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Signal transducer and activator of transcription 6 down-regulates toll-like receptor-4 expression of a monocytic cell line

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Summary

Background Toll-like receptor 4 (TLR4), part of the bacterial lipopolysaccharide (LPS) receptor, is an important bridge between innate and adaptive immunity. Our previous studies have indicated reduced expression of TLR4 and reduced responsiveness to LPS in nasal mucosa of atopic adults compared with non-atopic adults. IL-4 and signal transducer and activator of transcription 6 (STAT6), which are increased in atopic patients, may have a role in modulating TLR4.

Objective To examine direct effects of IL-4 and STAT6 on TLR4 expression of U-937 monocytic cells.

Methods LPS responsiveness, under different conditions of U-937 cells was measured by nuclear factor (NF)- κ B activation of transcription. TLR4 mRNA was quantified by real-time PCR and TLR4 surface expression was measured by flow cytometry. The promoter and 4.3 kb of the upstream region of TLR4 were cloned into a plasmid vector and transiently transfected into U-937 cells. Transfected cells were incubated with IL-4 and transcriptional activity was assayed by the luciferase assay. STAT6 was transfected to evaluate overexpression of this transcription factor. Cells were also incubated with Tyrphostin AG490 to inhibit tyrosine kinases.

Results NF- κ B activation by LPS was inhibited by IL-4 pre-incubation but not when IL-4 was added at the same time as LPS. TLR4 mRNA expression was inhibited by IL-4 as early as 6 h but the effect was lost by 24 h. Surface expression of TLR4 was inhibited by IL-4 at 12 and 24 h, but returned to baseline at 48 h. IL-4 inhibited activity of the TLR4 promoter as early as 6 h, but, like the mRNA, these effects were transient. STAT6 overexpression enhanced the inhibition of the TLR4 promoter and prolonged it. Inhibition of TLR4 by IL-4 was abolished by pre-incubation with the tyrosine kinase inhibitor Tyrphostin AG490.

Conclusion Our findings demonstrate that IL-4, through STAT6, can modulate TLR4 expression and suggests that Th2 cytokines can impact on the LPS responsiveness of cells.

Keywords atopy, IL-4, monocytes, STAT6, TLR4

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Introduction

The incidence of allergic diseases such as atopic asthma and rhinitis is increasing worldwide. The hygiene hypothesis suggests that exposure in early life to pathogen-associated molecular patterns (PAMPs) reduces potential allergic atopy through the development of immune regulatory mechanisms. One such PAMP, bacterial lipopolysaccharide (LPS), is a component of the outer membrane of Gram-negative bacteria, and binds to the Toll-like receptor 4 (TLR4) complexes, composed of TLR4, CD14, and MD2 proteins. LPS, also known as endotoxin, is a

potent immunostimulatory molecule found ubiquitously in nature. Several studies support the notion that exposure to endotoxin early in life is protective against the development of atopic diseases [1–3]. We have recently investigated the effects of LPS on explanted nasal mucosa of young children and found a decrease in allergen induced inflammatory cytokine production [4].

We have previously shown that children, regardless of their atopic state, express high levels of TLR4 positive cells in their nasal mucosa [4]. In comparison, adults displayed less TLR4-positive cells in their nasal mucosa compared with children. Moreover, atopic adults, compared with

non-atopic adults, had significantly less TLR4-positive cells in their mucosa suggesting some mechanism present in atopic individuals that down-regulates this receptor. It has been shown that the Thelper type 2 (Th2) cytokine IL-4 decreases surface expression of TLR4 on human peripheral blood monocytes [5]. IL-4 can also decrease LPS responsiveness *in vitro* in human monocytic cells [6]. As Th2 cytokines are also highly expressed in the nasal mucosa of atopic asthmatics [7], they may play a role in modulating responses to LPS *in vivo*.

IL-4 signal transduction involves Jak-mediated phosphorylation and activation of signal transducer and activator of transcription 6 (STAT6). STAT6 is a transcription factor that can activate many genes involved in allergic inflammation such as IgE and eotaxin. We have also shown elevated STAT6 expression in the nasal mucosa of allergic rhinitis (AR) [8]. Additionally, STAT6 can inhibit several inflammatory genes, including interferon regulatory factor-1 (IRF-1) [9], TNF- α and IL-12p40 [10]. However, it has not been investigated whether this transcription factor can inhibit expression of genes involved in innate immunity, such as TLR4.

This study was conducted to delve into the mechanism of IL-4 inhibition of TLR4. We used the U-937 monocytic cell line that expresses high levels of TLR4 and is responsive to IL-4. LPS activation of nuclear factor (NF)- κ B was partially inhibited by IL-4 pre-incubation but not when IL-4 was added at the time of LPS challenge, suggesting that IL-4 down-regulates LPS signal transduction machinery. It has been shown that the LPS response is proportional to the expression of TLR4 [11]. We indeed measured down-regulation of TLR4 by IL-4, but this effect was transient in our cell system at the level of promoter activity, mRNA expression and protein surface expression. Suppression of tyrosine phosphorylation inhibited IL-4's effects on TLR4, and overexpression of STAT6 led to prolonged and greater inhibition of TLR4. Together these findings show that STAT6 is required for IL-4's inhibition of TLR4 expression and may affect LPS responsiveness of U-937 cells.

Materials and methods

Reagents

Recombinant human IL-4 was purchased from R&D Systems (Minneapolis, MN, USA) and Tyrphostin AG 490 (2-Cyano-3-(3,4-dihydroxyphenyl)-N-(benzyl)-2-propenamide) from Sigma-Aldrich (St Louis, MO, USA). Cell viability was assessed by the trypan blue exclusion assay and was found to be $95 \pm 5\%$ for all experiments performed.

Cell line and treatment

U-937, a human monocytic cell line derived from a histiocytic lymphoma [12] was cultured in RPMI 1640

supplemented with 2 mM *L*-glutamine, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% fetal calf serum. Cultures were maintained between 0.5×10^5 and 1.5×10^6 cells/mL, and all experiments were conducted when the cells were in log phase of growth. For IL-4 stimulation U-937 cells were seeded into 12-well plates with a density of 1×10^6 cells/mL and incubated with 10 ng/mL of IL-4. Where AG490 tyrphostin was used, the cells were pre-treated with 25 μ M AG490 in DMSO for 16 h before IL-4 incubation, as previously described [13].

Plasmid constructs and purification

4.3 kb of U-937 genomic DNA, containing the promoter region upstream of the human TLR4 gene, was amplified using the TaqPlus Precision PCR System from Stratagene (La Jolla, CA, USA) and the primers 5'-CTC CAT GGC ACA TTC TGC AGT AAA CTT GGA GGC-3' (sense) and 5'-CTG AGC TCC ACA TTC TGC AGT AAA CTT GGA GGC-3' (antisense) similarly to a previously described method [14]. PCR fragments were cloned into the plasmid vector pGEM-T (pGEM[®]-T Vector System; Promega, Madison, WI, USA) for subcloning purposes. The 4.3 kb fragment was directionally cloned into pGL3-Basic luciferase reporter vector (Promega) using *Nco*I and *Sst*I restriction sites. A pVL1393 baculovirus vector containing the full-length human STAT6 was obtained as a generous gift from Dr James Ihle (Saint Jude Children's Research Hospital, Memphis, TN, USA). The pcDNA-STAT6 vector was formed by subcloning into the mammalian expression vector pcDNA3.1 Hygro(+) (generous gift of Dr Benoit Barbeau; Laval University, Quebec, Canada) with the primers 5'-GGG GTA CCC CGG CAC GAC GGG AGA G-3' (sense) and 5'-GTT TCT AGA AAC CAC ATG TCC AG-3' (antisense) and the restriction enzymes *Kpn*I and *Xba*I. All vector constructs were verified by sequencing (The McGill University and Genome Québec Innovation Centre) and restriction enzyme digestion. All plasmids were purified by the EndoFree Plasmid Maxi Kit (Qiagen, Mississauga, ON, Canada) and endotoxin levels were assessed to be less than 0.125 EU/mL by the Limulus Amebocyte lysate gel clot assay (Biowhittaker, Walkersville, MD).

Transient transfections and luciferase assay

U-937 cells were seeded in fresh media a day before transient transfection. Transfections were performed according to manufacturer's instructions. Briefly, 1×10^6 U-937 cells were transfected using 6 μ L of Superfect Transfection Reagent (Qiagen), 1 μ g of TLR4 reporter plasmid or pGL3-Basic plasmid co-transfected with 0.03 μ g of *Renilla* control vector. The cells were rested for 24 h and stimulated with 10 ng/mL of IL-4. When pcDNA-STAT6 was used, 1×10^6 U-937 cells were transfected with 6 μ L of Superfect, 2 μ g of STAT6 expression vector or empty

pcDNA3.1, the TLR4 reporter vector and *Renilla* control vector. The cells were rested for 24 h and stimulated with 10 ng/mL of IL-4. After each time-point the cells were harvested and lysates were assayed for firefly and *Renilla* luciferase activity using the Dual Luciferase Reporter Assay System (Promega Technology) on a Lumat LB9507 luminometer (Berthold, Oak Ridge, TN, USA). The relative light units (RLU) were calculated by normalizing firefly luciferase activity against *Renilla* luciferase activity for transfection efficiency, and values were expressed as percent of the unstimulated control.

Nuclear factor- κ B assay

1×10^6 U-937 cells were transiently transfected with 6 μ L of Superfect and 1.0 μ g of pNF- κ B Luc (BD Biosciences, Mississauga, ON, Canada) and 0.03 μ g of *Renilla* control vector. pNF- κ B Luc is a plasmid containing four tandem copies of the NF- κ B consensus sequence. The cells were rested for 24 h and incubated in 10% fetal calf serum (FCS) with or without 10 ng/mL IL-4 for 24 h, and stimulated with 0.25 μ g/mL of LPS (Sigma-Aldrich; *Escherichia coli* O111:B4) for 6 h. RLUs were calculated by normalizing firefly luciferase activity against *Renilla* luciferase activity, and values were expressed as fold expression over unstimulated controls.

Real-time -Polymerase chain reaction

Total RNA was extracted using the RNeasy mini kit (Qiagen). 1 μ g of RNA was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Burlington, ON, Canada) according to the manufacturer's directions. cDNA was quantified using QuantiTect SYBR Green PCR Kit (Qiagen) and the primers for TLR4, 5'-CAA CCA AGA ACC TGG ACC TG-3' (sense) and 5'-GAG AGG TGG CTT AGG CTC TG -3' (antisense); and the house-keeping gene ALAS1, 5'-CCC ATG AGT TTG GAG CAA TC -3' (sense) and 5'-ACC CTC CAA CAC AAC CAA AG-3' (antisense). The copy numbers were calculated from a standard curve, obtained by plotting known input concentrations of five different log dilutions of purified pGEM-T plasmid with the cloned insert. Primers were designed in separate exons to avoid amplification of genomic contamination. Ratios of the number of copies of TLR4 vs. the number of copies of ALAS1 were calculated. Melting curve analysis and DNA gel electrophoresis were used to control for amplification specificity and primer-dimer formation.

Flow cytometry

After culture and treatments, U-937 cells were washed with staining buffer (Phosphate buffered saline (PBS) containing 1% FCS). Mouse IgG2a anti-human TLR4,

clone HTA125 (BD Pharmingen, Mississauga, ON, Canada) was added at a concentration of 2.5 μ g of antibody/ 1×10^6 cells. After washing with staining buffer, the cells were incubated with goat anti-mouse IgG2a-FITC (Santa Cruz, Santa Cruz, CA, USA) and immediately analysed with a FACSCalibur flow cytometer system using CellQuest Software (BD Biosciences). Background staining was determined by staining the cells with the secondary antibody only. Relative TLR4 surface expression on the cells was quantified by determining the mean fluorescent intensity (MFI), subtracting the background MFI and normalizing with the MFI of unstimulated controls.

Statistical analysis

Results are given as the mean $\pm$ SE from at least three separate experiments. Data were compared using ANOVA. When overall differences were identified, multiple contrasts with a post hoc Bonferroni's adjustment were used to identify which groups were significantly different. Statistical significance was defined as $P < 0.05$.

Results

Interleukin-4 inhibits lipopolysaccharide activation of Nuclear factor- κ B

LPS responsiveness of U-937 cells under different conditions was evaluated by monitoring activation of NF- κ B signal transduction pathway. U-937 cells were transfected with pNF- κ B which contains the luciferase gene controlled by 4 tandem NF- κ B consensus sequences. IL-4 did not activate the NF- κ B reporter plasmid (1.1 ± 0.1 -fold of unstimulated cells, Fig. 1). LPS caused a strong increase in NF- κ B reporter activity (3.0 ± 0.3 -fold of unstimulated cells). Cells pre-incubated with IL-4 had activation of the reporter plasmid (1.8 ± 0.1 of unstimulated cells), but this was significantly less than LPS alone ($P < 0.05$). Conversely, if cells were only given IL-4 at the time of LPS challenge, the response was as high as with LPS alone (2.8 ± 0.5 -fold of unstimulated cells).

Interleukin-4 inhibits toll-like receptor4 mRNA and protein expression

A decreased response to LPS suggests down-regulation of LPS receptor components. U-937 cells showed a high basal expression of TLR4 mRNA (Fig. 2a). Cells were incubated with 0.1–50 ng/mL of IL-4 for 12 h and the lowest dose required to reach maximum suppression of TLR4 mRNA was found to be 10 ng/mL. This is the dose used in all other experiments. When the cells were incubated with the optimal dose of IL-4, there was a rapid and significant reduction in the expression of TLR4 mRNA

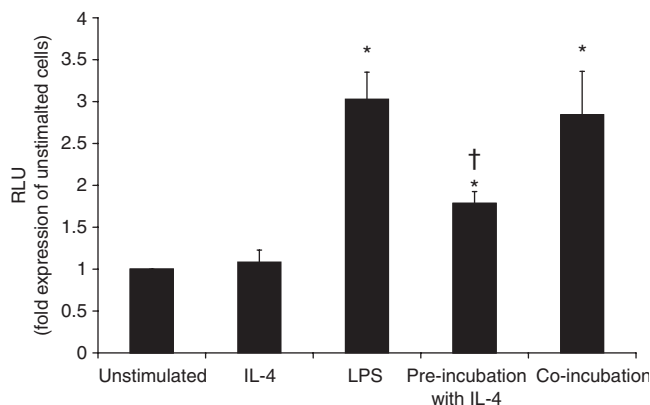


Fig. 1. IL-4 inhibits nuclear factor (NF)- κ B activation by bacterial lipopolysaccharide (LPS). U-937 cells were transiently transfected with pNF- κ B a vector containing four tandem copies of the NF- κ B DNA consensus sequence controlling the expression of the luciferase gene. A *Renilla* control vector was used to correct for transfection efficiency. Transfected cells were either left unstimulated, given 10 ng/mL of IL-4 (12 h), stimulated with 0.25 μ g/mL of LPS (6 h), incubated with IL-4 (12 h) followed by LPS stimulation (6 h) or stimulated with LPS and IL-4 (6 hours). Relative light units (RLUs) were obtained by performing the luciferase assay. Results are expressed as the RLU-fold expression over RLU of unstimulated cells. * P < 0.05 vs. baseline, † P < 0.05 vs. LPS treatment (n = 3).

as early as 6 h. Down-regulation of TLR4 mRNA was maintained at 12 h but was returned to baseline by 24 h.

Surface expression of TLR4 of U-937 cells was evaluated using flow cytometry. $94.8 \pm 5\%$ of the U-937 cells were positive for surface staining of TLR4 (Fig. 2b, histogram). IL-4 did not reduce the number of TLR4-positive cells, but reduced the individual cell surface TLR4 intensity measured by using MFI (Fig. 2b). TLR4 MFI was significantly reduced at 12 h ($91.9 \pm 0.9\%$ of MFI of unstimulated cells, Fig. 2b) and 24 h ($86.9 \pm 1.4\%$) but returned to basal levels by 48 h ($99.4 \pm 4.2\%$ of unstimulated cells).

Interleukin-4 and signal transducer and activator of transcription 6 inhibit toll-like receptor4 promoter transcriptional activity

To determine the effect of IL-4 on the promoter activity of the TLR4 gene, we cloned the proximal promoter region of the human TLR4 gene, ranging from 4.3 kb of the ATG start codon, into a luciferase reporter plasmid, forming the vector TLR4-pGL3. U-937 cells were transiently transfected with the reporter construct, and activity was five-fold higher than the promoter-less control vector pGL3-Basic. Incubation of transfected U-937 cells with IL-4 caused a decrease in reporter activity that occurred at 6 h ($67.0 \pm 13.7\%$ of cells without IL-4, Fig. 3a) and was maintained at 12 h ($71.7 \pm 15.4\%$) but was lost at 24 h ($87.6 \pm 12.7\%$).

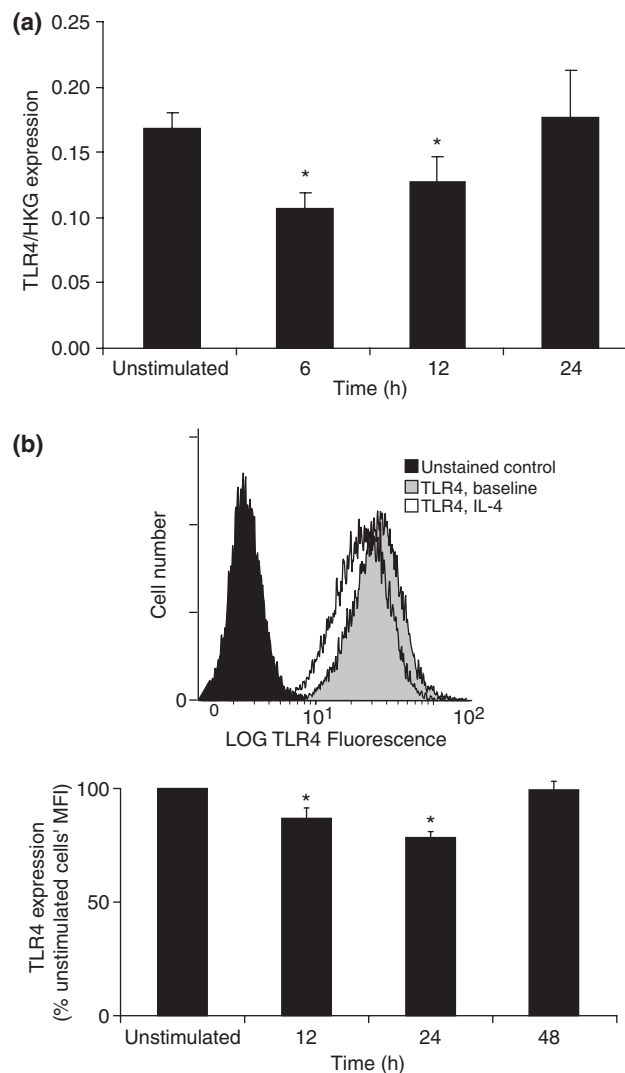


Fig. 2. IL-4 inhibits Toll-like receptor 4 (TLR4) mRNA and protein expression. (a) U-937 cells (1×10^6 cells/mL) were incubated with 10 ng/mL of IL-4 for 0–24 h. RNA was extracted at different time-points and TLR4 RNA and ALAS1 house-keeping gene were quantified by real-time quantitative PCR. Results are expressed as copies of TLR4 vs. copies of the house-keeping gene ALAS1. * P < 0.05 vs. baseline (n = 4). (b) U-937 cells (1×10^6 cells/mL) were incubated with 10 ng/mL of IL-4 for 0–48 h. Surface expression of TLR4 of U-937 cells was measured by flow cytometry using mouse IgG2a anti-human TLR4 and goat anti-mouse IgG2a FITC. The flow cytometry histogram shows a representative example of 24 h stimulation with IL-4 (open histogram) compared with unstimulated control (shaded histogram). Background fluorescence was determined by incubation of the cells with FITC-conjugated secondary antibody alone (filled histogram), and was subtracted from the sample mean fluorescent intensity (MFI)-values. Results are expressed as a percent of unstimulated cells for each time-point. * P < 0.05 vs. baseline (n = 3).

A human STAT6 expressing plasmid was created by subcloning STAT6 DNA into the constitutive CMV promoter containing vector, pcDNA3.1. This construct was transfected into U-937 cells to determine whether

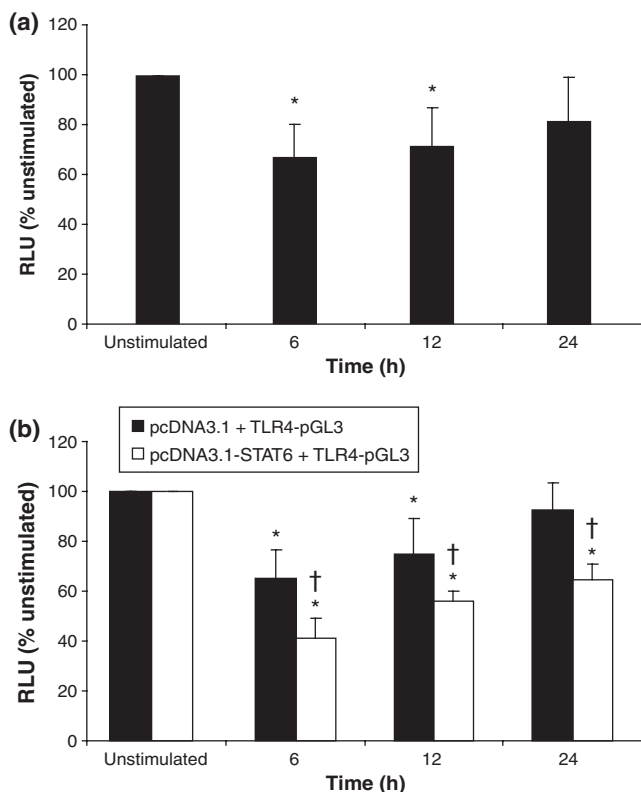


Fig. 3. IL-4 and signal transducer and activator of transcription 6 inhibits transcriptional activity of Toll-like receptor 4 (TLR4). (a) The 4.3 kb of the proximal promoter of the TLR4 gene was directionally inserted upstream of the luciferase gene in the vector pGL3-Basic. This new construct is defined as TLR4-pGL3. U-937 cells were transfected with the TLR4-pGL3 construct and the *Renilla* control vector and stimulated for 0–24 h with 10 ng/mL of IL-4. Relative light units (RLUs) were obtained by performing the luciferase assay. Results are expressed as a percent of RLUs of cells receiving IL-4 vs. the RLUs of unstimulated cells for each time-point. * $P < 0.05$ vs. unstimulated cells ($n = 4$). (b) pcDNA3.1 is a plasmid that contains no cloned sequences and is used as a negative control. pcDNA3.1-STAT6 is a vector that constitutively expresses wild-type human STAT6 mRNA. U-937 cells were transiently transfected with TLR4-pGL3 and pcDNA3.1 (solid bars) and with TLR4-pGL3 and pcDNA3.1-STAT6 (open bars). After transfections the cells were stimulated with or without 10 ng/mL of IL-4. Relative light units (RLUs) were obtained by performing the luciferase assay. Results are expressed as a percent of RLUs of cells receiving IL-4 vs. the RLUs of cells that were unstimulated for each time-point. * $P < 0.05$ vs. unstimulated cells, † $P < 0.05$ vs. pcDNA3.1 for respective time-points ($n = 4$).

overexpression of STAT6 would cause a greater inhibition of TLR4-pGL3 vector activity when the cells are incubated with IL-4. Empty pcDNA3.1 without STAT6 was used as a negative control. IL-4 caused a decrease in TLR4-pGL3 activity in cells receiving empty pcDNA3.1 at 6 h ($64.9 \pm 11.9\%$, Fig. 3b), at 12 h ($74.9 \pm 14.2\%$) but no decrease at 24 h ($94.5 \pm 10.9\%$). The effects of empty pcDNA3.1 were comparable with cells receiving TLR4-pGL3 alone (Fig. 3a). IL-4 caused a decrease in TLR4-pGL3 activity in cells transfected with pcDNA3.1-STAT6, and

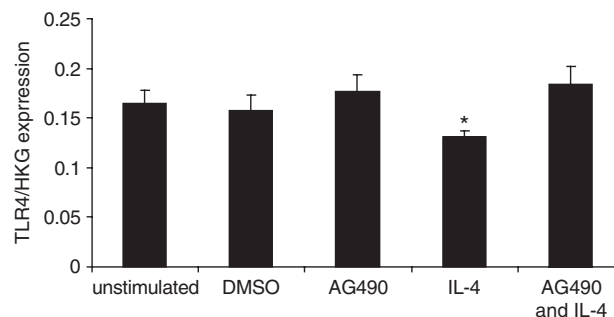


Fig. 4. Inhibiting STAT6 blocks IL-4 effects on Toll-like receptor 4 (TLR4). U-937 cells were pre-treated with Tyrphostin AG 490 for 16 h followed by 12 h of IL-4 stimulation. RNA was extracted and TLR4 RNA and ALAS1 house-keeping gene were quantified by real-time quantitative PCR. TLR4 RNA was normalized to ALAS1 RNA. * $P < 0.05$ vs. unstimulated cells ($n = 3$).

this was even greater than cells receiving empty pcDNA3.1. TLR4 reporter activity at 6 h was $41.0 \pm 8.1\%$ and at 12 h it was $55.7 \pm 4.5\%$ of unstimulated cells. Unlike pcDNA 3.1 vector transfections, at 24 h, pcDNA-STAT6transfected cells maintained lower activity ($64.3 \pm 6.6\%$) of the TLR4 reporter vector.

Tyrphostin AG490 blocks Interleukin-4 inhibition

Tyrphostin AG490, an inhibitor of Jak kinases, was used to block activation of endogenous STAT6. This allowed us to evaluate if blocking STAT6 would inhibit the effect of IL-4 on TLR4 transcription. Spiekermann et al. [15] have shown that AG490 can be toxic for U-937 cells, but with the dose of $25 \mu\text{M}$ that was used, the cell viability was $95 \pm 5\%$ as measured by Trypan blue exclusion. Incubation with DMSO (Fig. 4) or AG490 alone had no effect on TLR4 mRNA levels. As before there was a significant decrease in TLR4 mRNA at 12 h of incubation with IL-4. Pre-incubation of U-937 cells with AG490 abrogated the decrease caused by IL-4.

Discussion

In this report, we show that IL-4, present at high levels in atotics, can modulate the LPS responsiveness and TLR4 expression of U-937 cells. We have previously shown that cells of the nasal mucosa of atopic adults have a reduced expression of TLR4 and LPS responsiveness compared with non-atotics [4]. This suggests that factors, possibly Th2 cytokines, are present in atopic adults that down-regulate TLR4 expression. STAT6 a transcription factor activated by IL-4, is known to inhibit transcription of several inflammatory genes. In our model, IL-4 rapidly decreased the transcriptional activity of the upstream region of TLR4, TLR4 mRNA, TLR4 protein surface expression as well as LPS responsiveness. This suggests a

form of transcriptional regulation. IL-4's inhibitory effect on TLR4 was enhanced and prolonged if STAT6 was overexpressed and inhibition of tyrosine phosphorylation abolished the effect of IL-4 on TLR4 expression. This evidence points to the role of STAT6 in down-regulating TLR4 expression at the level of transcription.

In this study we used U-937 monocytic cell line provides many advantages as a cell model. They are easily transfectable, and are responsive to LPS and IL-4 similarly to primary blood monocytes. Other reports have shown that they produce TNF- α , IL-1 β , IL-10, MCP-1, and IL-6 upon LPS stimulation [6, 16–18]. IL-4 can reduce LPS-induced production of TNF- α , IL-10 and IL-1 β , but the mechanism has not been determined. Here, we show that cells pre-incubated with IL-4 have a reduced activation of NF- κ B upon LPS stimulation. The mechanism by which this occurs has not been determined, but it can be either through down-regulation of LPS receptor components, inhibition of signal transduction components or cross-talk between signal transduction pathways.

Substantial evidence from epidemiological studies has helped make headway in explaining the increasing prevalence of atopic diseases. Several studies have shown that bacterial LPS may be important for the development against atopy [2, 19, 20]. This suggests that exposure to LPS, or other PAMPs, may be necessary for optimal development of the immune system. We have shown that direct incubation of surgically resected human nasal mucosa with LPS inhibits allergen induced IL-4, IL-5 and IL-13 [4]. This effect seemed to be dependent upon induction of the cytokine IL-10. Down-regulation of TLR4 by IL-4, as in this report, may reduce the production of regulatory factors, like IL-10, thereby promoting allergic inflammation.

Other evidence shows that TLRs may have a role in the development of Th2 immune responses [21–23]. Low doses of LPS can promote immune responses, primarily through the activation of dendritic cells. In this context, IL-4 may limit inflammatory responses to LPS by negative feedback. LPS would induce IL-4, which would repress TLR4 and ultimately reduce responses to LPS.

In vitro, IL-4 can inhibit LPS-induced human monocyte production of IL-1, TNF- α , IL-6, IL-12 and IL-10 [6, 24–27]. *In vivo*, IL-4 has been extensively studied as an agent capable of preventing sepsis. One report showed that mice given recombinant IL-4 were capable of surviving lethal sepsis-causing doses of *Pseudomonas aeruginosa* [28]. We show here that the mechanism by which IL-4 mediates these functions may be related to down-regulation of TLR4. In other studies, the timing of incubation was shown to be essential for inhibition of LPS responsiveness [24, 28, 29]. Here we show that pre-incubation of cells with IL-4 reduced LPS responsiveness, but co-incubation has no effect. The time of incubation with IL-4 coincides to the period where there is a greatest decrease

in TLR4 surface expression. This suggests that time is required for IL-4 to inhibit LPS signaling machinery. TLR4 mRNA and transcriptional activity were decreased early, but it was only after 12 h that surface expression of TLR4 was significantly decreased. This may be because of the time it takes for existing TLR4 to be degraded and replaced by nascent TLR4. It is possible that IL-4 and STAT6 can inhibit other components of the LPS signaling complex and this remains to be clarified. It has been shown that IL-4 can reduce CD14 surface expression in monocytes but the mechanism has not been determined or the effects on LPS responsiveness [30].

IL-4 functions by binding to the IL-4 receptor, which consists of an IL-4R α subunit and the γ common (γ_c) chain subunit. Binding of IL-4 activates Jak kinases which phosphorylate tyrosine residues of the intracellular domain of the receptors. STAT6 is recruited to the receptor and is phosphorylated by Jaks. Phosphorylated STAT6 homodimerizes, migrates to the nucleus, binds to STAT6-response elements and once there it regulates transcription. Many genes activated by STAT6 are important for allergic inflammation, and STAT6-deficient animals have defective IL-4 responses [31]. As well as an activator of transcription, STAT6 has been shown to inhibit transcription of many genes [32]. STAT6 can inhibit transcription by competing for binding of DNA response elements with STAT1 [9] and NF- κ B [33]. STAT6 can also inhibit transcription by competing with other transcription factors for co-activators of transcription. STAT6 has been shown to play an important defense against sepsis, most probably by controlling expression of inflammatory genes. STAT6-deficient mice have uncontrolled production of cytokine and chemokine production when given LPS [34]. These mice also succumbed to sub-lethal doses of LPS compared with their wild-type counterparts. We have shown using the Jak inhibitor, AG490 tyrphostin, that tyrosine phosphorylation is important for IL-4 to inhibit TLR4 mRNA expression. By transfecting U-937 cells with STAT6 expressing plasmid, we have shown that STAT6 inhibits the 5'-upstream region of the TLR4 gene to activate transcription. Altogether this shows that IL-4 activates STAT6 to inhibit TLR4 expression, but by which mechanism STAT6 functions remains to be determined.

IL-13, another Th2 cytokine, sharing the IL-4 receptor components, can activate STAT6 and be protective against sepsis. Similarly, IL-13 has been shown to reduce LPS-induced IL-6, IL-8, IL-1 β and TNF- α [35–37]. Although this study was largely focused on IL-4, preliminary results showed that IL-13 was capable in decreasing U-937 cell expression of TLR4 mRNA in a time and dose-dependent manner (data not shown). These effects are most likely mediated by STAT6 as well. Other cytokines, such as GM-CSF and TGF- β , may have a role in the regulation of TLR4 with IL-4 and IL-13. When used in combination with IL-4, GM-CSF and TGF- β differentiate monocytes into

Langerhan cells, decrease TLR4 expression and LPS responsiveness [38].

IL-4 rapidly decreased but did not abolish TLR4 expression. Rapid decrease suggests direct inhibition by STAT6 rather than transcription of indirect inhibitors. Moreover, incomplete inhibition as well as a transient effect seen suggests that IL-4 and STAT6 fine tune responses rather than completely suppress TLR4. The effect on TLR4 surface expression was modest but significant and was smaller than what was observed in primary monocytes [5]. This effect may be related to cell-type differences; U-937 cells may be refractory to IL-4 treatment over time. Indeed, it has been reported that there is less STAT6 phosphorylation and less γ_c chain expression in monocytes after incubation with IL-4 [6]. This may explain why overexpressing STAT6 prolonged IL-4's inhibition of the upstream region of TLR4. Alternate mechanisms to down-regulation of signal transduction include activation of endogenous inhibitors of STAT6 such as Src homology-containing phosphatase-1 (SHP-1) [39], protein phosphatase 2A (PP2A) [40] and suppressor of cytokine signalling-1 (SOCS-1) [41]. Other mechanisms have been proposed for the regulation of STAT6 activity. Removal of a methyl group on Arg²⁷ of STAT6 decreases phosphorylation of STAT6 and nuclear translocation [42]. Regulation of STAT6 has shown to be more complex than once believed, and whether these play a role in the transient nature of IL-4 effects in our model has yet to be determined.

Increasing importance is being attributed to environmental factors in promoting or preventing the development of allergic inflammation. We show that allergic inflammation may affect the response to LPS. Detailed reports on the transcriptional regulation of TLR4 have not been done, with reports only on basal transcription factors [14] and transcription under conditions of hypoxia [43]. Here we show that STAT6 down-regulates TLR4 expression. This response is temporary and may be cell specific. Nevertheless, this may be another means by which Th2 cytokines can promote allergic inflammation; aside from stimulating pro-inflammatory mediators, IL-4 can down-regulate the innate immune receptor TLR4, potentially affecting the immunoregulatory effect of LPS in allergic disease.

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