TLR4 receptor and selected cytokines in development of food allergy in infants and small children

Rola receptora TLR4 i wybranych cytokin w rozwoju alergii pokarmowej u niemowląt i małych dzieci

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Słowa kluczowe: alergia, niemowlęta i małe dzieci, TLR4, cytokiny.

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Abstract

Introduction: Food allergy (FA) in infants and small children is an important and rising problem. TLR4 is the main receptor recognizing lipopolysaccharide (LPS) and belonging to the Toll-like receptor (TLR) family. Triggering this receptor leads to activation of nuclear factor κB (NF-κB) and interferon regulatory factors (IRFs) and expression of genes included in immunological reactions.

Material and methods: Fifty children (aged 1-36 months) were included in this study: 35 children with food allergy (17 of them with IgE-dependent allergy, 18 with IgE-independent allergy) and 15 healthy children without any allergy. In this work mRNA expression of TLR4 receptor on neutrophils and concentrations of selected Th1/Th2 cytokines were evaluated.

Results: We observed lower mRNA TLR4 expression in children with food allergy especially IgE-independent food allergy. Furthermore, higher concentrations of IL-4, IL-5 and IL-10 in children with food allergy compared with children from the control group were found. Estimated mRNA expression of TLR4 on neutrophil surfaces was negatively correlated with serum concentration of IL-4 and positively correlated with IFN-γ serum levels in children with IgE-dependent FA.

Conclusions: Results of this study suggest a role of innate immunity in the development of food allergy and evaluation of this immunity can be helpful in diagnosis of allergy in small children.

Streszczenie

Wstęp: Alergia pokarmowa u niemowląt i małych dzieci stanowi istotny i narastający problem. Receptor TLR4, będący głównym receptorem rozpoznającym lipopolisacharyd (LPS), należy do rodziny receptorów Toll-podobnych. Pobudzenie tego receptora powoduje aktywację jądrowego czynnika transkrypcyjnego (NF-κB) i czynników IRF, co następnie prowadzi do ekspresji licznych genów włączonych w odpowiedź immunologiczną.

Materiał i metody: Badaniami objęto 50 dzieci (wiek badanych mieścił się w granicach 1–36 mies.), w tym 35 z alergią pokarmową (17 z nich z alergią IgE-zależną, 18 z alergią IgE-niezależną) i 15 dzieci zdrowych bez objawów alergii. W przeprowadzonej pracy oceniono ekspresję mRNA TLR4 oraz stężenia wybranych cytokin Th1/Th2.

Wyniki: Stwierdzono niższą ekspresję mRNA TLR4 u dzieci z alergią pokarmową, bardziej zaznaczoną u badanych z grupy alergii IgE-niezależnej. Wykazano ponadto większe stężenia IL-4, IL-5 i IL-10 u dzieci z alergią pokarmową w porównaniu z grupą odniesienia. Przeprowadzona ocena korelacji pomiędzy ekspresją mRNA receptora TLR4 na neutrofilach krwi obwodowej a oznaczonymi stężeniami cytokin (IL-4, IFN-γ) w surowicy dzieci z alergią IgE-zależną wykazała ujemną korelację z IL-4, a dodatnią z IFN-γ.

Wnioski: Wyniki przeprowadzonych badań sugerują udział odporności naturalnej w rozwoju alergii pokarmowej oraz wskazują, że ocena tej odporności może być pomocna w diagnostyce alergii u niemowląt i małych dzieci.

Introduction

Food allergy is a major problem especially in infants and small children. Pathogenesis of allergic diseases is not yet very clear. It is determined by specific immune mechanisms. Such mechanisms can be classified into IgE-dependent allergy or IgE-independent allergy [1-6]. The prevalence of food allergy is not strictly defined; it is estimated from 0.5% to 12% in the general population and is most prevalent in infants and small children [6-9]. Efficiency and immaturity of different components of the gut barrier play a major role in this disease. Cow's milk allergy is most prevalent in early childhood [5-7, 10, 11]. The role of immunity in pathogenesis of allergic disease seems to be important, both innate immunity and acquired immunity [12-14]. Natural immunity is a germ line-encoded rapid response, the first line of host defence against invading pathogens [13]. Pathogen recognition receptors (PRRs) play a key role in this natural system [12, 13]. These receptors can recognize a wide range of ligands called PAMPs (pathogen-associated molecular patterns) [13, 15, 16]. Some of these receptors are transmembrane proteins like Toll-like receptors (TLRs), others are secreted such as collectins, and there are also recently identified intracellular microbial receptors: NOD-like receptors (NLR) [12, 17]. One of the most important groups of natural receptors is TLRs [12, 16, 18, 19]. TLR4 receptor recognizes endogenous and exogenous ligands such as Hsp60, Hsp70, RSV fusion protein, double-stranded RNA of viruses and lipopolysaccharide [19]. TLR4 is expressed on monocytes, neutrophils, lymphocytes and also in the spleen [20, 21]. Triggering TLRs leads to activation of transcription factors (nuclear factor κB – NF-κB) and (interferon regulatory factors – IRFs) as well as induction of cytokine genes included in immunological reactions [14, 18, 19, 22]. It is also noted that TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed extracellularly while TLR3, TLR7, TLR8 and TLR9 are localized intracellularly [12]. There are many data concerning TLRs [16, 19, 23] but not enough is known about expression of TLR4 on neutrophil surfaces in food allergy.

Cytokines play an important role in pathogenesis of allergic diseases, as they are important mediators of immunological reactions. Among them interleukin 4 (IL-4) plays a major role during the onset and course of allergic reactions, and IL-5 influences production, maturity and activation of eosinophils as well as IL-10, which is an important immunoregulatory cytokine [4, 6, 11, 18, 24, 25].

Aim

The aim of this study was to assess the role of TLR4 receptor and selected cytokines in pathogenesis of food allergy.

Material and methods

There were 50 children aged 1-36 months included in this study: 35 children suffered from food allergy, 15 children were healthy and were included in the control group. All the children were hospitalized in the 2nd Department of Paediatrics and Allergology, Polish Mother's Memorial Hospital, Research Institute, Lodz, Poland. The basis of diagnosis of cow's milk protein allergy was presence of clinical symptoms of allergy and positive results of elimination and provocation challenge with cow's milk proteins. Among them 17 children also had antigen-specific IgE antibodies (asIgE) against cow's milk proteins ≥ 2 class according to 4 degrees classification of atopy, and were included in the IgE-dependent allergy group. The remaining 18 children with correct results of asIgE against milk proteins were included in the IgE-independent allergy group.

The following research was approved by the Polish Mother's Memorial Hospital Research Institute Ethical Committee and each time the consent of parents or carers.

In each examined child the following was evaluated:

- concentration of total IgE – concentration of total IgE was immunochemically determined with kinetic nephelometry measured with Dade Behring equipment; results were compared with references prepared by the producer;
- total allergen-specific IgE (asIgE) – total allergen-specific IgE against milk proteins was measured in blood sera with the immunochemical method using IgE FAST PLUS test kit 3M "Diagnostic System"; concentration ≥ 2 class (≥ 0.76 IU/ml) was considered abnormal according to the four-degree classification of atopy [26];
- selected haematological blood indices – selected haematological blood indices were measured with a Bayer H1 analyzer; the results were compared with range norms prepared by Ochocka *et al.* [27];
- TLR 4 expression assay – whole blood granulocytes isolated in a mean amount of 10^6 (±4%) were resuspended in 30 µl of PBS and total RNA isolation was carried out using RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's procedure. A single-stage reaction was carried out with the help of Techne Flexi Gene (GB) thermocycler and One-step RT-PCR Kit (Qiagen, USA) using 10 µl of total RNA isolated was used to amplify the TLR4 specific product.

RT-PCR reaction steps: 52°C – 30 min RT; 95°C – 15 min denaturation; then 30 cycles for: 94°C – 35 s, 49°C – 45 s, 72°C – 1 min. RT-PCR reaction product analysis was carried out using agarose electrophoresis (1.7% gel, TAE buffer) followed by ethidium bromide staining and EDAS 290 (Kodak, USA) scanning system with

Table I. Average concentration of immunoglobulin E in blood sera of examined children
Tabela I. *Wartości średniego stężenia immunoglobuliny E w surowicy badanych dzieci*

Examined group	IgE [IU/ml]	
	x	SD
IgE-independent allergy (n = 18)	8.27	6.09
Value of p	< 0.001	
IgE-dependent allergy (n = 17)	119.77	112.69
Value of p	< 0.001	
Control (n = 15)	8.97	8.98

n – number of children

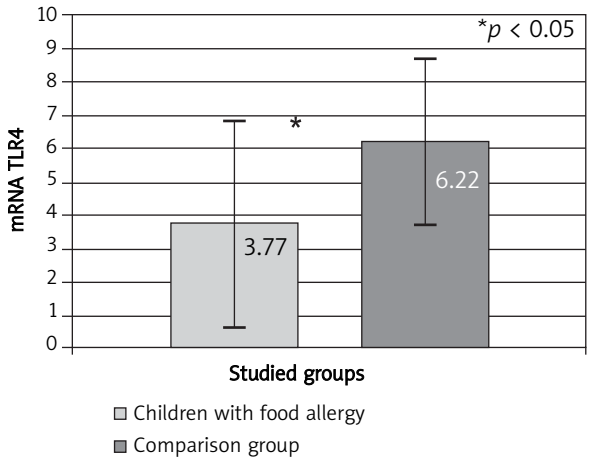

Fig. 1. Average mRNA TLR4 receptor expression on neutrophils
Ryc. 1. *Wartość średniej ekspresji mRNA TLR4 na neutrofilach*

Kodak 1D Image Analysis software. To evaluate the TLR4 expression rate "0" to "10" optical density (OD) was adopted, where "0" was the lowest OD and "10" the highest one. The values between this range show relative expression of TLR receptor.

Results

Studied children comprised 29 infants, 15 children in the second year of life and 6 in the third. Sixty percent of them were male and 40% were female. The concentration levels of total immunoglobulin E were significantly higher in the group with IgE-dependent allergy than in other groups: IgE-independent and control. The concentration levels of immunoglobulin E in children with IgE-independent allergy were comparable to IgE levels in children from the control group (Table I). Antigen specific IgE (asIgE) was elevated in all children from the IgE-dependent allergy group; most often it was class II (70.6%), class III (11.8%) and class IV (17.6%) according to the four-degree classification of atopy. Significantly lower expression of mRNA TLR4 receptor in children with food allergy was observed in comparison with the control group (Figure 1). However, the analysis of mRNA TLR4 receptor expression depending on type of allergy, IgE-dependent (4.2 ±2.7) or IgE-independent (3.42 ±3.45), revealed only small differences, not statistically significant ($p > 0.05$). Furthermore, the differences between the children with IgE-dependent allergy and the comparative group, as well as between the children with IgE-independent allergy and the comparative group, were not statistically significant. These results only indicate the course of changes and lack of statistical differences only underlines the need for further research. Next selected Th1/Th2 cytokines were estimated in sera of studied children. The results revealed higher average concentration of IL-4, IL-5, and IL-10 in children from IgE-dependent and IgE-independent allergy groups compared with children from the control group. It should be highlighted that the average concentration of IL-4 was significantly higher in children with IgE-dependent allergy whereas concentration of IL-5 was significantly higher in children with IgE-independent allergy compared with the control group. However, differences in concentration of IL-10 were statistically significant in children with IgE-dependent allergy as with IgE-independent allergy compared with children from the control group (Table II). The analysis of IL-2 and IFN-γ concentrations revealed higher average value of these cytokines in children from the control group. However, the difference was significant only in children with IgE-dependent allergy (Table III). Next the correlation between mRNA TLR4 expression on neutrophils and concentrations of IL-4 and IFN-γ in blood sera of children with IgE-dependent allergy were assessed.

The mRNA expression of TLR4 receptor on neutrophils was negatively correlated with serum concentration of IL-4 (r = –0.242, p = 0.500) but positively correlated with IFN-γ concentration (r = 0.396, p = 0.149). The observed correlations were not statistically significant (Figure 2).

Discussion

Natural immunity is an important factor influencing body homeostasis. Keeping all functions of this immunity is important to maintain host defence against invading pathogens. The TLR4 receptor was found to be the main receptor recognizing lipopolysaccharide (LPS) [14, 16]. Some studies are devoted to participation of TLR receptors in allergic disorders but not many of them

Table II. Average values of phenotype Th2 cytokine concentrations in sera of examined children
Tabela II. *Średnie wartości stężenia cytokin fenotypu Th2 w surowicy badanych dzieci*

Cytokine [pg/ml]	Examined group				
	IgE-depenent (n = 17)		Control (n = 15)		IgE-independent (n = 18)
	x ± SD	p	x ± SD	p	x ± SD
IL-4	61.63 ±75.91	+	29.39 ±21.70	–	45.69 ±28.89
IL-5	27.25 ±31.77	–	14.91 ±19.33	+	30.17 ±48.17
IL-10	654.07 ±596.01	+	234.96 ±185.27	+	584.50 ±391.06

n – number of children, "+" – statistical significance, p < 0.05, "–" – not statistically significant

Table III. Average values of phenotype Th1 cytokine concentrations in sera of examined children
Tabela III. *Średnie wartości stężenia cytokin fenotypu Th1 w surowicy badanych dzieci*

Cytokine [pg/ml]	Examined group				
	IgE-depenent (n = 17)		Control (n = 15)		IgE-independent (n = 18)
	x ± SD	p	x ± SD	p	x ± SD
IL-2	62.39 ±82.45	+	303.25 ±461.31	–	261.65 ±519.98
IFN-γ	603.01 ±569.17	+	1461.51 ±1231.67	–	1302.88 ±1218.9

n – number of children, "+" – statistical significance, p < 0.05, "–" – not statistically significant

concerning infants and very young children [16, 19, 23]. In this study the expression of mRNA TLR4 receptor on neutrophil surfaces was lower in children in both allergic groups, IgE-dependent and IgE-independent, than in the control group. These observations are comparative with other authors. Prescott *et al.* observed lower TLR4 expression on peripheral blood mononuclear cells (PBMC) in allergic children with simultaneously hyperresponsiveness of these receptors after the ligand stimulation [23]. Other authors reported lower TLR4 expression and even lower TLR2 in nose mucosa patients with allergic rhinitis [28]. It should be noted that nowadays it is difficult to explain whether this lower expression is the reason or cause of this allergic reaction.

There is much evidence that cytokines mediate allergic reactions, especially IL-4, IL-5 and IL-10. The evaluation of IL-4 serum concentrations revealed a significantly higher level in children with IgE-dependent allergy in comparison with the control group and it was comparative with other authors [7, 27, 29]. However, IL-5 concentrations were significantly higher in children with IgE-independent allergy in comparison with children from the control group. The study results of Crittender *et al.* showed that the IgE-independent mechanism of allergic reaction can be manifested by presentation from the digestive system with possible eosinophilic enteropathy [7]. These observations suggest that IL-5 can contribute to IgE-independent reactions. This investigation reveals significantly higher serum levels of IL-10 in children with IgE-dependent allergy and IgE-independent allergy in comparison with the control healthy children, which may indicate the important role of IL-10 in an allergic reaction. These observations are consistent with our previous observations [29]. Lamblin *et al.*

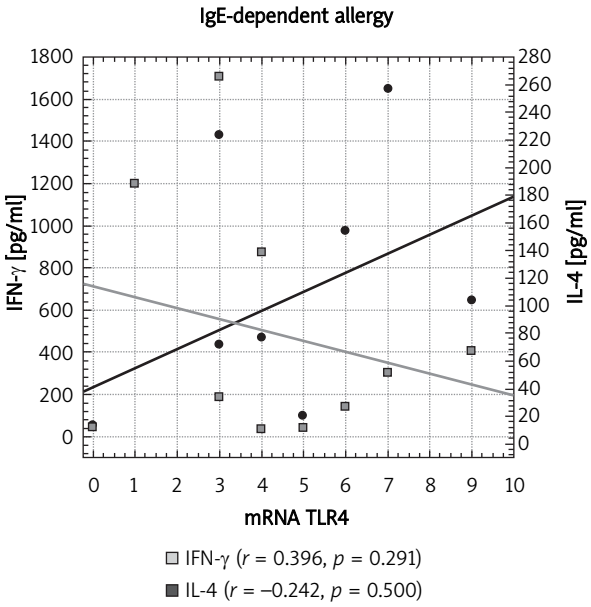

Fig. 2. Correlation between mRNA TLR4 expression on neutrophil surfaces and IL-4, IFN-γ concentration in sera of children with IgE-dependent allergy
Ryc. 2. *Wartości korelacji pomiędzy ekspresją mRNA TLR4 na neutrofilach a stężeniami IL-4, IFN-γ w surowicy dzieci z alergią IgE-zależną*

observed increased mRNA expression of IL-10 in intestinal mucosa of patients with asthma [25]. Other authors suggest that IL-10 is excreted by CD4 lymphocytes with the highest CD25 expression, which can prevent an adverse reaction to cow's milk proteins [30]. Many authors suggest that production of IL-4, IL-5 and IL-13 with lower IFN-γ production can contribute to allergic reactions. Such relations were observed in children with atopic dermatitis and cow's milk allergy [24] or with allergic rhinitis [31]. The results of our study showed lower production of IFN-γ in children with allergy, statistically significantly lower in the IgE-dependent allergy group compared with the control group. However, Tiemessen *et al.* revealed higher concentration of IL-4, IL-13 and IFN-γ in children with persistent cow's milk allergy, which may suggest involvement of this cytokine in delayed allergic reactions [30]. At the same time they observed lower concentration of IL-10, which may be connected in these children with lack of tolerance to cow's milk proteins. It is thought that in IgE-independent allergy IFN-γ can activate proinflammatory cytokines, which is why an increased level of this cytokine is possible in IgE-independent reactions [7]. Our study showed that levels of IFN-γ were higher in children from the IgE-independent group than in children from the IgE-dependent group. Paajanen *et al.* observed an increased level of IL-4, IL-5 and IFN-γ from duodenal biopsy in children with delayed cow's milk allergy when an elimination diet was not introduced [32]. The results of our study showed lower concentration of IL-2 in allergic groups (especially in children with IgE-dependent allergy). This observation is in accordance with other studies [33]. Lower concentration of IL-2 can contribute to disturbances in regulatory mechanisms including Th1/Th2 balance.

Correlations between mRNA TLR4 expression on neutrophils and IL-4 and IFN-γ serum concentrations were comparable with those revealed by O'Mahony *et al.* [21]. Mita *et al.* observed reduced mRNA TLR4 expression caused by IL-4 on monocytes and decreased mRNA TLR4 expression on B lymphocytes [34]. Investigation of mRNA TLR expression on eosinophil surfaces also showed TLR7 and TLR8 increased expression caused by IFN-γ but not by IL-4 and IL-5 [35].

Conclusions

1. Lower TLR4 receptor expression in children with allergic disease may indicate an important role of this receptor in the process.
2. Inverse correlation between TLR4 expression and IL-4 concentration and positive correlation with IFN-γ in children with IgE-dependent allergy may suggest involvement of this receptor in development of food allergy.
3. The results of our research suggest that natural immunity may be important in pathogenesis of allergy and assessment of this immunity may by helpful in diagnosis of allergy in small children, especially in clinically difficult cases.

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