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Inflammation Research

Thymoquinone suppressses *in vitro* production of IL-5 and IL-13 by mast cells in response to lipopolysaccharide stimulation

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Abstract. *Objective:* Activated mast cells produce Th2 cytokines that regulate allergic inflammation. We have previously shown that thymoquinone (TQ) attenuated airway inflammation in a mouse model of allergic airway inflammation. The present study investigated whether TQ affects Th2 cytokine response in vitro in lipopolysaccharide (LPS)-activated rat mast cells, RBL-2H3.
Materials and methods: RBL-2H3 cells were stimulated for 12 h with 0.1 μg/ml LPS in the presence or absence of 10 μM TQ. Th2 cytokine production was measured in the culture supernatants by ELISA. The mRNA expression of IL-5, IL-13 and GATA transcription factors was determined by RT-PCR. The expression of the transcription proteins c-Fos, c-Jun and phospho-c-Jun were determined by western blotting. The in vivo binding of GATA, AP-1 and NF-AT transcription factors to IL-5 promoter was assessed by chromatin immunoprecipitation analysis.
Results: TQ significantly ($p < 0.05$) inhibited LPS-induced IL-5 and IL-13 mRNA expression and protein production. However, TQ did not affect IL-10 production. GATA transcription factors are involved in the transcription of IL-5 and IL-13. TQ had no effect on the expression of AP-1 protein subunits, c-Jun and c-Fos, but markedly reduced the transcription of GATA-1 and -2 genes. Chromatin immunoprecipitation revealed that GATA, AP-1 and NF-AT binding to IL-5 promoter was induced by LPS stimulation and that TQ inhibited GATA binding at the IL-5 promoter but did not affect AP-1 and NF-AT binding.
Conclusions: These results suggest that TQ inhibits LPS-induced proinflammatory cytokine production in RBL-2H3 cells by blocking GATA transcription factor expression and promoter binding which demonstrates the anti-inflammatory effect of TQ.

Key words: Th2 cytokines – Thymoquinone – Inflammation-mast cell – Transcription factors

Introduction

Mast cells and their cytokines play a pivotal role in establishing the characteristic inflammatory processes occurring in many diseases such as allergic asthma [1], inflammatory bowel disease [2], and rheumatoid arthritis [3]. These cells produce several inflammatory mediators involved in allergic inflammation as wells as neutrophilic inflammation in response to bacterial infection [4, 5].

Mast cell activation is triggered by the cross-linking of the high affinity IgE receptor and by the bacterial lipopolysaccharide (LPS) [6, 7]. It has recently been reported that many of the T helper (Th)2-mediated inflammatory responses are influenced by LPS exposure [8]. LPS activates mast cells to produce Th2-associated cytokines such as interleukin (IL)-5, IL-13 and IL-10, which play a role in the pathogenesis of allergic inflammation [5, 6, 9, 10]. In addition, it has been shown that low level exposure to LPS exacerbates Th2-mediated inflammation of allergic airways [11, 12]. IL-5 and IL-13 mediate eosinophilic inflammation and mucus production [13-15], while IL-10 plays a regulatory and protective role [1]. Therefore, regulation of Th2 cytokines determines the characteristics of the immune and inflammatory response in allergic asthma.

Previous studies reported that thymoquinone (TQ), the main active principle of the volatile oil extract of *N. sativa*, has anti-inflammatory activities [16–18]. In addition, we have previously shown that thymoquinone (TQ) attenuated eosinophilic inflammation and Th2 cytokine production in the airways of allergic mice [19]. The present study demonstrates that TQ affects Th2 cytokine response in LPS-activated mast cells, RBL-2H3. TQ suppressed IL-5 and IL-13, but not IL-10, mRNA expression and protein production. TQ inhibited IL-5 and IL-13 cytokine production by blocking GATA transcription factor expression and binding to the cytokine gene promoter.

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Material and methods

Cell Culture and Reagents

The RBL-2H3 cells were cultured in minimal essential medium (MEM) with 10% fetal calf serum (Invitogen, Grand Island, NY) at 37ºC under 5% CO2 atmosphere, as described previously [20]. Cells were cultured overnight at 5×10^5 cells/well in 24-well plates and then stimulated for 12 h with 0.1 µg/ml LPS (*Escherichia coli* 055:B5; Sigma, St. Louis, MO) in the presence or absence of 10 µM of TQ dissolved in 1% DMSO (Sigma). An equal volume of DMSO was added to the control cultures.

Cytokines Assay

The levels of IL-5, IL-13, and IL-10 were measured in the cultures supernatant using enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Recombinant IL-5, IL-13, and IL-10 were diluted and used as standards. Samples and standards were added to the coated plates and incubated at room temperature for 2 h. After washing, the secondary antibody-horseradish peroxidase conjugate was added for 1 h at room temperature. Following washing, the substrate solution was added and left at room temperature for 30 min. The reaction was stopped and then read at 450 nm. The samples were assayed in duplicates. The minimum detectable limit of the assay was 7 pg/ml for IL-5, 1.5 pg/ml for IL-13, and 4 pg/ml for IL-10.

Reverse Transcription (RT)- PCR

Total RNA was isolated from RBL-2H3 cells using TRIzol reagent, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Total RNA (2 µg) was reverse transcribed to cDNA using 200U Superscript transcriptase (Invitrogen), 100 pmol random primers, and dNTPs. The reaction was incubated at 42ºC for 1 h and then at 98ºC for 5 min. PCR was performed using primers sets corresponding to the murine IL-5, IL-13, IL-10, GATA-1, GATA-2 and GATA-3 genes, as follow: IL-5 forward, 5'-TCACCGAGCTCTGTTGACAA-3' and reverse, 5'-CCACACTTCTCTTTTTGGCG-3'; IL-13 forward, 5'-GACCCAGAGGATATTGCATG-3' and reverse, 5'-CCAGCAAAGTCTGATGTGAG-3'; IL-10 forward, 5'-ATGCCTGGCTCAGAC-3' and reverse, 5'-GTCCTGCATTAAGGTC-3'; GATA-1 forward, 5'-ATGGATTTTCCTGGTCTAGGGGC-3' and reverse, 5'-TCAAGAACTGAGTGGGGCGATCACG'; GATA-2 forward, 5'-GCAGAGAAGCAAGGCTCGC-3' and reverse, 5'-CAGTTGACACACTCCCGGC-3'; GATA-3 forward, 5'-CAGTCCGCATCTCTTCAC-3' and reverse, 5'-TAGTGCCCAGTACCATCTC-3'. The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was also amplified as an internal control: GAPDH forward, 5'- TTCACCACCATGGAGAAGGC-3' and reverse, 5'- GGCATGGACTGTGGTCATGA-3'. The PCR reaction contained 5 µl cDNA, 1 µM of each primer, 2.5 mM $MgCl_2$, 0.2 mM each dNTP and 0.04 U/µl Taq DNA polymerase. The PCR cycling conditions were: 1 cycle at 94ºC for 5 min; 35 cycle at 94ºC for 30 sec, 60ºC for 30 sec and 72ºC for 30 sec, and a final cycle at 72ºC for 10 min. Ten micrograms of each product were run on a 1.2% agarose gel and visualized by ethidium bromide staining. Images were scanned and densitometric analysis was performed by measuring the signal intensity of each band using National Institutes of Health Scion Image Software. The data were normalized to GAPDH expression and are expressed as fold change relative to medium control.

Western Blot Analysis

Expression of c-Jun, c-Fos and phosphorylated c-Jun was determined by western blotting. Unstimulated or stimulated cells were harvested and washed with PBS and resuspended in lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, and 1% Triton X-100). Cell lysates were cleared by centrifugation at 15,000 rpm for 15 min at 4 °C. Protein concentration was determined by the Bradford assay (Bio-Rad). Equal amounts (50 µg) of proteins were heated to 100ºC for 5 min in sample buffer, chilled on ice and then separated on 10% SDS-polyacrylamide gel. The proteins were transferred to polyvinylidene difluoride membranes (Pierce Biotechnology, Rockford, IL) and probed for 2 h with polyclonal antibodies specific to c-Jun and c-Fos (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. HRP was detected using a chemiluminescent detection reagent (Amersham Biosciences, Piscataway, NJ). The membranes were stripped and reprobed with monoclonal antibody specific to phosphorylated c-Jun (Santa Cruz Biotechnology).

Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed to assess the in vivo relative binding of transcription factors to the IL-5 promoter. Briefly, cross-linked chromatin was isolated, sheared, and then immunoprecipitated with antibodies against GATA-1, GATA-2 (R&D Systems), NF-AT1, NF-AT2, or c-Jun/AP-1 (Gene Tex, San Antonio, TX), using ChIP a assay kit according to

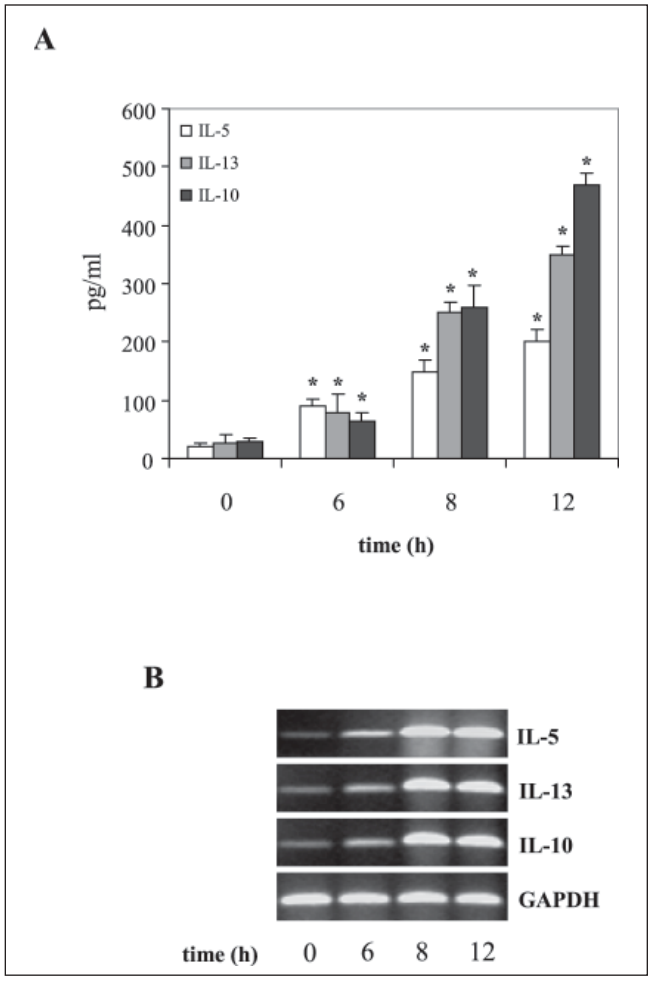

Figure 1. Th2 cytokine expression in RBL-2H3 cells. *A*, cells were stimulated with 0.1 µg/ml LPS for the indicated times and IL-5, IL-13 and IL-10 protein levels were measured in the culture supernatants by ELISA. Data are the mean ± SEM of three independent experiments. *, $p < 0.05$ compared with medium control. *B*, total RNA was isolated, reverse-transcribed to cDNA and analysed by PCR. The data are representative of three experiments.

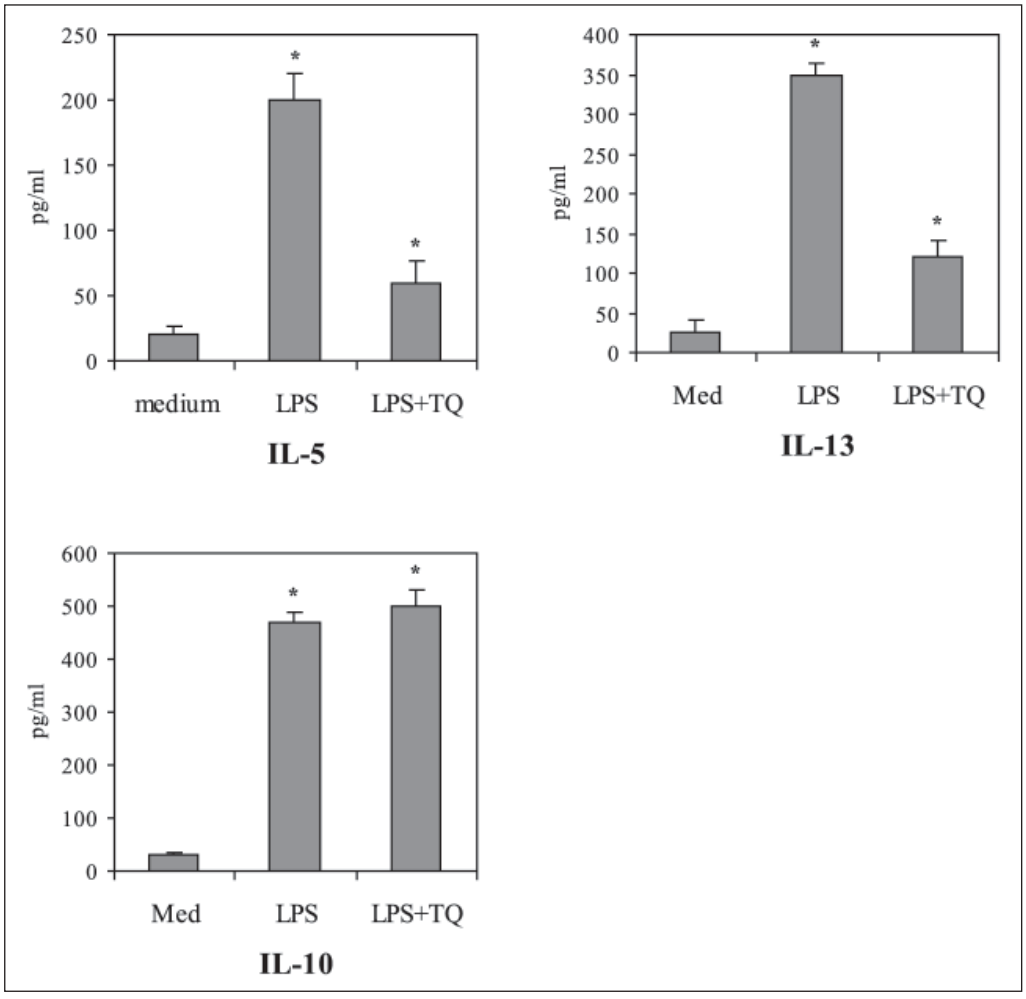

Figure 2. Effects of TQ on Th2 cytokine response in LPS-activated mast cells. RBL-2H3 cells were stimulated with 0.1 μg/ml LPS for 12 h in the presence or absence of 10 μM TQ in DMSO. An equal volume of DMSO was added to the control culture (medium). Culture media were collected and the levels of IL-5, IL-13 and IL-10 were measured by ELISA. Values are the mean ± SEM of three independent experiments. *, $p < 0.05$ compared with medium control.

the manufacturer's instructions (Upstate, Lake Placid, NY). An IgG-immunoprecipitated sample was included as a negative control. Cross-links were reversed overnight at 65 ºC and the DNA was purified and assayed for the presence of the IL-5 promoter sequences by PCR under the condition described for RT-PCR. Equal (μl) amounts of PCR products were run on 1.5 % agarose gel and visualized by ethidium bromide staining. The bands were quantitated as described above. The immunopreciptated DNA was normalized to the input DNA and the data are presented as fold change relative to medium control. The primers used for detecting the IL-5 promoter sequences were: IL-5 forward (5'-TAAGATATAGGCATTGGAA-3') and reverse (5'-5'AACGTTCTGCGTTTGC-3'. These primers amplified 165 bp sequences containing GATA, NF-AT and AP-1 binding sites of the IL-5 promter.

Statistical Analysis

Data were analysed by Student's *t* test and are expressed as mean ± SEM. Results with $p < 0.05$ were considered statistically significant.

Results

Th2 cytokine production in RBL-2H3 cells.

To investigate the effects of LPS stimulation on the kinetics of Th2 cytokine response in RBL-2H3 cell line, cells were stimulated with 0.1 μg/ml of LPS for 0, 6, 8 and 12 h. The IL-5, IL-13 and IL-10 protein production was measured by ELISA. Cytokine production was significantly induced by 6 h and reached a higher levels by 12 h (Fig. 1A). While the three cytokines were induced, cells produced more IL-10 compared to IL-5 and IL-13. Analysis of mRNA expression indicated that the IL-5 and IL-13 were induced by 6 h and peaked at 8–12 h (Fig. 1B). IL-10 followed a similar pattern, which increased and remained high by 12 h.

TQ suppresses LPS-induced IL-5 and IL-13 production in RBL-2H3 cells

To examine the effect of TQ on Th2 cytokine production, RBL-2H3 cells, a model of rat mucosal mast cells [21], were stimulated for 12 h with 0.1 μg/ml LPS. The IL-5, IL-13 and IL-10 proteins were measured in the culture supernatant by ELISA and the mRNA expression was determined by RT-PCR. LPS significantly induced production of the three cytokines (Fig. 2). When TQ was present in the culture for 12 h, the levels of IL-5 and IL-13 decreased markedly, while IL-10 level remained elevated, indicating that TQ does not influence the IL-10 production in RBL-2H3 cells.

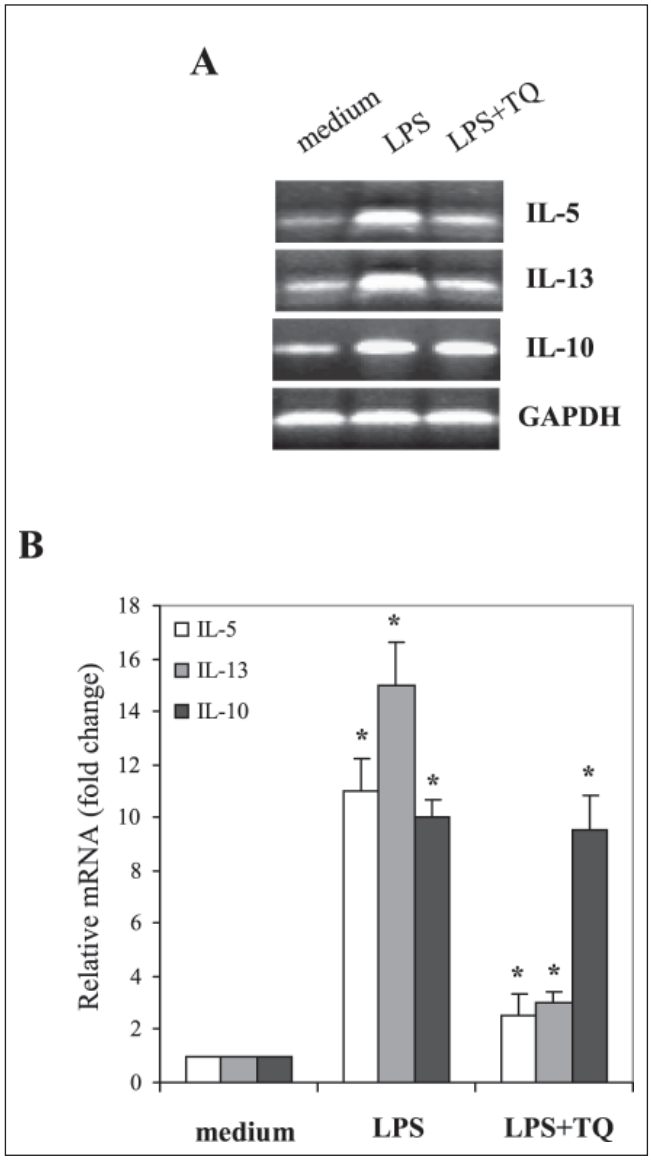

Figure 3. Effects of TQ on IL-5, IL-13 and IL-10 gene expression. *A*, RBL-2H3 cells were cultured and stimulated as described in the legend to Fig. 1. Total RNA was extracted and the levels of IL-5, IL-13 and IL-10 mRNA expression were determined by RT-PCR. The GAPDH gene was amplified as an internal housekeeping control. The data shown are representative of three independent experiments. *B*, densitometric analysis of the data presented in A. The cDNA bands were quantified, and the data were normalized to GAPDH expression and are presented as fold change relative to medium control (1 fold). Data are mean ± SEM of three experiments. * $p < 0.05$ compared with medium control.

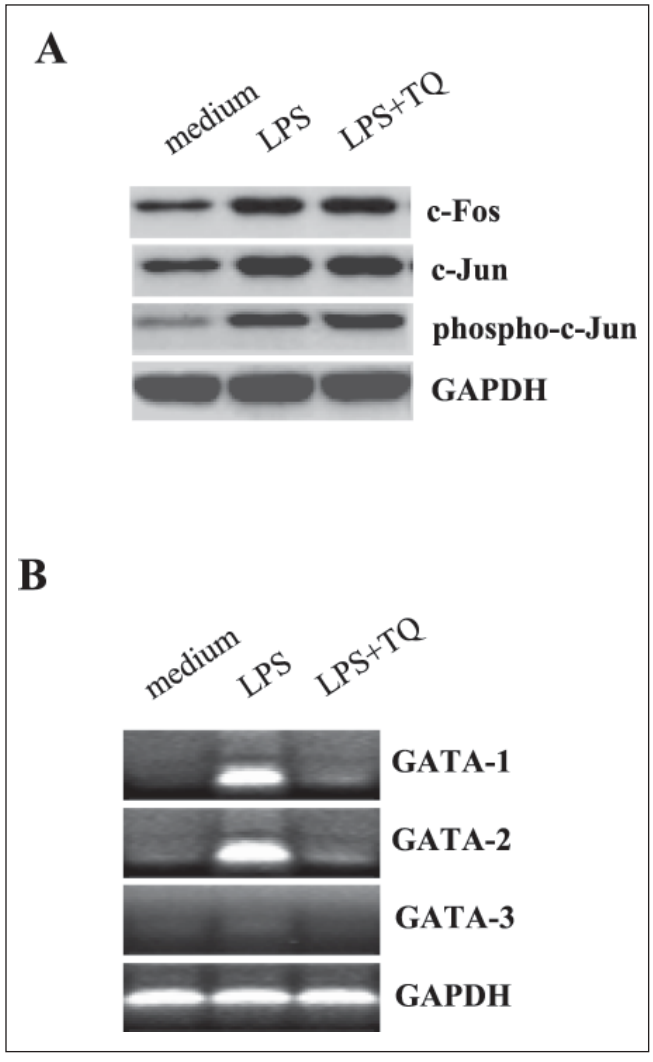

Figure 4. *A*, western blot analysis of c-Fos, c-Jun and phosphorylated c-Jun protein expression in LPS-stimulated RBL-2H3 cells. Cells were stimulated with 0.1 μg/ml LPS for 12 h in the presence or absence of TQ. Cell lysates were electrophoresed and immunoblotted with anti-c-Jun or anti-c-Fos antibody. The membranes were stripped and reprobed for phospho c-Jun. GAPDH protein was blotted as a control. The results are representative of two independent experiments. *B*, GATA transcription factor mRNA expression. Cells were treated as described in the legend to Fig. 2. The mRNA expression of GATA-1, -2 and -3 was determined by RT-PCR. The results are representative of three independent experiments.

To investigate whether the inhibition of IL-5 and IL-13 by TQ was mediated at the level of their gene transcription, mRNA expression of IL-5 and IL-13 was assessed by RT-PCR. As shown in Figure 3A, mRNA levels were increased following LPS stimulation. TQ markedly reduced IL-5 and IL-13 mRNA levels. However, TQ failed to affect IL-10 mRNA level. These results indicated that TQ affected IL-5 and IL-13 production at the level of their gene transcription

TQ does not affect AP-1 transcription factor.

The data presented above indicated that TQ inhibited the cytokine expression through a transcriptional mechanism. IL-5 and IL-13 promoters have binding sites for the AP-1 and GATA transcription factors [1, 22–25]. The two major components of the AP-1 protein complex are the c-Jun and c-Fos, which form a heterodimer and regulate transcription through the AP-1 binding site [26]. The effect of TQ on AP-1 was investigated by western blot analysis of c-Jun and c-Fos protein expression. The result (Fig. 4A) demonstrated that c-Jun and c-Fos protein expression was induced by LPS stimulation and that LPS activated phosphorylation of c-Jun.

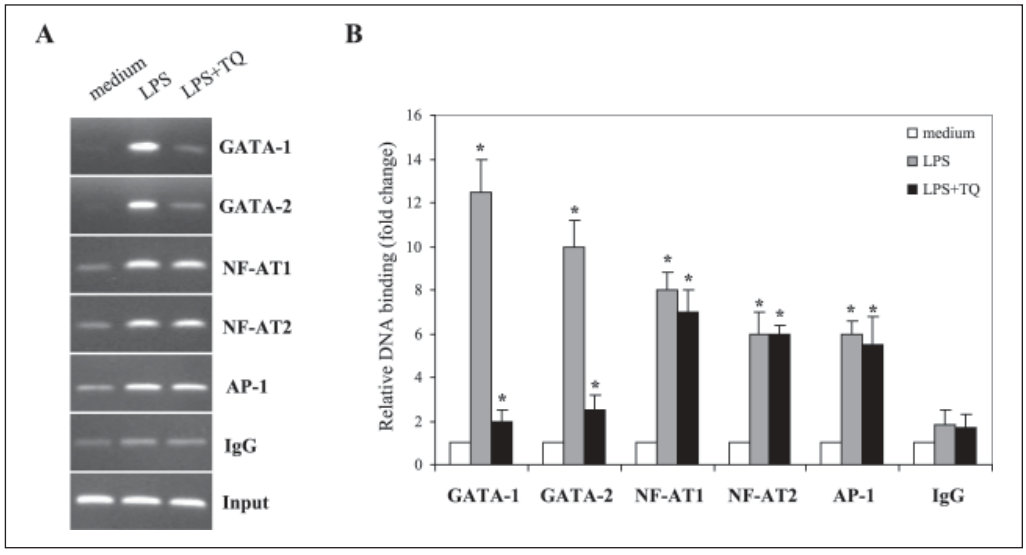

Figure 5. Chromatin immunoprecipitation analysis of GATA, AP-1 and NF-AT factor binding to IL-5 promotes. *A*, ChIP assay was performed as described in Material and methods. The immunoprecipitated DNA, along with the input DNA, was amplified by PCR. Equal amounts of DNA were separated on 1.2% agarose gel and visualized by ethidium bromide staining. IgG-immunoprecipitated DNA was included as a control. *B*, The band intensities were quantitated and normalized to input DNA. The data shown are the mean ± SEM of three independent experiments and are presented as fold change relative to medium control (1 fold). *p <0.05 compared with medium control.

TQ treatment did not notably affect expression of c-Jun or c-Fos. TQ also did not affect the phosphorylation and activation of c-Jun that was induced by LPS stimulation, as the amount of phosphorylated c-Jun remained unchanged after treatment with TQ.

TQ inhibits mRNA expression of GATA transcription factor

IL-5 and IL-13 gene expression has been shown to be regulated transcriptionally by GATA proteins in mast cells [22, 23]. To examine whether TQ inhibited IL-5 and IL-13 by affecting GATA gene expression, total RNA was isolated from control or LPS-stimulated RBL-2H3 cells and the level of GATA-1, -2, and -3 mRNA expression was measured by RT-PCR. LPS stimulation markedly induced GATA-1 and -2 mRNA expression (Fig. 4B). Meantime, GATA-3 was undetectable in RBL-2H3 cells. When TQ was added to the culture, the levels of GATA-1 and -2 were decreased markedly. These results indicate that TQ suppressed expression of GATA-1 and GATA-2 transcription factors which could, in turn, block proinflammatory cytokine gene expression.

TQ inhibits GATA, but not AP-1 or NF-AT, transcription factor binding to IL-5 promoter

The data presented above showed that TQ may attenuate IL-5 and IL-13 gene expression and protein production by targeting GATA transcription factors. Chromatin immunoprecipitation was performed to determine the effects of TQ on the in vivo binding of GATA to IL-5 promoter. The results (Fig. 5) demonstrated that GATA-1 and GATA-2 were not bound to the promoter in unstimulated cells. Upon LPS stimulation, a significant amount of both proteins was detected at the promoter. In addition, AP-1 and NF-AT, which have been shown to regulate IL-5 and IL-13 promoters [22, 27], were constitutively bound to the IL-5 promoter at very low levels. Their binding was significantly induced by LPS and remained bound in the presence of TQ. Together, these results suggest that TQ attenuates Th2 cytokine gene trancription by targeting GATA, but not AP-1 or NF-AT binding to the IL-5 promoter.

Discussion

The present study shows that TQ inhibits production of the pro-inflammatory Th2 cytokines, IL-5 and IL-13, in LPS-activated mast cells. TQ exerted its effect by blocking the GATA transcription factor gene expression and binding to these cytokine promoters.

The Th2-associated inflammatory cytokines, IL-5, IL-13 and IL-10, produced by mast cells modulate the immune and inflammatory response [1, 5, 8] and therefore are pivotal to the development of chronic allergic inflammation [28]. Recent studies have reported that Th2 inflammatory responses are influenced by LPS exposure [8]. Inhalation of antigen with LPS enhanced the inflammatory response in a mouse model of allergic airway inflammation [29, 30]. In addition, it has been reported that bone marrow-derived mast cells produce Th2 cytokines in response to LPS stimulation [1]. The current results show that IL-5, IL-13 and IL-10 mRNA and proteins are significantly induced in RBL-2H3 by LPS in a time-dependent manner, with a more robust increase in IL-10 seen at 12 h.

Regulation of Th2 cytokines production determines the characteristic immune and inflammatory responses observed during allergic reactions [31, 32]. Our previous study [19] demonstrated that TQ attenuated Th2 cytokine production and lung inflammation in a mouse model of allergic airway inflammation. The present study shows that TQ inhibited LPS-induced IL-5 and IL-13 mRNA expression and protein production. IL-5 plays a critical role in allergic inflammation by regulating the growth, activation and recruitment of eosinophils [13, 32]. IL-5 mRNA expression and protein production has been shown to be increased in the airways of asthmatics [33] and anti-IL-5 treatment was reported to have a profound effects on airway eosinophilic inflammation [34]. IL-13 plays a role in allergic inflammation [35, 36] by en-

hancing the effect of other inflammatory cytokines [32], regulating eosinophil functions and increasing airway epithelial cell mucus production [37, 38]. IL-5 production is increased in LPS-stimulated mast cells [1]. IL-10, on the other hand, has an immunomodulatory functions. IL-10 was shown to inhibit airway eosinophilic inflammation and to induce isotype switch from IgE to IgG4 [39–41], and LPS induced IL-10 expression in mast cells [1]. The current results show that TQ inhibited LPS-induced expression of IL-5 and IL-13, but not that of IL-10, thus demonstrating its differential anti-inflammatory effects in RBL-2H3 cells. Previous studies have demonstrated that IL-10 induction by LPS inhibited the production of proinflammatory cytokines in macrophages [42–44]. The finding that TQ did not block IL-10 expression further supports the protective role of TQ.

TQ inhibited IL-5 and IL-13 expression in RBL-2H3 cells by a mechanism involving GATA transcription factors. A GATA family member, GATA-3, has been shown to be critical for IL-5 and IL-13 transcription in T cells [45, 46]. GATA-3, however, is not expressed by bone marrow-derived mast cells [11], and the current results indicated that it is expressed at a very low level in activated RBL-2H3 cells. GATA-1 and GATA-2, on the other hand, are expressed in mast cells [47] and their gene expression is induced by LPS stimulation [11, 23]. Previous studies have reported that GATA-1 and -2 were critical for IL-5 and IL-13 gene transcription in mast cells [22, 23]. GATA proteins have been shown to be increased in the airways of asthmatics. Such increase was associated with upregulation of Th2 cytokine gene expression [48]. GATA inhibition has been shown to prevent airway inflammation in allergic mice [49]. These previous studies demonstrated the role of GATA transcription factors in the development of Th2-mediated inflammation. Here TQ inhibited GATA-1 and -2 mRNA induction by LPS. Such inhibition was concomitant to the decrease in IL-5 and IL-13 gene expression. It is not clear, however, whether TQ inhibited GATA expression through a direct effect on the gene transcription or by inhibiting an upstream regulator of GATA expression. The transcription factor STAT6 functions upstream of GATA and its activation is sufficient for the induction of Th2 cytokine expression in T cells [50], and STAT6 knock out mice do not produce Th2 cytokines [51, 52]. It is yet to be seen whether TQ attenuates GATA-1 and -2 expression by targeting STAT6. In addition, in vivo binding of GATA to the IL-5 promoter was markedly decreased after TQ treatment. This finding, however, does not necessarily indicate that TQ directly targets GATA binding to the cytokine promoter, because GATA mRNA expression was already inhibited by TQ and subsequently there should be less GATA protein available.

In addition to GATA, AP-1 and NF-AT transcription factors have been shown to be involved in the regulation of IL-5 and IL-13 expression in mast cells [22, 24, 53, 54]. AP-1 and NF-AT proteins functionally interact with GATA and regulate IL-5 and IL-13 promoters [22, 23, 54, 55]. While their synergistic interaction is required for cytokine gene induction, the specificity of transcriptional regulation is governed, at least in part, by GATA [56]. The results here showed that TQ did not affect the AP-1 protein subunit expression or AP-1 binding to the IL-5 promoter. Neither did TQ alter NF-AT binding to the IL-5 promoter. Although AP-1 and NF-AT bound to the IL-5 promoter in vivo in the presence of TQ, they were unable to drive cytokine transcription, likely because of the absence of GATA expression and binding due to the inhibition by TQ.

The present study shows that TQ inhibits IL-5 and IL-13 production in LPS-activated RBL-2H3 cells by inhibiting their gene transcription through blockade of GATA transcription factors, thus demonstrating its anti-inflammatory action in mast cells.

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