

Decreased levels of soluble Toll-like Receptor 2 in patients with asthma

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Abstract

Background: Recently, reports have indicated a role for the membrane form of Toll-like Receptor 2 (TLR2) in asthma pathogenesis. In this study we examined soluble TLR2 levels in serum and sputum of asthmatic and healthy subjects.

Methods: Serum and sputum samples were obtained from 33 asthmatic and 19 healthy subjects. The asthmatics were classified into four groups according to the Global Initiative for Asthma. A sandwich ELISA was developed to measure soluble TLR2 (sTLR2) in serum and sputum. TLR2 mRNA expression was determined by semi-quantitative RT-PCR of all sputum samples.

Results: The mean sTLR2 levels from serum and sputum of asthmatics were significantly lower than those from healthy subjects. Moreover, sTLR2 concentration decreased concomitantly with asthma severity. The differences observed, however, were not statistically significant. TLR2/GAPDH mRNA of sputum leukocytes was also significantly lower in asthmatics than in healthy subjects.

Conclusion: This study demonstrated for the first time that TLR2 levels are lower in serum and sputum samples from asthmatic than from healthy subjects, and this could be an indicator of TLR2 expression. We also found that sTLR2 concentration in serum decreased concomitantly with an increase of asthma severity clinical score.

Keywords: Asthma, Expression, TLR2 mRNA, Soluble Toll-like receptor

Introduction

Recently, the prevalence of asthma and allergies has increased dramatically in industrialized countries, and this increase cannot be explained by genetic changes. Respiratory infections have been linked to asthma in both preventative and exacerbating roles (1-2); hence, investigating the system that initially recognizes respiratory system pathogens seems imperative to the understanding of this inflammatory disease. Innate immunity is the first line of defense against pathogens such as bacteria and viruses. Recent reports have indicated the role of innate immune mechanisms in the regulation of allergic reactions (3). Toll-like receptor 2 (TLR2) as a pattern-recognition receptor plays a central role in the primary recognition of a

broad range of microbial components (4), and has been recently shown to have an important role in the prevention and exacerbation of asthma and other airway allergic inflammations.

In German farmers' offspring, TLR2 polymorphism was demonstrated as a major determinant of the susceptibility to asthma and allergies (5). Sukkar et al. (6) showed that airway smooth muscle cells (ASMCs) expressed TLR2 and other TLR mRNAs. Other reports verified the existence of functionally active TLR2 on human primary type II alveolar epithelial cells (7), in addition to the overexpression of TLR2 in subjects with neutrophilic asthma vs. other asthma subtypes and controls (8). LeBouder et al. found that blood monocytes released soluble forms of TLR2 (sTLR2) constitutively into human plasma and breast milk and that the

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kinetics of sTLR2 release increased upon cell activation. They also showed that depletion of sTLR2 from serum resulted in increased cellular responses to bacterial lipopeptide, the ligand for TLR2 (9). They suggested that sTLR2 has a regulatory effect on the immune system. In the present study, we investigated whether the complex humoral and cellular milieu of asthmatic lung affects TLR2 expression in peripheral blood and sputum leukocytes. To test this hypothesis, sTLR was measured in the serum and sputum of asthmatic and normal subjects. We also examined whether the sTLR2 level reflects TLR2 expression in sputum leukocytes.

Materials and Methods

Study subjects

Thirty-three adults with newly-diagnosed bronchial asthma who were not treated with inhaled steroids were selected for the study at the Respiratory Disease outpatient clinic of the Quaem Hospital of Mashhad, Iran. They were defined as asthmatic if they were diagnosed with asthma by a physician and had increased responsiveness to inhaled methacholine. The asthmatics had current respiratory symptoms and forced expiratory volumes in 1 s (FEV₁) above 70% of the predicted value. Asthmatics with histories of atopy and total serum immunoglobulin E (IgE) concentrations >200 IU/ml were defined as having allergic asthma (Table 1). Asthma was classified as mild intermittent (step 1), mild persistent (step 2), moderate persistent (step 3), or severe persistent (step 4), according to the Global Initiative for Asthma (GINA) (10).

The healthy subjects (N=19) were compared to the asthmatics accounting for age and gender. They underwent general and systemic clinical examinations to exclude current illnesses. We excluded subjects who had evidence of peripheral blood eosinophilia or elevated serum total IgE for age. After getting informed consent, peripheral blood samples were obtained from all participating subjects. This study was approved by the institutional review board.

Sputum Induction, Sputum Processing and Biochemical Assays

All subjects received 200 µg salbutamol before sputum induction. Sputum was induced with an aerosol of 3% hypertonic saline generated by an ultrasonic nebulizer (Heyer Mono, Carl. Heyer GmbH, Germany) for 10 min. Subjects were instructed to blow their noses and rinse their mouths with water before the procedure to avoid squamous cell contamination. The samples were collected into sterile plastic containers as soon as possible after induction.

Four volumes of 0.1% dithiothreitol (DTT) were added to the sputum. The sputum samples plus DTT were then mixed gently and centrifuged at 400 x g for 15 min. The suspension was next filtered through 48 µm nylon gauze (BNSH Thompson, Scarborough, Ontario, Canada) to remove cell debris and mucus. The resulting suspensions were centrifuged at 1,400 x g for 10 min at 4°C. The supernatants were aspirated, cell pellets were resuspended in one milliliter phosphate-buffered saline (PBS), and the supernatants and cell pellets were frozen at -70°C until use (11).

Total IgE was measured in sputum supernatants and sera using a commercially available ELISA kit according to the manufacturer's instructions (Radim, Pomezia Terme, Italy). Peripheral blood eosinophil and sputum cell counts were determined. sTLR2 in sputum supernatants and sera was measured using the ELISA method described below.

ELISA

To measure sTLR2 in sera, a sandwich ELISA was developed in our laboratory. Briefly, 0.2 µg of anti-human TLR2 mAb clone TL2.3 (Hbt; HyCult Biotechnology b.v., Uden, The Netherlands) in 100 µl carbonate buffer (pH 9.3) was incubated at 4°C overnight per well of a 96-well microtiter plate (Nunc MaxiSorp™, Fisher Scientific, Pittsburg, PA). Each well was then blocked for 1 h at 37°C with 150 µl of 2% Bovine Serum Albumin (BSA) in PBS, aspirated, and washed four times with 300 µl of PBS. Each well was then incubated for 1 h with 100 µl of sputum supernatant or serum at room temperature without shaking. Wells were

aspirated and washed three times with 300 µl of PBS. Each well was then incubated with 100 µl of 1% BSA containing 0.2 µg of a biotin-conjugated antihuman TLR2 (Hbt; HyCult Biotechnology b.v., Uden, The Netherlands). Wells were then washed under the same conditions followed by the addition of 100 µl of a 1:500 dilution of HRP-conjugated streptavidin (Bio-Rad, USA). After four washes, 100 µl of chromogenic substrate were added to each well and the plate was incubated for 30 min in the dark. Color development was stopped by the addition of 20 µl of 3 N HCl and the plate was read at 450 nm on an ELISA reader.

Isolation of total RNA and synthesis of cDNA

RNA was isolated from processed sputum samples using TriPure Isolation Reagent (Roche, Germany) according to the manufacturer's instructions. Total RNA was reverse transcribed (RT) into cDNA with MBI RevertAid (Fermentase, Life. Sciences, Lithuanian) according to the manufacturer's instructions, and cDNA samples were stored at -20°C.

RT-PCR

A semiquantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was carried out to determine of TLR2 mRNA expression. The oligonucleotide primer sequences used for PCR were TLR2 sense primer, 5'-GCT TAG TTC ATT AGG AAA CAG CAC A-3'; and TLR2 antisense primer, 5'-GCC CCA AAT ACT TTG CCT TG-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA was co-amplified with the target sequence and used as an internal PCR control. The GAPDH sense primer was 5'-GGC CAA GAT CAT CCA TGA CAA CT-3', and the antisense primer was 5'-ACC AGG ACA TGA GCT TGA CAA AGT-3'. The predicted sizes of the amplified genes were 172 bp for TLR2 and 461 bp for GAPDH. The RT-PCR mixture (final volume of 20 µl) contained 3 µl of cDNA, 10 µl of Qiagen Multiplex PCR Master Mix 2x (Qiagen, Germany) and 10 pmols of each complementary primer specific for TLR2 and GAPDH. All samples from

asthmatic and healthy subjects were amplified simultaneously and in duplicate with the same reagent master mix.

The samples were denatured at 95°C for 15 min and amplified using 35 cycles of 95°C for 30 s, 60°C for 80 s, and 72°C for 45 s, followed by a final elongation at 72°C for 3 min on a Corbett Research thermocycler (Sydney, Australia). Five microliters of the final amplification product were electrophoresed on 2% ethidium bromide-stained (EtBr) agarose gels and documented by the G-Box gel documentation system (Syngene, Cambridge, UK). The expression ratio of TLR2/GAPDH was estimated by Kodak 1D Image (Eastman Kodak) analysis. TLR2/GAPDH ratios were calculated for each sample.

Statistical analysis

Data are presented as mean±SE. The t test was used to analyze differences between the two groups. Comparison of three groups was performed using analysis of variance and Tukey test. A p-value<0.05 was considered significant.

Results

We investigated whether asthma is accompanied by changes in sTLR2 concentrations in serum and sputum.

Subject characteristics

Thirty-three newly-diagnosed bronchial asthmatics who were not treated with inhaled steroids and 19 healthy subjects were included in our analysis; 33.3% of asthmatics had severe persistent asthma (step 4), 33.3% had moderate persistent asthma (step 3), 21.2% had mild persistent asthma (step 2) and 9.4% had mild intermittent asthma (step 1). Total serum IgE concentrations in asthmatics were significantly higher than in control subjects ($p<0.001$); however, among asthmatics, there were no significant differences in total serum IgE or peripheral blood eosinophil counts between different steps of asthma severity.

Detection of sTLR2 in serum and sputum

We measured sTLR2 in sera in duplicate for each sample and calculated the average optical

densities (mean ODs). The mean sTLR2 levels in sera of asthmatics were significantly lower than those in healthy controls (mean ODs were 0.43 ± 0.076 and 1.19 ± 0.14 respectively, $P < 0.000$) (Fig. 1); however, there was no significant difference between allergic and non-allergic groups (mean ODs were 0.39 ± 0.08 and 0.49 ± 0.14 , respectively).

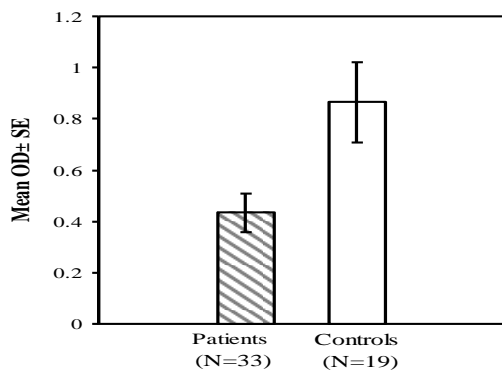


Fig. 1: The mean sTLR2 levels in serum samples of asthmatic and healthy subjects. Plots show the mean OD±SE.

There was no correlation between sTLR2 levels and FEV₁. Although lower sTLR2 levels were associated with the clinical progression of asthma, differences in sTLR2 ODs between clinical steps of asthma were not statistically significant (Fig. 2).

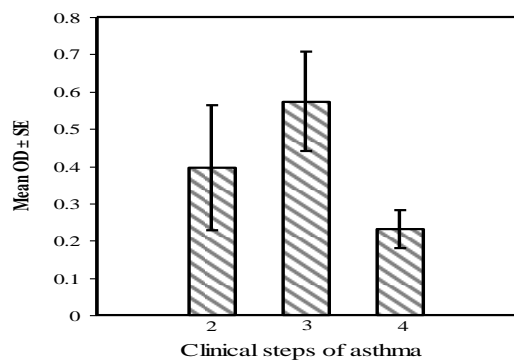


Fig. 2: The mean TLR2 levels among asthmatics at Steps 2, 3, and 4. Plots show the mean OD±SE.

The mean sTLR2 OD in sputum samples of asthmatics was significantly lower than that of healthy controls (mean ODs were 0.3319 ± 0.027 and 0.64 ± 0.06 respectively, $P < 0.05$). All subjects with detectable amounts of sTLR2 in sputum had high levels of sTLR2 in serum,

while sTLR2 in sputum was undetectable in subjects with moderate or low levels of sTLR2 in serum. There was no correlation between sTLR2 concentrations and the numbers of leukocytes in sputum.

Semiquantitative RT-PCR Results

To determine TLR2 gene expression, we established a semi-quantitative RT-PCR protocol using oligonucleotide primers specific for TLR2 and GAPDH. We measured TLR2 transcripts relative to GAPDH transcripts in sputum leukocytes.

The TLR2 mRNA expression ratios (mean ± SE) were 2.734 ± 0.62 for asthmatic patients and 11.5 ± 2.12 for healthy controls. Mean relative TLR2 expression ratios in asthmatics was significantly lower than that in normal subjects ($P = 0.0013$).

The abundance of TLR2 transcripts in sputum was similar to GAPDH transcripts in 26 of 33 asthmatics; however, in seven of the asthmatic subjects, TLR2 mRNA was 2- to 10-fold greater than that of GAPDH (Fig. 3). Interestingly, these seven patients also had relatively high levels of sTLR2 in serum. Furthermore, in 14 of 19 normal samples, TLR2 mRNA was 4- to 32-fold more abundant than GAPDH (Fig. 3). TLR2 mRNA/GAPDH mRNA in sputum leukocytes was also significantly lower in asthmatic than in healthy subjects.

Discussion

In this study, we provide evidence that the complex humoral and cellular milieu of asthmatic lung decreases TLR2 expression in peripheral blood and sputum leukocytes. This was demonstrated in two ways: first, sTLR2 in serum and sputum was lower in asthmatic than in healthy subjects; second, our semi-quantitative RT-PCR demonstrated that TLR2 mRNA expression in sputum leukocytes was significantly lower in asthmatic than in healthy subjects.

The finding that significantly less TLR2 mRNA is present in peripheral blood and sputum leukocytes of asthmatics than in healthy subjects agrees with the observations that less

TLR2 mRNA is present in allergic conjunctiva (12) and chronic rhinitis mucosa (13) than in comparable tissues from healthy subjects.

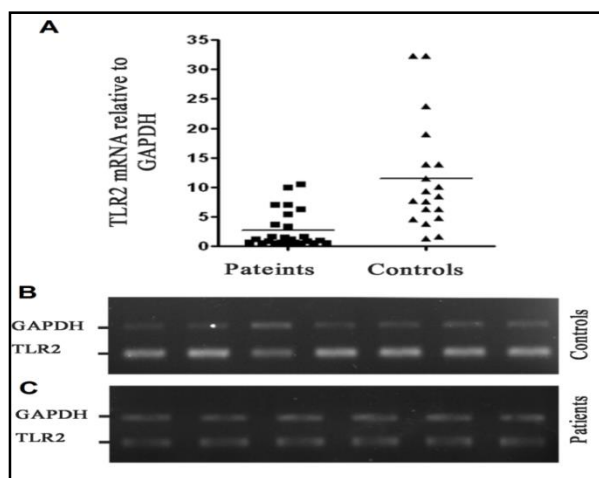


Fig. 3: TLR2 mRNA expression was determined relative to that of GAPDH mRNA in sputum of asthmatic and normal subjects. The graph shows each OD ratio obtained from the asthmatic and healthy subjects (A). EtBr-stained agarose gels of TLR2 and GAPDH RT-PCR products amplified from mRNA isolated from sputum leukocytes of normal (B) and asthmatic (C) subjects.

Down-regulation of TLR2 expression that leads to decreased TLR2 in body fluids could be explained by the humoral and cellular milieu of asthmatic lung. Cytokine milieu in the lung shows a bias towards Th2-related (14-16) as well as inflammatory, cytokines such as IL-1, TNF- α , and IL-6 (17), which down-regulate TLR2 expression on monocytes (18) and isolated granulocytes (19). In addition, Pipe *et al.* demonstrated that interleukin (IL)-1 β -stimulated human airway smooth muscle cells express and produce chemokines such as eotaxin and monocyte chemoattractant protein-1 (MCP-1) *in vitro* (20) through the mitogen activated protein kinase (MAPK) signaling pathway (21), which has a negative effect on TLR2 expression (22). Consistent with these data, we found that sTLR2 concentrations in serum decreased concomitantly with asthma severity according to the GINA (Fig. 2), although these differences did not reach statistical significance. However, further research is needed to clarify the involvement of sTLR2 in the pathogenesis and clinical manifestations of

allergic asthma. Another study revealed the same association between sTLR concentrations and progression of HIV and mycobacterium infections (23).

There is some evidence, however, that decreased TLR2 expression could be due to elevated microbial exposure (5, 24). Lauener *et al.* reported that TLR2 and CD14, but not TLR4, mRNAs were increased in Swiss farmers' children who had fewer allergies than non-farmers' children (3).

In the present study, we provide evidence that sTLR2 concentration could be an indicator of TLR2 expression. As indicated in Figure 3, our experiments show that a decrease in sTLR2 in sera is concomitant with a decrease in TLR2 in sputum leukocytes; therefore, if the clinical relevance of TLR2 expression in asthmatic patients is confirmed in future studies, soluble TLR2 could be a marker for asthma severity.

In conclusion, this study has shown for the first time that sTLR2 levels are lower in serum and sputum samples from asthmatics than from healthy subjects. These results were confirmed by RT-PCR. Thus, we hypothesize that the complex humoral and cellular milieu of asthmatic lung affects TLR2 expression in peripheral blood and sputum leukocytes. However, it remains unclear whether reduced sTLR2 contributed to asthma severity, or conversely, whether increased asthma severity leads to reduced sTLR2 expression.

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