

Mechanisms of disease

Role of toll-like receptor 4 in protection by bacterial lipopolysaccharide in the nasal mucosa of atopic children but not adults

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Summary

Background Exposure to bacterial products in early life could protect against development of atopy. We examined the effect of bacterial lipopolysaccharide on allergic inflammation and expression of cytokines and lipopolysaccharide receptor (toll-like receptor 4; TLR4) in nasal mucosa of 15 atopic children and ten atopic adults.

Methods Explanted mucosa was cultured with allergen with or without lipopolysaccharide (0.1 mg/L) for 24 h. Immunocytochemistry and in-situ hybridisation were used to phenotype the cells and cytokines.

Findings In explants from atopic children, lipopolysaccharide prevented allergen-induced T-helper type 2 (Th2) inflammation and upregulated Th1 cytokine reactivity and expression. These effects were blocked by antibody to interleukin 10. In children but not in adults, lipopolysaccharide caused increases of three times in T-cell reactivity, five times in T-cell proliferation, and four times in expression of interleukin 10 compared with mucosa stimulated with allergen alone. This difference in response was mirrored by lipopolysaccharide-induced increases in TLR4 reactivity in children but not adults. TLR4 receptor was expressed by CD3-positive T cells, and TLR4-positive cells contained interleukin 10. Lipopolysaccharide increased expression of cells positive for both CD3 and TLR4; both TLR4 and interleukin 10; and both CD4 and CD25.

Interpretation Lipopolysaccharide inhibits allergic inflammation in nasal mucosa of atopic children by skewing local immune responses from Th2 to Th1 and upregulating production of interleukin 10. These effects are mediated by TLR4. Our results emphasise an important difference between adults and children in their ability to respond to bacterial products. These differences could have a role in normal maturation of the immune system.

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Introduction

The **HYGIENE HYPOTHESIS** suggests that decreasing exposure to infections or bacterial products is the cause of the increasing incidence of atopy and asthma.^{1–3} Epidemiological studies have consistently shown that the farming environment is protective in young children against development of hayfever, atopy, and wheeze. Bacterial **LIPOLYSACCHARIDE** has been suggested as a potential mediator of these protective effects.^{4–7} Although epidemiological evidence supports the role of bacterial lipopolysaccharide in the hygiene hypothesis, the mechanisms of any lipopolysaccharide-induced immunomodulation or modification of an allergic immune response during early life are poorly understood.

We have shown previously that although exposure of rats to lipopolysaccharide during early sensitisation protects against their development of ovalbumin-specific IgE,⁸ coexposure of lipopolysaccharide with allergen results in dose-dependent inhibition of acute and late-phase allergic inflammation and bronchial hyper-responsiveness,⁹ exposure to lipopolysaccharide after allergen challenge further exacerbates the allergic response.⁸ The effect of lipopolysaccharide depended on the timing of exposure and the dose given. We have shown lately that administration of lipopolysaccharide to explanted nasal mucosa of young non-atopic children shifts the immune response towards a T-helper 1 (Th1) phenotype by causing inflammatory cell activation and promoting strong proliferation of T cells.¹⁰ This model shows many of the features of in-vivo lipopolysaccharide-induced inflammation previously described in both animals and human beings, including cellular activation, production of

RELEVANCE TO PRACTICE

BACKGROUND

The incidence of asthma has increased in recent years. Researchers have suggested that reduced exposure to bacteria during early life could make children more susceptible to developing asthma or atopy (the tendency to be allergic) at a later age.

This study looked at whether lipopolysaccharide, a major component of the cell walls of many bacteria, could reduce the inflammation that normally occurs in the nasal mucosa of young atopic children when they are exposed to an allergen. The researchers found that lipopolysaccharide prevented allergen-induced inflammation in children, but not in atopic adults. This effect was achieved by shifting from a T-helper-2 (Th2) immune response, which contributes to the tendency to be atopic, towards a Th1 immune response. Lipopolysaccharide also increased expression of lipopolysaccharide receptor, toll-like receptor 4 (TLR4) on the T cells in the mucosa of atopic children but not atopic adults, which suggests that TLR4 could have a role in mediating the Th2 to Th1 shift in young children.

IMPLICATIONS

These findings suggest a mechanism by which reduced exposure to bacterial products at an early age could lead to skewed development of the immune system, making children more prone to development of asthma or atopy.

GLOSSARY**CYTOKINES**

A family of small, low-molecular-weight proteins involved in intercellular signalling, growth, proliferation, and cell death.

HYGIENE HYPOTHESIS

The idea that low exposure to bacterial products, especially during early life, might be the reason for the increasing incidence of asthma.

LIPOLYSACCHARIDE

A major component of the cell wall of gram-negative bacteria.

TOLL-LIKE RECEPTOR 4

A receptor involved in lipopolysaccharide-induced signal transduction.

CYTOKINES, and mast-cell degranulation. Importantly, these effects of lipopolysaccharide were mediated locally without recruitment of inflammatory cells from the systemic circulation.

To investigate further the effect of lipopolysaccharide on allergic inflammation in the nasal mucosa of young atopic children, we used the above explant model to compare the responses with those in atopic adults. We decided to stimulate the allergic mucosa simultaneously with allergen and lipopolysaccharide to mimic the normal environmental exposure more closely, because both of these antigens are ubiquitous contaminants in the environment. We hypothesised that lipopolysaccharide can downregulate the allergen-induced local inflammation in the nasal mucosa of atopic children by causing a shift from a Th2 towards a Th1 immune response and inducing production of the immunoregulatory cytokine interleukin 10.

Methods**Participants**

22 children and 17 adults were recruited for this study. 15 atopic children (mean age 3.2 years [SD 0.4]) and seven non-atopic children (mean age 3.8 years [0.7]) of both sexes were recruited from the ear, nose, and throat clinic at the Montreal Children's Hospital. Atopy was confirmed by skin-prick testing to a panel of common allergens including housedust mite, grass pollen, ragweed extract, and cat and dog antigens. Non-atopic children had no history of asthma or other allergic diseases and had negative results on skin-prick testing. Ten adults sensitive to ragweed (mean age 40.4 years [5.3]) with seasonal allergic rhinitis and seven non-atopic adults (mean age 35.2 years [6.8]) were recruited from the Notre-Dame Hospital and the Department of Otolaryngology, McGill University Health Centre. Nasal mucosal tissue was resected from the inferior turbinate of adults and children undergoing tonsillectomy, adenoidectomy, nasal septal surgery, or sinus surgery. All children and adults were asymptomatic at the time the samples were taken; biopsies in adults were done outside of the ragweed season. Individuals were excluded if they had had any viral or bacterial infections during the month before surgery or were taking anti-inflammatory medication regularly. Informed consent was obtained from parents of children or from adult participants before the procedure. The study was approved by the ethics committees of the McGill University Health Centre and Research Centre, University of Montreal Hospital.

Procedures

Each biopsy sample was placed in bicarbonate-buffered culture medium immediately after excision and

transported to the Meakins-Christie Laboratories for processing. The time between removal of tissue and culture of nasal mucosa was less than 1 h in all cases. Serial sections of tissue were each placed in 30 mm, filtered-well inserts (Millicell CM, Millipore Corporation, Bedford, MA, USA) with a 0.4 µm pore diameter within a six-well culture plate (Costar, Corning Inc, NY, USA) containing 2 mL fresh bicarbonate medium. The tissue was oriented with epithelium exposed to the air, forming an air-liquid interface to mimic the in-vivo situation. Tissue from atopic children was incubated with: medium alone; 250 µL specific allergen (10–2000 protein nitrogen units); 0.1 mg/L lipopolysaccharide (*Escherichia coli* serotype 026:B6; Sigma-Aldrich, St Louis, MO, USA); or allergen and lipopolysaccharide, added directly into the culture medium. The dose of lipopolysaccharide used (0.1 mg/L) is the optimum dose previously used in this model.¹⁰ In a subset of three atopic children and three atopic adults, specificity of the TOLL-LIKE RECEPTOR 4 (TLR4) was tested with a commercial purified lipopolysaccharide preparation (1 µg/L; *E coli* serotype R515; Alexis Biochemicals, San Diego, CA, USA). This preparation is a strong activator of TLR4 and has no effect on TLR2.

Ten of the 15 atopic children were positive on skin-prick testing to housedust mite (*Dermatophagoides farinae* or *D pteronyssinus*), two were positive to ragweed allergen, two to dog allergen, and one to the cat allergen extract. The concentration of allergen used for stimulation was 2000 protein nitrogen units for *D farinae*, 1000 protein nitrogen units for ragweed, and 10 protein nitrogen units for dog and cat extracts (Hollister-Stier, Spokane, WA, USA). To examine the functional role of specific cytokines, in four experiments, samples of atopic mucosa from children stimulated with allergen and lipopolysaccharide were also coincubated for 24 h with neutralising antibodies to human interleukin 12 (0.9 mg/L), interleukin 10 (0.03 mg/L), interferon γ (0.06 mg/L; all R&D Systems, Minneapolis, MN, USA), or isotype-matched controls. All tissue was incubated overnight at 37°C in 5% carbon dioxide, 95% air.

After 24 h of culture, tissue was fixed in 4% paraformaldehyde, washed in 15% sucrose/phosphate-buffered saline, and blocked in optimum-cutting-temperature embedding medium (Tissue-Tek, Somagen Diagnostics, Edmonton, Alberta, Canada) by snap freezing in isopentane cooled in liquid nitrogen. The tissue was sectioned at 5 µm thickness onto glass slides (Microm HM500, Microm International GmbH, Walldorf, Germany) coated with poly-L-lysine (0.1%) and baked overnight at 37°C.

By use of alkaline phosphatase/anti-alkaline phosphatase immunocytochemistry as previously described,^{10,11} we identified macrophages with anti-CD68 (1 in 50 dilution; DAKO Diagnostics, Mississauga, Ontario, Canada), eosinophils with anti-major basic protein (1 in 30; DAKO), neutrophils with anti-elastase (1 in 100; DAKO), mast cells with anti-tryptase (1 in 250; Cedarlane Laboratories, Hornby, Ontario, Canada), T cells with anti-CD3 (1 in 100; DAKO), activated T cells with anti-CD25 (1 in 5; DAKO), TLR4 expression (1 in 200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and interleukin 2 (1 in 25; R&D Systems), interleukin 5 (1 in 25; DAKO), interleukin 12 (1 in 100; Santa Cruz Biotechnology), interferon γ (1 in 50; DAKO), and interleukin 10 (1 in 100; DAKO) proteins. All primary antibodies, except those to TLR4 (rabbit antibody to human antigen; 1 in 10) and interleukin 2 (goat antibody to human antigen; 1 in 25),

were monoclonal mouse antibodies to human antigens, so the secondary antibody was rabbit antibody to mouse immunoglobulin (DAKO; 1 in 60) and the tertiary antibody was mouse alkaline phosphatase/anti-alkaline phosphatase (1 in 60; DAKO). For the polyclonal antibodies to TLR4 and interleukin 2, the secondary antibodies used were biotinylated swine antibody to rabbit immunoglobulin (1 in 300; DAKO) and biotinylated rabbit antibody to goat immunoglobulin (1 in 500; DAKO), respectively; streptavidin-alkaline phosphatase (1 in 200; DAKO) was the tertiary antibody. All immunostaining was developed with fast red (Sigma Chemical Company, Toronto, Ontario, Canada) dissolved in substrate solution (0.5 mmol/L naphthol AS-MX phosphate, 2% dimethylformamide, 1 mmol/L levamisole, and 0.1 mol/L Tris base, pH 8.2). Slides were counterstained with Gill II haematoxylin and coverslips applied. Positive cells appeared red under bright-field illumination. For negative controls, the primary antibody was replaced by an isotype-matched control antibody.

T-cell proliferation was studied by incubation of tissue with 5-bromo-2'-deoxyuridine (10 μ mol/L; Roche Diagnostics GmbH, Mannheim, Germany) in the presence or absence of 0.1 mg/L lipopolysaccharide for 24 h at 37°C in 5% carbon dioxide, 95% air. After incubation, tissue was fixed, blocked, and sectioned as described above. Cellular incorporation of bromodeoxyuridine was detected by use of a specific antibody and visualised with antibody to mouse immunoglobulin conjugated to fluorescein isothiocyanate (Roche Diagnostics). T cells were identified with goat antibody to human CD3 conjugated to red phycoerythrin (Cedarlane Laboratories) as previously described.¹⁰ Slides were incubated with anti-bromodeoxyuridine (1 in 10) for 30 min at 37°C, rinsed three times with phosphate-buffered saline, then incubated with secondary antibody to mouse immunoglobulin labelled with fluorescein (1 in 10), rinsed in phosphate-buffered saline, and finally incubated with goat antibody to human CD3 labelled with red phycoerythrin (undiluted) for 30 min at 37°C. Proliferating bromodeoxyuridine-positive cells were identified as green, CD3-positive cells as red, and colocalisation of proliferating cells with CD3-positive T cells as yellow immunostaining.

To examine whether T cells expressed TLR4, slides were incubated with red-phycoerythrin-labelled goat antibody to human CD3 (undiluted) for 30 min, washed, and incubated with TLR4 conjugated to fluorescein isothiocyanate (undiluted, Santa Cruz Biotechnology). CD3-positive T cells were identified as red, TLR4-positive cells as green, and colocalisation of T cells with TLR4 as yellow immunostaining. To examine whether the TLR4-positive cells contained interleukin 10, slides were incubated with TLR4 conjugated to fluorescein isothiocyanate followed by incubation with mouse antibody to human interleukin 10 conjugated to red phycoerythrin (1 in 25; Cedarlane Laboratories). TLR4-positive cells were identified as green, interleukin-10-positive cells as red, and colocalisation of TLR4 with interleukin 10 as yellow. The expression of cells positive for CD4 and CD25 was examined by use of mouse antibody to human CD4 labelled with allophycocyanin (1 in 60, BD Pharmingen, San Diego, CA, USA) followed by incubation with mouse antibody to human CD25 labelled with fluorescein isothiocyanate (1 in 50; BD Pharmingen). CD4-positive cells were identified as dark red, CD25-positive cells as green, and colocalisation of CD4 with CD25 as yellow. Isotype-matched control antibody was used for each marker. All slides were

covered with PermaFluor mounting medium (Shandon, Pittsburgh, PA, USA) and examined at $\times 660$ magnification with a fluorescence microscope (LSM410, Carl Zeiss, Jena, Germany).

Cytokine mRNA was detected in the nasal tissue by use of in-situ hybridisation as previously described.^{12,13} Tissue was permeabilised with 0.3% Triton (BDH, Toronto, Ontario, Canada) and proteinase K (1 mg/L; Sigma Chemical Company). Prehybridisation was done in 50% formamide (Sigma) in $2\times$ standard sodium citrate solution for 15 min at 37°C, and sections were then hybridised overnight at 42°C with sulphur-35-labelled antisense and sense probes coding for human interleukins 4, 5, 13, 12, or 10 or interferon γ (0.75×10^6 cpm per slide). Non-specific binding was removed by a series of washes in decreasing concentrations of standard sodium citrate solution at 42°C. Slides were subsequently treated with RNase A (20 mg/L) to destroy excess, unbound RNA probe. Slides were dipped in LM-2 emulsion (Amersham LM-2, Oakville, Ontario, Canada), stored at 4°C, and developed 2 weeks later in Kodak D19 solution prepared according to the manufacturer's instructions (Eastman Kodak, Toronto). All slides were counterstained with Gill II haematoxylin and coverslips were applied. Positive signal was identified as silver grains over the cells.

For immunocytochemistry and in-situ hybridisation, positive cells were counted by two observers unaware of experimental origin on an Olympus light microscope (Carson Group Inc, Markam, Ontario, Canada) with $\times 400$ magnification and an eyepiece graticule of 0.202 mm². In the airway epithelium and subepithelium, the number of positive cells was averaged from six to eight random, non-overlapping grids and expressed as the mean number of positive cells per field. Immunofluorescence was semiquantified by use of ImageJ software (<http://rsb.info.nih.gov/ij/>; accessed Feb 10, 2004) and expressed in optical density units.

Statistical analysis

The differences in mean number of immunopositive cells from baseline for a particular cell type or cytokine or receptor expression in presence and absence of lipopolysaccharide were analysed by two-way ANOVA, which used treatments (baseline, lipopolysaccharide, allergen, allergen plus lipopolysaccharide) as one factor and participant as a blocking factor. We used Dunnett's post-hoc test for comparison of all four treatments versus common control and Tukey's test for the other pairwise comparisons. Dunnett's test was applied by a researcher using the residual mean square for the two-way ANOVA as estimator of the common residual variance. Results in figure 1 are expressed as mean and 95% honest CI (HCI), which represents the error term in a multiple comparison; thus, if the two-sided error bars do not intercept, the means differ significantly at a family-wise confidence limit of 95%. All other results are expressed as mean and 95% CI.

Role of the funding source

The funding sources had no role in study design; collection, analysis, or interpretation of data; or in the final decision to submit this report for publication.

Results

In non-stimulated atopic mucosa of children, we detected constitutively expressed CD68, major basic protein, elastase, tryptase, CD3, interleukin 2, and CD25. Similarly, constitutive expression of mRNA for both Th2 (interleukins 4, 5, 13, and 10) and Th1 (interferon γ , interleukin 12) cytokines was detected in this tissue.

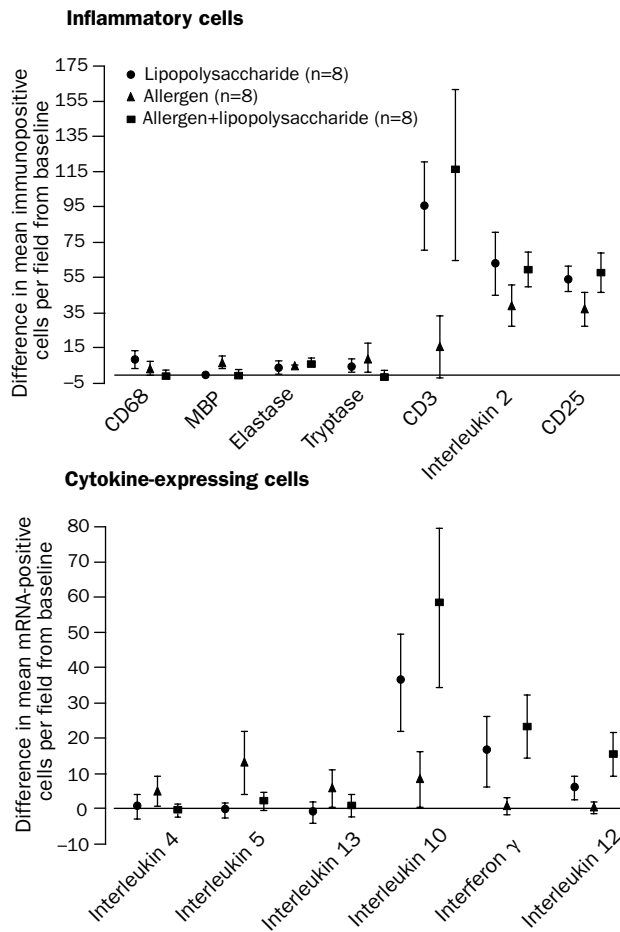


Figure 1: Effect of lipopolysaccharide on allergen-induced inflammation and cytokine production in nasal mucosa of atopic children after 24 h stimulation

Data are presented as difference in mean number of cells/field from baseline (unstimulated atopic mucosa) with 95% HCl. MBP=major basic protein.

Exposure of atopic mucosa to lipopolysaccharide without allergen significantly increased the number of cells immunoreactive for CD68 ($p=0.0009$), elastase ($p=0.01$), tryptase ($p=0.05$), CD3 ($p=0.0008$), interleukin 2 ($p=0.0009$), and CD25 ($p=0.0008$) compared with unstimulated atopic tissue at baseline (figure 1). The numbers of CD68-positive and elastase-positive cells were increased by about 2.5 times, whereas

the numbers positive for CD3, interleukin 2, and CD25 were increased four to five times compared with unstimulated atopic tissue. The cells present in the greatest number after exposure to lipopolysaccharide were CD3-positive T cells, followed by those positive for interleukin 2 and CD25 (figure 1). By double immunostaining, we found that about 60% of CD3-positive cells were immunoreactive for CD25. Lipopolysaccharide caused an increase of more than six times in the expression of Th1 cytokines (interferon γ $p=0.0007$, interleukin 12 $p=0.0009$) and cells positive for interleukin 10 mRNA ($p=0.0009$; figure 1). Expression of cells immunoreactive for major basic protein ($p=0.34$) and Th2 cytokines remained unchanged after lipopolysaccharide stimulation (interleukin 4 $p=0.78$; interleukin 5 $p=0.62$; interleukin 13 $p=0.31$; figure 1).

Stimulation of atopic mucosa with allergen alone caused a significant increase in the number of cells immunoreactive for major basic protein ($p=0.0008$), tryptase ($p=0.02$), CD3 ($p=0.03$), interleukin 2 ($p=0.0008$), and CD25 ($p=0.0007$) compared with unstimulated atopic mucosa (figure 1). A smaller, although significant, increase was also observed in the numbers of CD68-positive ($p=0.01$) and elastase-positive cells ($p=0.04$) after allergen stimulation. Allergen caused increases of three to five times in the expression of Th2 cytokines (interleukin 4 $p=0.01$; interleukin 5 $p=0.01$, interleukin 13 $p=0.04$) compared with non-stimulated atopic mucosa and a smaller but significant increase in interleukin 10 mRNA ($p=0.02$). Expression of Th1 cytokines remained unchanged after allergen challenge (interferon γ $p=0.48$; interleukin 12 $p=0.85$; figure 1).

Exposure of atopic mucosa to lipopolysaccharide in the presence of allergen prevented the allergen-induced increase in immunoreactivity for CD68 ($p=0.52$), major basic protein ($p=0.07$), and tryptase ($p=0.54$), and the numbers of these cells were similar to those in unstimulated atopic mucosa (figure 1). Lipopolysaccharide had no effect on the allergen-induced increase in elastase immunoreactivity ($p=0.09$). In addition, lipopolysaccharide further increased the numbers of cells positive for CD3 ($p=0.0009$), interleukin 2 ($p=0.01$), and CD25 ($p=0.0008$) compared with atopic mucosa stimulated with allergen alone. These increases were of similar size to those seen in atopic mucosa stimulated with lipopolysaccharide alone. The cells present in atopic mucosa in greatest number after allergen and lipopolysaccharide stimulation were CD3-positive T cells, followed by cells positive for interleukin 2 and for CD25. A similar proportion of CD3-positive cells were also CD25-positive (about 60%). Incubation of atopic mucosa with

	Allergen+ lipopolysaccharide, mean (95% CI)	Anti-interleukin 10 Mean (95% CI)	p	Anti-interleukin 12 Mean (95% CI)	p	Anti-interferon γ Mean (95% CI)	p	Anti-interleukin 12 and anti-interferon γ Mean (95% CI)	p
Cytokine mRNA									
Interleukin 4	3.8 (0.9 to 6.6)	7.8 (4.0 to 11.6)	0.03	6.6 (2.5 to 10.8)	0.04	5.1 (3.1 to 7.1)	0.73	8.2 (5.1 to 11.4)	0.03
Interleukin 5	4.5 (2.6 to 6.4)	14.1 (8.7 to 19.5)	0.01	13.0 (9.4 to 16.5)	0.02	10.1 (6.4 to 13.7)	0.04	17.2 (12.7 to 21.7)	0.01
Interleukin 13	5.8 (3.7 to 8.0)	12.4 (10.1 to 14.8)	0.02	11.5 (7.0 to 15.9)	0.02	8.2 (4.1 to 12.4)	0.04	12.0 (9.0 to 15.1)	0.02
Interferon γ	20.3 (15.0 to 25.5)	13.7 (11.5 to 15.8)	0.04	10.2 (7.9 to 12.5)	0.02	17.2 (13.2 to 21.2)	0.73	9.3 (6.0 to 12.5)	0.02
Interleukin 12	17.8 (9.5 to 26.0)	5.9 (3.6 to 8.2)	0.01	16.5 (6.5 to 26.5)	0.81	9.4 (7.2 to 11.5)	0.03	3.5 (1.6 to 5.3)	0.01
Interleukin 10	55.3 (37.2 to 73.4)	50.3 (29.9 to 70.8)	0.92	22.1 (18.2 to 25.9)	0.02	40.0 (30.7 to 49.2)	0.04	18.1 (13.0 to 23.2)	0.01
Cytokine protein									
Interleukin 5	3.1 (1.1 to 5.1)	13.1 (-5.6 to 31.7)	0.03	12.2 (0.1 to 24.2)	0.04	15.1 (-6.2 to 36.3)	0.04	12.9 (4.0 to 21.9)	0.03
Interleukin 12	11.7 (7.5 to 15.9)	6.2 (3.1 to 9.4)	0.04	13.5 (-0.5 to 27.6)	0.94	8.5 (5.8 to 11.2)	0.04	4.2 (-2.2 to 10.5)	0.03
Interferon γ	17.3 (2.5 to 32.1)	11.2 (7.0 to 15.4)	0.04	10.2 (8.2 to 12.3)	0.04	14.2 (4.9 to 23.5)	0.79	8.4 (7.0 to 9.8)	0.03
Interleukin 10	52.9 (23.9 to 81.9)	49.7 (21.4 to 77.9)	0.98	30.0 (13.1 to 46.9)	0.04	40.0 (34.9 to 45.1)	0.04	19.6 (1.9 to 37.2)	0.03

Effects of antibodies to interleukin 10, interleukin 12, and interferon γ on cytokine mRNA expression and immunoreactivity in atopic mucosa from children stimulated with allergen and lipopolysaccharide

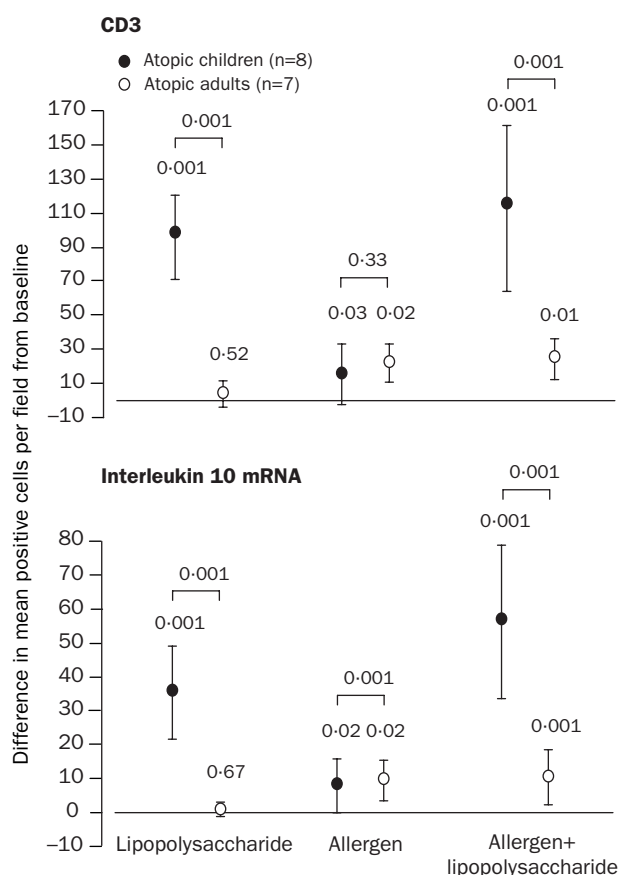


Figure 2: **Lipopolysaccharide-induced increase in CD3 and interleukin 10 mRNA in mucosa from atopic children but not atopic adults**

Difference in mean number of positive cells per field from baseline (unstimulated atopic mucosa) with 95% CI.

lipopolysaccharide and allergen prevented the allergen-driven increase in Th2 cytokine expression (interleukin 4 $p=0.84$, interleukin 5 $p=0.13$, interleukin 13 $p=0.68$) but significantly increased expression of Th1 cytokines (interferon γ $p=0.0007$, interleukin 12 $p=0.0009$) and interleukin 10 mRNA ($p=0.0007$) compared with atopic mucosa stimulated with allergen alone. The expression profile was similar to that seen in atopic mucosa stimulated with lipopolysaccharide alone.

Incubation of atopic mucosa with neutralising antibody to interleukin 10 in the presence of allergen and lipopolysaccharide restored Th2 cytokine expression and lowered the mRNA and protein expression of Th1 cytokines compared with atopic mucosa stimulated with allergen and lipopolysaccharide (table). The values were similar to those seen in atopic mucosa stimulated with allergen only. Similar increases in expression of Th2 cytokines and a reduction in expression of Th1 cytokines were observed when tissue was incubated with anti-interleukin 12. In addition, anti-interleukin 12 abolished the increase in the number of cells expressing interleukin 10 mRNA and interleukin 10 protein in this tissue. Although antibody to interferon γ had similar effects, the magnitude was smaller. Anti-interferon γ had no effect on the number of cells positive for interleukin 4 mRNA in atopic mucosa stimulated with allergen and lipopolysaccharide. The addition of antibodies to both interleukin 12 and interferon γ in the presence of allergen and lipopolysaccharide in atopic mucosa had a greater effect than either antibody alone. Antibodies to

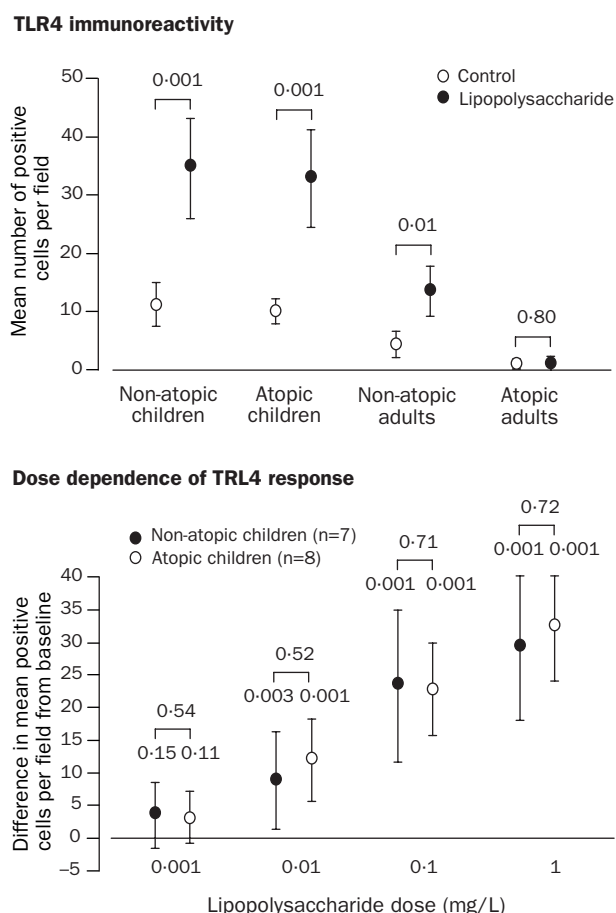


Figure 3: **Lipopolysaccharide increased TLR4 immunodetection in mucosa from atopic children but not atopic adults**

Difference in mean number of TLR4-positive cells per field from baseline with 95% CI.

interleukin 10, interleukin 12, and interferon γ had no effect on the number of cells positive for the respective mRNA in the explant (table). The changes in mRNA expression were also translated into changes in cytokine protein immunoreactivity. Isotype-matched controls for antibodies to interleukin 10, interleukin 12, and interferon γ had no effect on Th1 or Th2 cytokine mRNA or protein expression (data not shown).

Lipopolysaccharide significantly increased the number of bromodeoxyuridine-positive cells in atopic tissue from 9.0 (95% CI 6.2–12.1) per field in unstimulated atopic tissue to 47.0 (29.0–76.2) per field 24 h after lipopolysaccharide exposure ($p=0.0008$). A similar increase in the number of bromodeoxyuridine-positive cells was observed in atopic mucosa 24 h after stimulation with both allergen and lipopolysaccharide (from 7.0 [5.4–9.2] per field to 54.0 [43.8–71.8] per field after lipopolysaccharide; $p=0.0008$). This proliferation of CD3-positive T cells is likely to be driven by interleukin 2, immunoreactivity to which was significantly increased after lipopolysaccharide stimulation (figure 1).

In explants from atopic children, lipopolysaccharide caused an increase of about five times in the number of CD3-positive T cells and an increase of more than six times in the number of cells positive for interleukin 10 mRNA (figure 2). Similar numbers of CD3 and interleukin 10 mRNA immunoreactive cells were observed in mucosa of these atopic children after stimulation with lipopolysaccharide in the presence of allergen. In explants from atopic adults, allergen significantly increased the

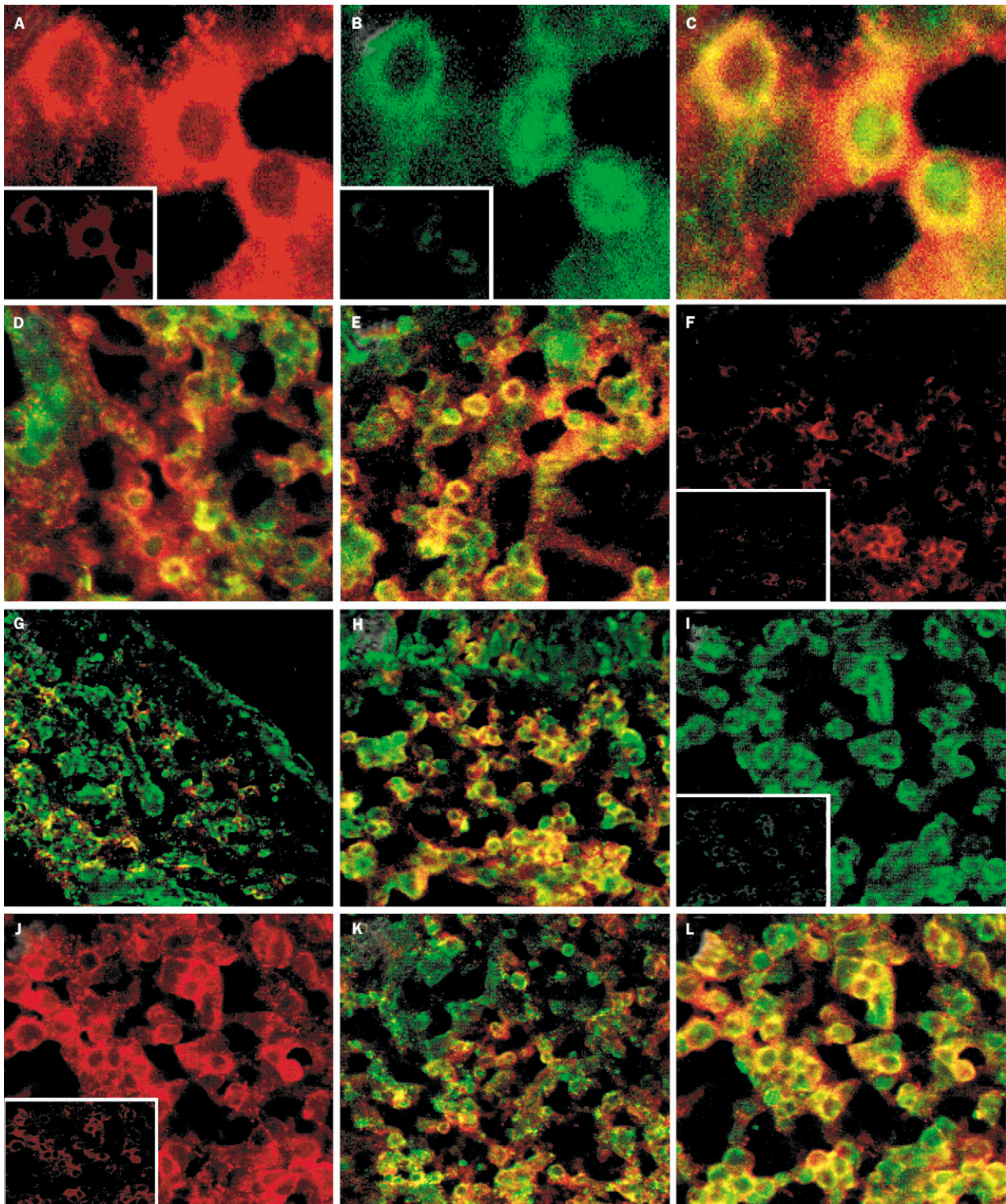


Figure 4: Immunodetection of TLR4 on T cells and TLR4-positive cells containing interleukin 10 in nasal mucosa of atopic children stimulated with lipopolysaccharide

A: CD3-positive T cells (red). B: TLR4-positive cells (green). C: colocalised CD3-positive TLR4-positive cells (yellow). D: CD3-positive TLR4-positive cells stimulated with allergen alone. E: CD3-positive TLR4-positive cells stimulated with lipopolysaccharide. F: interleukin-10-positive cells (red). G: colocalised interleukin 10 protein with TLR4-positive cells in atopic mucosa stimulated with allergen alone. H: colocalised interleukin 10 protein with TLR4-positive cells in atopic mucosa stimulated with allergen and lipopolysaccharide. I: CD25-positive cells (green). J: CD4-positive cells (red). K: CD25-positive CD4-positive cells in atopic mucosa stimulated with allergen alone. L: CD25-positive CD4-positive cells stimulated with allergen and lipopolysaccharide. Inserts are isotype controls for CD3 (A), TLR4 (B), interleukin 10 (F), CD25 (I), and CD4 (J) antibodies.

numbers of cells positive for CD3 and interleukin 10 mRNA compared with baseline unstimulated atopic mucosa; however, lipopolysaccharide alone had no effect.

Stimulation of atopic adult mucosa with lipopolysaccharide in the presence of allergen significantly increased the number of cells positive for CD3 and

interleukin 10 mRNA compared with control, but the size of this increase was similar to that seen in atopic adults stimulated with allergen alone. To address specifically the problem of potential contaminating substances in the lipopolysaccharide preparation, such as lipoproteins which are known to stimulate TLR2 receptors, we used a purified lipopolysaccharide preparation that activates only TLR4. Similar response profiles in CD3 immunoreactivity and interleukin 10 mRNA expression were seen in adults' and children's mucosa 24 h after stimulation with purified lipopolysaccharide, clearly supporting the involvement of TLR4 in this model.

Constitutive expression of TLR4 was detected in nasal mucosa from both children and adults irrespective of atopy status; however, baseline TLR4 expression was five to ten times greater in children than in atopic adults (figure 3). There was no difference in baseline expression of TLR4 between non-atopic and atopic children ($p=0.42$). Lipopolysaccharide significantly increased TLR4 immunoreactivity in the mucosa of non-atopic adults but had no effect in atopic adults. The increase in the number of TLR4-positive cells in both non-atopic and atopic children was dependent on the dose of lipopolysaccharide (figure 3).

We have shown that TLR4 is expressed by CD3-positive lymphocytes (figure 4). Stimulation of atopic mucosa from children with allergen and lipopolysaccharide increased the number of CD3-positive TLR4-positive cells (figure 4) compared with mucosa stimulated with allergen alone. TLR4-positive cells contained interleukin 10 protein, and stimulation with lipopolysaccharide increased the number of TLR4/interleukin-10-colocalised cells compared with stimulation by allergen alone. In mucosa stimulated with allergen and lipopolysaccharide there was increased expression of CD25-positive CD4-positive cells compared with mucosa stimulated by allergen alone.

Discussion

We have extended our previous studies with an ex-vivo human model of lipopolysaccharide-induced inflammation in explanted nasal mucosa from non-atopic children to show that administration of lipopolysaccharide can skew the local allergic response in young atopic children away from a Th2 towards a Th1 immune response. The shift in cytokine profile is associated with increased production of immunoregulatory interleukin 10, proliferation of CD3-positive T lymphocytes and increased numbers of CD3-positive TLR4-positive cells, colocalised interleukin 10 protein with TLR4-positive cells, and CD25-positive CD4-positive immunoreactive cells in the tissue. These effects are mediated by TLR4 and were not observed in atopic mucosa from adults after lipopolysaccharide stimulation. Although TLR4 is generally thought to be expressed by dendritic cells, macrophages, and monocytes, the receptor is also expressed in murine B cells¹⁴ and murine regulatory T cells.¹⁵ We have shown that TLR4 is also highly expressed by the CD3-positive T lymphocytes in the nasal mucosa of young children.

We acknowledge that the study had limitations. The advantage of an explant technique is that the ex-vivo role of bacterial lipopolysaccharide in modifying allergic inflammation can be studied without the influence of cellular recruitment from systemic circulation. However, the small size of the biopsy samples limits the ability to measure mRNA or protein production quantitatively in this tissue after lipopolysaccharide stimulation. The explant technique is suitable for both in-situ hybridisation

and immunocytochemistry; although these techniques are only semiquantitative, they yielded consistent results for both mRNA and protein. These techniques are sufficiently robust for measurement of relative expression of cytokine mRNA and protein and thus the effect of lipopolysaccharide on local Th1 and Th2 immune responses can be investigated. Second, in our system allergen and lipopolysaccharide are added directly into the culture medium, so the submucosa and not the epithelium is the first point of contact. Nevertheless, the aim of the study was to examine the direct local immune interactions between allergen and lipopolysaccharide, and we have previously shown effective uptake of the stimulants into the submucosa and into the nasal epithelium of explanted nasal mucosa.¹⁶ Thus, it is a useful model to study such processes.

The ability of lipopolysaccharide to prevent allergen-induced inflammation in vivo has previously been described in animal models of allergic inflammation^{8,17,18} but not in human beings. In adults, lipopolysaccharide is well known as an occupational hazard among workers in cotton mills and poultry and pig farms. Inhalation of high concentrations of lipopolysaccharide in these environments is associated with acute reductions in lung function, respiratory symptoms, and nasal inflammation.¹⁹ In atopic asthmatic adults, increased airway eosinophilia is seen 4 h after local, low-dose nasal lipopolysaccharide challenge.²⁰ In contrast to those studies, we found that bacterial lipopolysaccharide skews the allergen-induced local allergic immune response in the nasal mucosa of atopic children away from the Th2 phenotype by preventing expression of mRNA for Th2 cytokines (interleukins 4, 5, and 13) and increasing expression of mRNA for Th1 cytokines (interferon γ and interleukin 12) and cytokine immunoreactivity. These effects depend on the presence of allergen; stimulation with lipopolysaccharide alone induced a local Th1 immune response and increased CD68 and elastase immunoreactivity.

Addition of neutralising antibodies to interleukin 10 or interleukin 12 showed the influence these cytokines have on lipopolysaccharide-induced prevention of Th2 responses in atopic children. The antibodies were equally effective and each could downregulate mRNA expression and immunoreactivity of the other, which suggests that they act synergistically to prevent the allergen-driven Th2 allergic response. Despite previous reports of interleukin 10 causing inhibition of interleukin 12, we observed a reproducible decrease in interleukin 12 after incubation with anti-interleukin 10, which suggests that interleukin 10 has a role in upregulating interleukin 12 in our model. This effect needs further investigation to find out whether it is explant specific. Furthermore, the number of interleukin-10-immunoreactive cells after lipopolysaccharide stimulation was almost five times higher than the number of interleukin-12-immunoreactive cells (table); thus, interleukin 10 may be the dominant cytokine in lipopolysaccharide-induced inhibition of allergic inflammation.

Our results support the notion that the mucosa of young children, whatever their atopic status, is primed to respond quickly to bacterial products. We found that only 24 h after lipopolysaccharide stimulation, there was rapid proliferation and activation of CD3-positive T lymphocytes in the mucosa, which was associated with increased production of immunoregulatory interleukin 10. These results suggest that resident CD3-positive T cells in the mucosa of children are an important source of interleukin 10; previous studies have shown that T cells are the predominant source of this cytokine.²¹ As we have

suggested in non-atopic children,¹⁰ we now have evidence that these cells are naturally occurring, local CD4-positive CD25-positive immunoregulatory T cells, which in our model are likely to be the major source of interleukin 10. These cells are thought to be involved in controlling Th2 responses.²² Furthermore, Caramalho and colleagues¹⁵ have shown that TLR4 is present on murine CD4-positive CD25-positive T regulatory cells. Interleukin 10 is a strong inhibitor of allergen-specific T-cell proliferation,²³ and interleukin 10 from T cells downregulates Th2-induced airway hyper-responsiveness and inflammation in mice.²⁴ We postulate that the increase in immunoregulatory interleukin 10 along with upregulation in the expression of CD4-positive CD25-positive and TLR4-positive interleukin-10-positive cells after lipopolysaccharide exposure controls allergen-specific immune responses in atopic children. This innate ability to develop a quick response to lipopolysaccharide may be the mechanism for the normal maturation of the immune response, and the lack of stimulation with lipopolysaccharide might lead to the development of an allergic phenotype.

The lipopolysaccharide-induced increase in CD3 immunoreactivity and associated increase in interleukin 10 mRNA expression in atopic mucosa from children was not observed in atopic adults. The nasal mucosa of atopic adults had very low basal expression of TLR4 compared with atopic or non-atopic children or non-atopic adults. This finding is interesting because TLR4 expression has been associated with lipopolysaccharide sensitivity²⁵ and could explain the lack of response to lipopolysaccharide in atopic adults and the greater lipopolysaccharide responsiveness in children than in adults. Our results also accord with the findings of Renshaw and colleagues²⁶ that TLR expression and function decline with age in murine macrophages.²⁶ Although previous studies have suggested an important role for TLR4 in lipopolysaccharide responsiveness in human beings²⁷ and an association between mutations in TLR4 and differences in responsiveness to lipopolysaccharide,²⁸ these studies did not differentiate between responsiveness among atopic and non-atopic adults.

Our findings suggest that the protective effect of lipopolysaccharide in atopic children is likely to be driven by TLR4. This conclusion is supported by our finding of a similar response with a purified lipopolysaccharide preparation selective for TLR4 and not TLR2 or any other TLRs. In mammals, TLR4 is the main receptor for lipopolysaccharide-induced signal transduction and forms a receptor cluster with CD14 on stimulation with lipopolysaccharide.²⁹ There is evidence of low expression of TLR4 in adult T cells;³⁰ TLR4 expression in children has not been studied previously. We found that TLR4 is expressed by CD3-positive T cells in atopic nasal mucosa from children and that TLR4 colocalises with interleukin 10 protein. The dose-dependent increase in TLR4 immunoreactivity after lipopolysaccharide stimulation is further evidence that the TLR4-dependent pathway is activated in children. Indeed, Cohen and colleagues³¹ have shown that adults can respond to lipopolysaccharide via both CD14-dependent and CD14-independent pathways, but children respond only via the CD14-dependent pathway,³¹ possibly because serum from neonates and young children lacks or has a non-functional non-CD14 plasma protein required for activation of the CD14-independent pathway.³¹ Thus, there are important differences in the capacity of adults and children to respond to bacterial lipopolysaccharide, which may have a crucial role in maturation of the immune system.

Two studies with TLR4 knockout mice have shown that lipopolysaccharide is involved in normal development of Th2-driven immune responses to allergen.^{32,33} Moreover, they found that although a low dose of lipopolysaccharide (0.1 µg) in sensitised mice was necessary to induce normal Th2 responses to allergen, high doses (100 µg) induced neutrophilia and a Th1-like immune response.³² Although we have used a similar low dose of lipopolysaccharide in our explant model, there are several possible reasons for the difference between these observations and ours. In addition to species effects, one explanation is that we used tissue from atopic children, whereas those studies used sensitised and allergen-challenged TLR4-deficient adult mice. As we have shown, there is a clear difference between adults and children in the ability to respond to lipopolysaccharide. Furthermore, although the role of TLR4 in the development of the normal innate immune response has been firmly established, little is known about the effects of genetic deletion of TLR4 on the normal maturation of the developing immune system. Our model is separated from the systemic circulation so does not allow cellular recruitment to occur; the findings reflect a locally mediated inflammatory process. We have used tissue obtained by nasal biopsy, not bronchoalveolar lavage washings from the lungs; the lipopolysaccharide administered in those studies was inhaled whereas in ours it was given in a culture medium and incubated simultaneously with the tissue.

We have shown that bacterial lipopolysaccharide can prevent local allergen-induced allergic inflammation in the nasal mucosa in atopic children by downregulation of local Th2 cytokines and upregulation of Th1 cytokines in the tissue, proliferation and activation of T cells positive for CD4, CD25, and TLR4, and increased expression of interleukin 10. These events are brought about through the TLR4-dependent pathway. TLR4 is an important bridge between innate and adaptive immunity, potentially driving the molecular mechanisms governing the "hygiene hypothesis" and helping to explain why low exposure to bacterial products might lead to delayed or skewed development of the immune system and more atopic disease.

Contributors

M K Tulic, D H Eidelman, and Q Hamid developed and designed the study. M K Tulic carried out laboratory work, data collection and analysis, and wrote the report. P O Fiset contributed to interpretation of data and assisted in writing of the discussion. D H Eidelman made constructive comments and helpful suggestions throughout this study. J J Manoukian, S Frenkiel, and F Lavigne are the ear, nose, and throat surgeons who collaborated with us to obtain nasal biopsy samples from both adult and paediatric patients.

Conflict of interest statement

None declared.

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