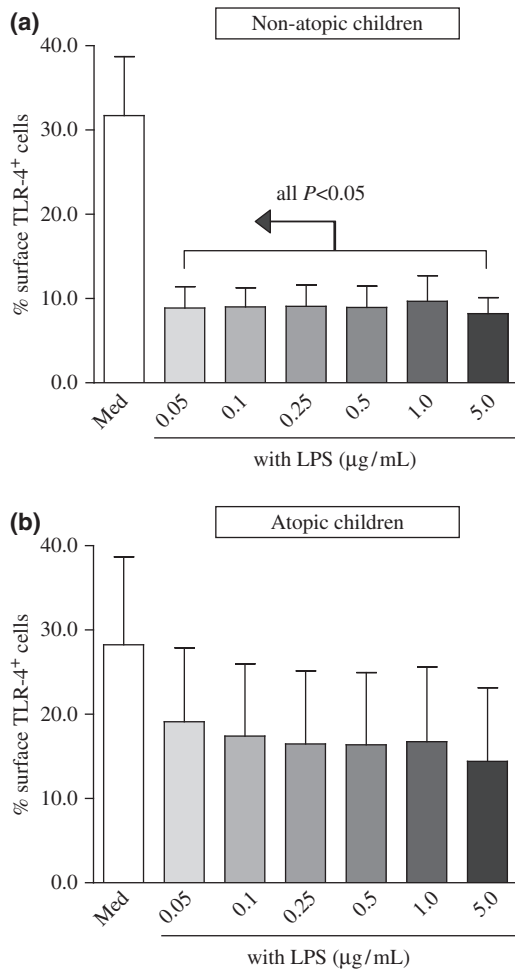


Fig. 4. Lipopolysaccharide (LPS) exposure decreases surface Toll-like receptor (TLR)-4 detection on monocytes from non-atopic children. Peripheral blood mononuclear cells were exposed to LPS (0.05–5 µg/mL) for 24 h followed by flow cytometric analysis of CD4/TLR-4 expression. LPS-induced decrease in TLR-4 expression was significantly greater on monocytes from non-atopic children [(a) $n = 4$, age: 9 ± 2.9] compared with cells from children with atopic histories [(b); $n = 5$, age: 10.2 ± 4.4].

of these cells. The ability of CD4⁺ monocytes to phosphorylate MAPK upon LPS exposure was dependent on the atopic status of the patient. LPS failed to trigger p42/44ERK and p38 phosphorylation in monocytes from children with atopic histories compared with cells from non-atopic children. These results are in line with Saghalian-Hedengren et al. [26] who similarly observed impaired LPS-induced p38 MAPK phosphorylation in 24-month-old children from allergic mothers compared with children from non-allergic mothers. The differences between atopic and non-atopic children were not due to differences in ages, as suggested by Boehmer et al. [24], because we carefully age-matched all subjects in the signal transduction experiments. This decrease in signaling in monocytes may help to explain the observations that PBMC from asthmatic patients released significantly

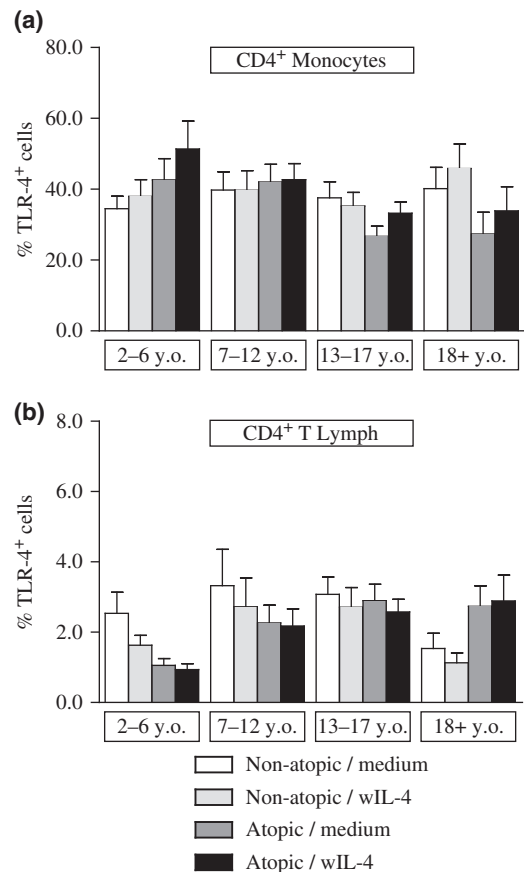


Fig. 5. Cross-sectional Toll-like receptor (TLR)-4 expression by circulating CD4-expressing leucocytes during childhood. Peripheral blood mononuclear cells were cultured for 24 h with/without IL-4 (13.5 ng/mL) before flow cytometric analysis of surface TLR-4 expression on (a) CD4⁺ monocytes and (b) CD4⁺ T 'helper' lymphocytes from children with/without atopic histories. All analyses were stratified by age: 2–6 ($n = 14/17$), 7–12 ($n = 18/19$), 13–18 ($n = 28/34$) and ≥ 18 ($n = 12/13$).

less IFN- γ , IL-10, IL-1 β and TNF- α compared with cells from controls [30, 33], or reduced IL-12 production in subjects with atopic dermatitis compared with controls, although no change was observed in the TLR-4 expression between the patients and control subjects [34]. Our data provide an explanation for differential cytokine production in atopy via defective LPS-induced signalling in peripheral cells from children with atopic diseases.

Allergic diseases including rhinitis and asthma typically feature Th2-type immune bias directed against innocuous allergens. In our study, the impaired ability of monocytes from children with a history of atopic conditions to recognize LPS and trigger signal transduction could be mimicked by pre-incubating cells from non-atopic donors with IL-4. This implies that TLR-4 functions are negatively influenced by a Th2-type cytokine environment [35, 36]. The mechanism by which IL-4 modifies LPS signalling was beyond the scope of this study; potential explanations include a direct modification of

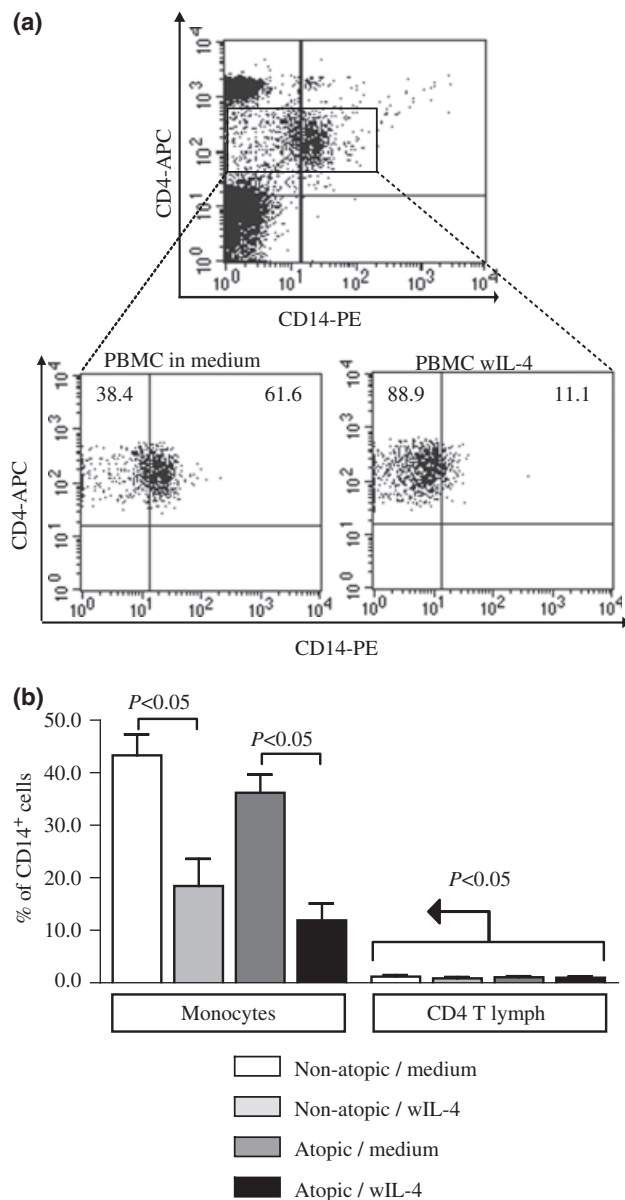


Fig. 6. CD14 expression on monocytes is decreased by IL-4 exposure, but its baseline expression is not affected by a history of atopy. (a) Peripheral blood mononuclear cells (PBMC) were analyzed for surface CD14 on monocytes following 24 h culture with (right) and without (left) IL-4 (13.5 ng/mL). (b) Analysis of CD14 expression on CD4⁺Low monocytes (left) and CD4⁺High T 'helper' lymphocytes (right) from children with/without atopic histories ($n = 13/10$, age: $13.8 \pm 2.8/12.1 \pm 4.7$).

the TLR-4 complex gene expression by IL-4 [35] via activation of downstream signal transducer and activator of transcription-6 [37]. Atopy may also induce the expression of other immuno-regulatory molecules, like the suppressor of cytokine signalling-1 (SOCS-1) molecule. SOCS-1, an IL-4-inducible molecule [39], has been associated with an inhibition of LPS signalling [40, 41].

We addressed the possibility of an atopy-specific variation in the TLR-4 expression using a large paediatric

cohort, stratified by age and atopic status. We assessed both baseline TLR-4 and expression following exposure to exogenous IL-4. No major variation in the TLR-4 expression could be observed across the whole cohort. However, we determined that age differentially affected TLR-4 expression on CD4⁺ leucocytes in children with/without atopic histories. Correlation analyses revealed that TLR-4 expression by monocytes from children with atopic histories slightly but consistently diminished as a function of age compared with non-atopic children. In contrast, TLR-4 expression by T 'helper' lymphocytes tended to increase with age in atopic but not in non-atopic children. The trends towards decreasing TLR-4 expression by monocytes from older children with a history of atopy and increasing TLR-4 on CD4⁺ T lymphocytes in older children with atopic histories compared with non-atopic children could be a consequence of the Th-2 immune bias associated with atopy. Children with atopic histories display elevated proportions of circulating activated T 'helper' cells (D. Préfontaine et al., in preparation), and activated CD4 T cells display elevated surface TLR-4 compared with naïve cells [9]. Elevated IL-4-producing Th2 cells may affect TLR-4 expression on circulating monocytes in the long term [35, 37].

This hypothesis may also be supported by the IL-4-mediated decrease in CD14 expression [36]. However, we could not detect differences in the percentages of CD14-expressing cells in non-atopic healthy children compared with children diagnosed with atopic conditions, and both groups were equally affected by culture with IL-4. Zdzilek and Jenmalm [38] reported reduced levels of soluble CD14 in children with atopic children, which can contribute to defective LPS-triggered signalling. In the light of our results and other studies [26, 27], negative regulation of LPS signalling in individuals with atopic histories does not appear to be caused by the down-regulation of TLR-4 complex elements.

We observed that TLR-4-expressing CD4⁺ monocytes from non-atopic subjects could be discriminated from those children with atopic histories on the basis of their loss of surface TLR-4 expression upon LPS exposure. Decrease in TLR-4 surface expression [42] due to internalization was shown to occur within minutes, and in a dynamin- and clathrin-dependent manner, and is then trafficked to early/sorting endosomes [12]. Our data demonstrate a pronounced and prolonged LPS-induced reduction of surface TLR-4 expression only in non-atopic monocytes. Potentially, atopy may interfere with LPS/TLR-4 complex internalization, and consequently, limit LPS-induced signal transduction. While LPS/TLR-4 complex internalization and endosomal trafficking were reported to be neither necessary nor sufficient for the activation of the NF- κ B signaling pathway [12, 43], there are no data regarding the impact of diminished internalization on the MAPK pathway. The consequences of LPS-

induced decreases in TLR-4 expression due to receptor internalization, and its effect on signal transduction and downstream immune responses in monocytes from atopic and non-atopic individuals, deserve further investigation.

In summary, we studied the ability of circulating CD4-expressing leucocytes from children with and without histories of atopic diseases, especially monocytes, to signal following exposure to LPS. We clearly demonstrate that monocytes from non-atopics respond to LPS through p42/44ERK and p38 MAPK protein phosphorylation and decrease in surface TLR-4 expression; these processes are highly impaired in cells from children with atopic conditions. This study provides a novel context for the hygiene hypothesis, which suggests that bacterial LPS has immuno-regulatory effects during childhood and may limit the allergic phenotype later in life [1]. Our results come in support to studies associating atopy with impaired LPS recognition by immune cells [26, 29, 30, 33], therefore suggesting that allergic histories may dampen TLR-4-mediated innate immune function early in life.

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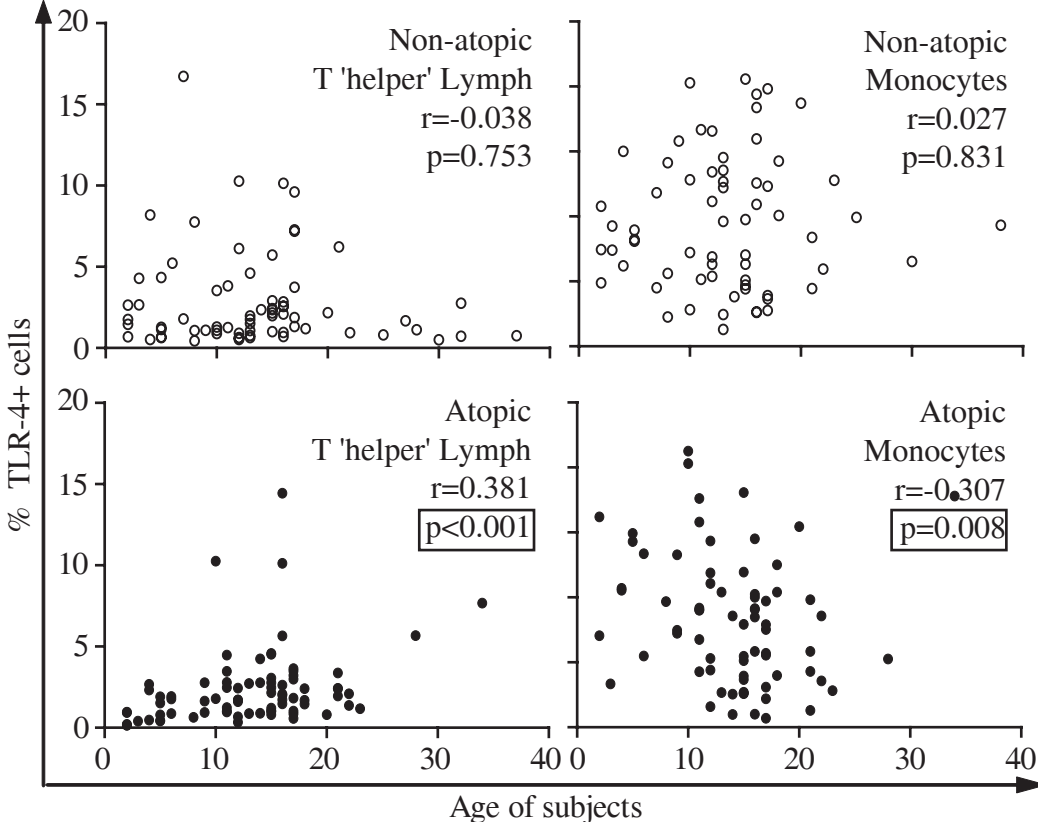
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Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1. Correlation analyses of TLR-4 expression as a function of subject's age are presented for both monocytes and T 'helper' lymphocytes (right and left graphs, respectively) from children without or with atopic histories (upper and bottom graphs, respectively). Spearman's rank correlation coefficients appear as '*r*', and *P* values < 0.05 were considered significant.

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Figure E1. Correlation analyses of TLR-4 expression as a function of subject's age are presented for both monocytes and T 'helper' lymphocytes (right and left graphs, respectively) from children without or with atopic histories (upper and bottom graphs, respectively). Spearman's rank correlation coefficients appear as 'r', and p-values < 0.05 were considered significant.