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Original Article

Correlation of TLR2 and TLR4 expressions in peripheral blood mononuclear cells to Th1- and Th2-type immune responses in children with henoch-schönlein purpura

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Abstract: We discussed the correlation of TLR2 (Toll-like receptor) and TLR4 expressions in peripheral blood mononuclear cells (PBMCs) to Th1- and Th2-type immune responses in children with Henoch-Schönlein Purpura (HSP). The role of TLR2 and TLR4 in the pathogenesis of HSP was analyzed. Sixty-four HSP children treated at our hospital from October 2011 to November 2012 were enrolled and divided into NHSPN group (complicated by renal impairment, 36 cases) and HSPN group (not complicated by renal impairment, 28 cases). In the meantime, 30 normal children receiving physical examination at our hospital were recruited as controls. Peripheral blood T cell subgroups and TLR2 and TLR4 expressions in PBMCs were detected by using flow cytometry; relative expression levels of TLR2 and TLR4 mRNA in PBMCs by real-time quantitative fluorescence PCR, and plasma levels of IFN-γ, IL-4 and IL-6 by ELISA method. Relative expression levels of TLR2 and TLR4 mRNAs in PBMCs and TLR2 and TLR4 protein expressions in children with HSP were significantly higher than those of the controls (P<0.01). The relative expression levels of TLR2 and TLR4 mRNAs in PBMCs and TLR2 and TLR4 protein expressions in HSPN group were obviously higher than those in NHSPN group (P<0.05; P<0.01; P<0.01; P<0.01); $CD3^+$ T cells and $CD3^+CD4^+$ T cells in HSP group were significantly decreased, while $CD3^+CD8^+$ T cells and $CD3^+HLADR^+$ T activated cells were considerably increased (P<0.01); The plasma levels of IL-4 and IL-6 in HSP group were significantly higher than those of the normal controls (P<0.01, P<0.01); IFN-γ level in the former was much lower than in the control group (P<0.05); IFN-γ/IL-4 ratio in the former was also lower than that in the control (P<0.01); TLR2 and TLR4 expressions in HSP group showed significantly positive correlation with the plasma levels of IL-4 and IL-6 (P<0.01, P<0.05; P<0.01, P<0.01) and significantly negative correlation with IFN-γ/IL-4 ratio (P<0.01; P<0.01). TLR2 and TLR4 activation may be involved in the pathogenesis of HSP. TLR2 and TLR4 overactivation may induce HSP-related renal impairment; Children with HSP showed T-cell disorders and Th1/Th2 imbalance. Activated TLR2 and TLR4 possibly mediate the pathogenesis of HSP by upregulating Th2-type immune responses.

Keywords: Henoch-Schönlein Purpura (HSP), children, toll-like receptor (TLR), inferno-γ (IFN-γ), interleukin-4 (IL-4)

Introduction

Henoch-Schönlein Purpura (HSP) is the most common form of systematic vasculitis in children. Though the pathogenesis is not fully understood, it is generally believed that infection is the main inducing factor. During acute phase Th1/Th2 imbalance is usually noted, leading to abnormal synthesis of immunoglobulins and ANCA-associated vasculitis [1]. Toll-like receptors (TLRs) are a novel class of transmembrane signaling receptors that recognize and bind to pathogen-associated molecular patterns. Triggering intracellular signal transduction pathway in host, TLRs can achieve the goals of promoting cytokine synthesis, activating T cells and maintaining Th1/Th2 balance. Therefore, TLRs play an important role in immune response and inflammatory response. But few reports have discussed whether TLRs are involved in the pathogenesis of HSP.

We determined the expressions of TLR2 and TLR4 proteins and their mRNAs in PBMCs in children with HSP, along with the plasma levels of IL-4 and IFN-γ. The correlation of TLR2 and

Table 1. Primer in real-time fluorescent polymerase chain reaction

Gene	Sequence	Annealing temperature (°C)	Product size (bp)
TLR2	Upstream: 5'-ATGCTGCCATTCTCATTCTTCT-3'	55	101
	Downstream: 5'-CTCCAGGTAGGTCTTGGTGTTC-3'		
GAPTH	Upstream: 5'-TCATGGGTGTGAACCATGAGAA-3'	57	146
	Downstream: 5'- GGCATGGACTGTGGTCATGAG-3'		
TLR4	Upstream: 5'-TGTCCTCCCACTCCAGGTAAGT-3'	55	144
	Downstream: 5-GATTGCTCAGACCTGGCAGTT-3'		
GAPTH	Upstream: 5'-TCATGGGTGTGAACCATGAGAA-3'	57	111
	Downstream: 5'-GGCATGGACTGTGGTCATGAG-3'		

Table 2. The levels of IFN-γ, IL-4, IL-6, and the ratio of IFN-γ/IL-4 in plasma from HSP children and the control group

Group	n	IFN-γ (pg/ml)	IL-4 (pg/ml)	IFN-γ/IL-4	IL-6 (pg/ml)
HSP group	64	22.37±6.05	33.99±9.25	0.74±0.36	27.10±5.17
normal control	30	28.22±12.57	14.85±4.54	1.64±0.62	7.76±0.72
t′		2.42	13.45	7.37	29.33
P		<0.05	<0.01	<0.01	<0.01

TLR4 expression in HSP to functions of Th1 and Th2 subgroups was identified. The findings shed a new light on the pathogenesis of HSP in children and the treatment measures.

Materials and methods

Subjects and grouping

From October 2011 to November 2012, 64 pediatric cases with HSP hospitalized at Pediatric Department of Affiliated Hospital of Qingdao University Medical College were recruited. They all conformed to 2005 EULAR/PReS diagnostic criteria for HSP [2]. There were 33 males and 31 females, aged 2-14 years old with an average of 6 years old. All cases belonged to first onset without the use of glucocorticoids, immunosuppressors or heparin in the recent 4 weeks. They were divided into NHSPN group (complicated by renal impairment, 36 cases) and HSPN group (not complicated by renal impairment, 28 cases) depending whether the cases were complicated by renal impairment [3]. For the control group, 30 normal children receiving physical examination at our hospital were enrolled, including 16 males and 14 females (aged 3-12 years old, average 6.4). Informed consent was obtained from the relatives of all cases, and the protocol was approved by the hospital ethnics committee.

Experimental methods

PBMC isolation: Using heparin as anticoagulant under sterilized conditions, 2-3 ml of venous blood was collected. Ficoll-Hypaque density gradient centrifugation was performed to isolate PBMCs and 1 mL of RNAiso Plus was added (TaKaRa Biotechnology (Dalian) Co., Ltd). The sample was preserved at -80°C.

cDNA synthesis: Total RNA extraction and quantification in lymphocytes: PBMCs added with RNAiso Plus solution were used for total RNA extraction according to the instruction of kit (TaKaRa). RNA concentration and purity were measured by using UV-spectrophotometer; RT-PCR: Extracted RNAs were reversely transcribed to cDNA according to the instruction (TaKaRa, DRR037A). Using 1 μl of cDNA as template, 30-40 cycles of PCR were performed. Primers were designed based on the mRNA sequences of target genes in Genebank. For relevant parameters, see **Table 1**. All primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd.; Identification of RT-PCR products: The amplified products of TLR3 and TLR4 (10 μl) were analyzed by 2% agarose gel electrophoresis at 90v for 30min. The purified proteins were recovered from the gel. The sequencing results were aligned with mRNA sequences of target gene in Genebank as a verification.

Real-time quantitative fluorescence PCR: All amplifications were done on ABI 7000 RT-PCR System, and the melting curves were plotted. Δct was calculated as Ct value of target gene minus that of internal reference GAPTH. Relative expression of the target genes was calculated by $2^{-\Delta\Delta ct}$ [4] (**Table 1**).

Table 3. T lymphocytes subset from HSP and the control group

Group	n	$CD3^+T$ (%)	$CD3^+CD4^+T$ (%)	$CD3^+CD8^+T$ (%)	$CD3^+HLADR^+T$ (%)
HSP group	64	56.17±10.22	26.43±9.93	29.76±6 .68	6.99±1.49
normal control	30	65.80±7.24	34.94±7.53	23.85±5.17	1.09±0.75
T or t' value		4.64	4.16	4.28	25.52
p value		<0.01	<0.01	<0.01	<0.01

Table 4. Expression of TLR-2, TLR-4 in peripheral blood mononuclear cells (PBMC) in HSP and the control group

		HSP group	normal control	T or t' value	*P* value
TLR2 mRNA	Relative expression quantity	1.24±0.26	1.04±0.18	4.283	<0.01
TLR2 flow result	Positive percent (%)	49.5±21.0	29.1±12.6	5.847	<0.01
	mean fluorescent intensity	24.71±8.07	11.5±5.7	15.46	<0.01
TLR4 mRNA	Relative expression quantity	1.68±0.73	1.04±0.49	5.00	<0.01
TLR4 flow result	Positive percent (%)	34.0±18.0	15.0±8.0	7.47	<0.01
	mean fluorescent intensity	17.85±7.36	7.57±2.70	9.85	<0.01
The number of cases		64	30		

Table 5. Expression of TLR-2, TLR-4 in PBMC in HSPN and NHSPN group

Group	The number of cases	TLR2 mRNA	TLR4 mRNA	TLR2 (MFI)	TLR4 (MFI)
HSPN group	28	1.763±0.698[a,b]	1.94±0.66[a,b]	27.85±8.11[a,b]	21.28±8.71[b,c]
NHSPN group	36	1.467±0.621[d]	1.48±0.74[d]	22.72±7.15[b]	15.18±4.71[b]
normal control	30	1.114±0.355	1.04±0.49	11.5±5.7	7.57±2.70
F value		9.32	14.25	21.522	41.12

Note: a comparison with NHSPN group, P<0.05; b and the normal control group, P<0.01; c and NHSPN group, P<0.01; d and the normal control group, P<0.05.

Flow cytometry for measuring the expressions of TLR2 and TLR4 on $CD14^+$ cells and T-cell subgroups in peripheral blood: 0.4 mL of venous blood was collected with heparin anticoagulation and split into 4 tubes. For each tube 100 μl of blood sample was added with 5 μL of anti-CD14-FITC antibody. For No. 1 tube, 5 μL of anti-TLR4-PE antibody was added for isotype control (PE-labeled mouse IgG2a, K isotype); for No. 2 tube, 5 μL of anti-TLR4-PE antibody was added; for No. 3 tube, 5 μL of anti-TLR2 antibody was added for isotype control (PE-labeled mouse IgG1, K isotype); for No. 4 tube, 5 μL of anti-TLR2-PE antibody was added. Staining was carried out in all tubes for 15 min in the dark at room temperature. Then the tube was added with hemolysin, properly mixed and stood for 15 min. After centrifugation at 2000 r/min for 5 min, the supernatant was discarded, and the cells were washed with PBS twice. The cells were resuspended in 500 μL of PBS. The positive expression rates of TLR2 and TLR4 on $CD14^+$ cells in peripheral blood were detected using CD14 to set the threshold. CellQuest software was employed to select mononuclear cells by setting FCS and SSC. The results were expressed as percentages of cells positive for both CD14 and TLRs; Detection of T-cell subgroups: 100 μl of blood sample was added with 20 μL of CD3-FITC, CD4-FITC, CD8-PE, CD3-FITC/HLADR-PE and FITC/PE-labeled monoclonal antibody, respectively, and mixed well. After incubation at room temperature in the dark for 15-20 min, 250 μL of sodium carbonate buffer was added to initiate hemolysis for 10 min. The cells were washed with PBS and then centrifuged at 1500 r/min for 5 min. Supernatant was discarded, and the cells were fixed in 0.5 mL of 1% paraformaldehyde. Flow cytometer (Beckman Coulter, USA) was used for the detections. All antibodies were purchased from eBioscience, Inc. (USA).

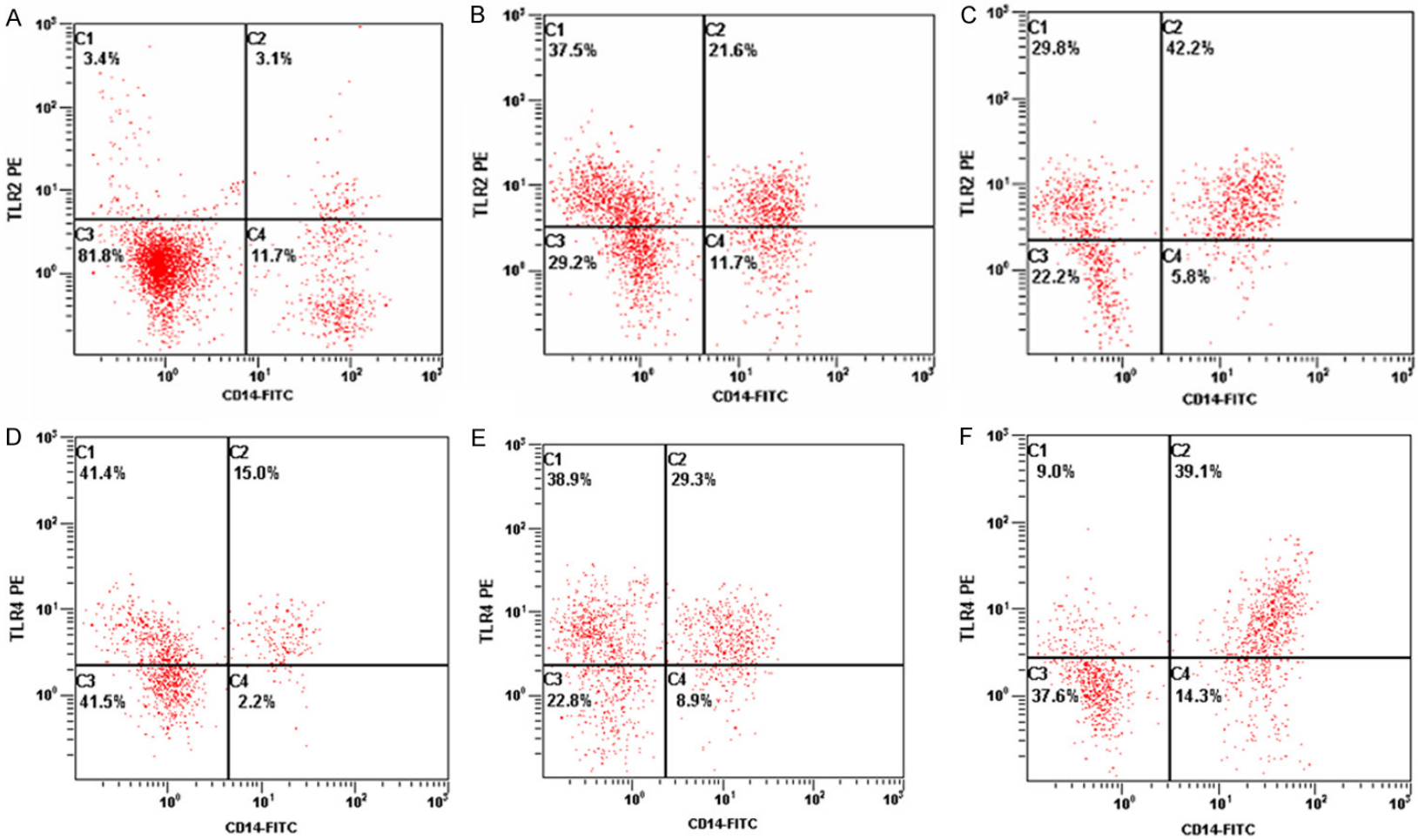

Figure 1. Percentages of cells positive for surface TLR2 andTLR4 staining in PBMCs in healthy control group, HSPN group and NHSPN group. A. Percentage of cells positive for TLR2 in control group. B. Percentage of cells positive for TLR2 in NHSPN group. C. Percentage of cells positive for TLR2 in HSPN group. D. Percentage of cells positive for TLR4 in control group. E. Percentage of cells positive for TLR4 in NHSPN group. F. Percentage of cells positive for TLR4 in HSPN group.

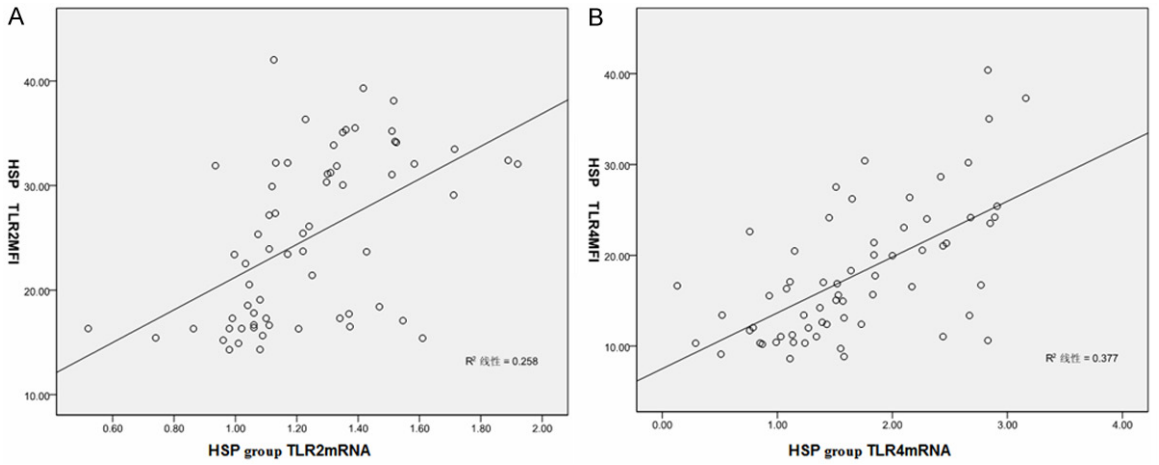

Figure 2. Correlation between protein and mRNA expressions of TLR2 and TLR4 in PBMCs in HSP group. A: Correlation between protein and mRNA expressions of TLR2 in PBMCs in HSP group. B: Correlation between protein and mRNA expressions of TLR4 in PBMCs in HSP group.

Table 6. Correlation of TL2 and TLR4 MFI to plasma levels of IFN-γ, IL-4, IL-6, and IFN-γ/IL-4 ratio

Group	IFN-γ	IL-4	IFN-γ/IL-4	IL-6
TLR2	r=0.17 P=0.184	r=0.42 P=0.001	r=-0.44 P=0.000	r=0.29 P=0.019
TLR4	r=-0.68 P=0.000	r=0.74 P=0.000	r=-0.80 P=0.000	r=0.72 P=0.000

ELISA method for measuring plasma levels of IFN-γ, IL-4 and IL-6: Model 550 microplate reader (BIO-RAD) and IFN-γ, IL-4 and IL-6 ELISA kits (Shanghai Senxiong Technology Industry Co., Ltd.) were used.

Statistical analysis

Statistical analysis was performed using SPSS17.0 software. When the original data did not conform to the assumption of homogeneity of variances or normal distribution, the square roots of the values were calculated. The converted data conforming to homogeneity of variances or normal distribution were expressed as $\bar{x} \pm s$. Independent-sample t test (conforming to homogeneity of variances) or t-test (not conforming to homogeneity of variances) was used for intergroup comparisons. For multi-group comparisons, one-way ANOVA was adopted; and for multi-group two-sample comparisons, LSD-t was used. Pearson correlation coefficients were calculated, with $P<0.05$ indicating significant difference and $P<0.01$ extremely significant difference.

Results

Comparison of plasma levels of IFN-γ, IL-4 and IL-6 and IFN-γ/IL-4 ratio

Plasma level of IFN-γ and IFN-γ/IL-4 ratio in HSP group decreased significantly, while the plasma level of IL-4 and IL-6 increased significantly (**Table 2**).

Levels of T-cell subgroups: $CD3^+$ T cells and $CD3^+CD4^+$ T cells in HSP group decreased obviously, while $CD3^+CD8^+$ T cells and $CD3^+HLADR^+$ activated T cells increased significantly (**Table 3**).

Relative expression levels of TLR2 and TLR4 mRNAs, percentages of cells positive for TLR2 and TLR4 and variations of mean fluorescence intensity (MFI): Relative expression levels of TLR2 and TLR4 mRNAs, percentages of cells positive for TLR2 and TLR4 and MFI in HSP group were obviously higher than those of the control group (**Table 4**).

Relative expression levels of TLR2 and TLR4 mRNAs as well as percentages of cells positive for TLR2 and TLR4 in HSPN group and NHSPN group were all higher than those of the control group. Relative expression levels of TLR2 and TLR4 mRNAs, percentages of cells positive for TLR2 and TLR4 and MFI in HSPN group were higher than those in NHPSN group, showing a statistically significant difference (**Table 5**; **Figure 1**).

Correlation between TLR2 mRNA and TLR2 protein expressions and between TLR4 mRNA and TLR4 protein expressions in PBMCs in cases with HSP: TLR2 mRNA and TLR2 protein expressions (MFI) in PBMCs were positively correlated in cases with HSP ($r=0.51$, $P<0.01$); TLR4 mRNA and TLR4 protein expressions (MF1) were also positively correlated ($r=0.61$, $P<0.01$) (**Figure 2**).

Correlation of TLF2 and TLR4 protein expressions (MFI) to plasma levels of IFN-γ, IL-4, IL-6 and IFN-γ/IL-4 ratio: TLR2 MFI was positively correlated to plasma levels of IL-4 and IL-6 in HSP group, but not correlated to plasma level of IFN-γ; it showed a negative correlation with IFN-γ/IL-4 ratio (**Table 6**). TLR4 MFI was positively correlated to plasma levels of IL-4 and IL-6 in HSP group, and negatively correlated to plasma level of IFN-γ and IFN-γ/IL-4 ratio (**Table 6**).

Discussion

HSP is a systematic disease featuring small-vessel necrotizing vasculitis in children and can affect multiple organs. Renal impairment occurs in 20-60% of children with HSP and is a predictor of prognosis [5, 6]. Pathogenesis of HSP may involve functional mutations of T cells, abnormal cytokine secretions, inflammatory media, coagulation and fibrinolytic disorders, or susceptibility genes [7]. Abnormal humoral immunity in HSP may be manifested as polyclonal B cell activation with an increase of IgA. Other immune abnormalities associated with HSP include: imbalance of T-cell subgroups, $CD4^+/CD8^+$ T lymphocytes imbalance, Th2 cell over-activation, over-expression of Th17 cells, reduced regulatory T cells or insufficient immune response which leads to B cell over-activation, increased synthesis of IgA and over-expression of pro-inflammatory cytokines. All these will aggravate inflammatory response and induce autoimmune diseases [8, 9]. We found that $CD3^+$ T cells and $CD3^+CD4^+$ T cells in peripheral blood during acute phase of HSP were obviously decreased, with a significant increase of $CD3^+CD8^+$ T cells and $CD3^+HLADR^+$ activated T cells. The plasma level of IFN-γ decreased, while that of IL-4 increased, accompanied by an obvious reduction of IL-4 ratio. This indicated cell-mediated immune disorder in children with HSP, which presented as T-cell disorders and hypofunction, T-cell over-activation and Th1/Th2 imbalance (TH2 dominance). The results agreed with the existing reports and our preliminary research [10].

However, the causes of immune imbalances in HSP are still unclear. Epidemiological statistics indicated that infection is an important precipitating factor of HSP. About 80% of children with HSP are combined with infections. TLRs offer the first line of defense during immune response in host, acting as the bridge between innate immunity and adaptive immunity. TLRs activation induced by infections may play an important role in initiating abnormal immune responses to HSP.

TLRs are type I transmembrane proteins, mainly expressed in immunocytes such as mononuclear cells, macrophages, lymphocytes and dendritic cells and also in nonimmune cells such as epidermal cells, fibroblasts, kidney podocytes and mesangial cells. TLRs cannot only recognize exogenous ligands such as bacteria and viruses, but also endogenous ligands such as heat shock proteins, fibronectin, fibrinogen and hyaluronic acid. By recognizing these endogenous and exogenous pattern recognition receptors, TLRs can trigger intracellular signal transduction and activate the innate immune cells. A variety of pro-inflammatory cytokines (eg., TNF-α, IL-1 and IL-6) will be expressed to induce inflammatory response. This will further promote antigen presentation and cause variations of Th1/Th2 balance in auxiliary T cells. Adaptive immune response can be also triggered by inducing the expression of co-stimulatory molecules [11-13]. Most ligands of TLRs can induce Th1-type immune responses, or Th2-type immune responses in some circumstances. TLRs regulate Th/Th2 responses via multiple mechanisms so that the host can generate an optimal immune response to pathogens and maintain stability of internal environment.

Abnormal activation of TLRs contributes to the pathogenesis of autoimmune diseases, hypersensitivity diseases and inflammatory diseases [14, 15]. TLR2 is upregulated in PBMCs and macrophages of synovial tissues in patients with juvenile idiopathic arthritis. Moreover, secretions of cytokines such as TNF-α, IL-1β and IL-6 are increased, which can be reversed

by the transfusion of anti-TLR2 antibodies, or partially through the transfusion of anti-TLR4 antibodies [16]. For children in the acute phase of Kawasaki disease, TLR4, MD-2 and MyD88 are obviously upregulated. TLR4 activation may be an important cause of immune imbalance in Kawasaki disease [17]. Pawar et al. [18] found that bacterial lipopeptides activated TLR2- and TLR4-mediated signaling pathways in renal tubular epithelial cells in mouse model of lupus nephritis. The secretions of inflammatory factors by renal tubular epithelial cells were enhanced through these pathways, which further activated TLR2- and TLR4-mediated signaling pathways. Thus the vicious cycle began, causing persistent damage to kidney, in which TLR signaling pathways play an important role. Coppo et al. [19] showed that TLR4 was upregulated in PBMCs for patients in active phase IgA nephropathy, indicating the mediating role of TLR4 in immune disorders related to IgA nephropathy.

Our results indicated that TLR2 and TLR4 proteins and mRNAs were upregulated in PBMCs in children with HSP. For HSPN group, the expressions of TLR2 and TLR4 proteins and mRNAs were increased compared with those of NHSPN group. The increased TLR2 and TLR4 protein expressions in children with HSP were positively correlated with IL-6 level. It was inferred that TLR4 and TLR4 on the surface of PBMCs, when stimulated by endogenous ligands, activated downstream signal transduction molecules via MyD88-dependent or non-MyD88-dependent pathways in HSP cases. As a result, the release of pro-inflammatory cytokines such as IL-6 was enhanced, facilitating the occurrence and progression of HSP and HSP-related renal impairment.

Li et al. [20] discovered that when PBMCs collected from children in acute phase of HSP were cultured in vitro without the addition of mitogen, several TLRs (TLR1, TLR2, TLR6, TLR3, TLR7, TLR9) were obviously upregulated except TLR4. Their results were different from ours, possibly due to different pathogens across the regions and seasons and TLR4 gene polymorphism among different populations.

We found that increased TLR2 expression was positively correlated with plasma level of IL-4 in HSP cases, and significantly negatively correlated with IFN-γ/IL-4 ratio; increased TLR4 expression was significantly positively correlated with plasma level of IL-4, and significantly negatively correlated with plasma level of IFN-γ and IFN-γ/IL-4 ratio. Several mechanisms may be involved in the regulation of Th1/Th2 differentiation, especially the differentiation into Th2 cells, through TLR2 and TL4: TLR signaling pathways, TLR ligands, regulation of Th0 differentiation by TLR, and regulation of Th0 differentiation by interactions between members of IL-1R/TLR superfamily. As a result of this, IL-4 is released in large quantities, while IFN-γ production is reduced, causing Th1/Th2 imbalance and the occurrence of HSP.

To conclude, cell-mediated immune disorders in HSP usually present as hypofunction of T-cell subgroups, T-cell overactivation and Th1/Th2 imbalance. TLR2 and TLR4 in host can activate innate immunity and regulate adaptive immunity. By binding to exogenous ligands, TLR2 and TLR4 activate TLR signaling pathways and promote the release of pro-inflammatory cytokines such as IL-6. Th2 dominant immune responses are induced through multiple mechanisms, resulting in the release of a large number of IL-4 and the reduction in IFN-γ production, and hence Th1/Th2 imbalance and HSP. As the endogenous ligands are constantly activated, TLR signaling pathways are further triggered. TLR2 and TLR4 overactivation will promote the release of pro-inflammatory factors, chemotactic factors and adhesion molecules in large quantities. The ensuing inflammatory cascade finally leads to HSP-related renal impairment.

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Disclosure of conflict of interest

None.

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