Association between toll-like receptor 6 expression and auxiliary T cells in the peripheral blood of pediatric patients with allergic purpura

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Abstract. The aim of the present study was to investigate the correlations between toll-like receptor 6 (TLR6) expression in the peripheral blood mononuclear cells (PBMCs) and auxiliary T cells of children with purpura. A total of 42 children with acute Henoch-Schönlein purpura (HSP) were selected for the study, and a further 30 healthy children were selected as a control group. Enzyme-linked immunosorbent assays were performed to detect the levels of plasma interferon (IFN)-γ, interleukin (IL)-4 and IL-17, and flow cytometry was performed to detect the TLR6 protein expression levels in PBMCs. The plasma levels of IL-4, IFN-γ and IL-17 in the HSP group were significantly higher compared with those in the normal control group. TLR6 protein expression was significantly increased in the PBMCs of the HSP patients. The TLR6 protein expression levels in the monocytes of the HSP group significantly positively correlated with the serum IL-4 and IL-17 levels, but not with the serum levels of IFN-γ. Therefore, the results of the present study suggest that the activation of TLR6 may be involved in the immunopathogenesis of HSP, and that the activated TLR6 may mediate this process by upregulating the immune responses of type 2 T helper (Th2) and Th17 cells.

Introduction

Henoch-Schönlein purpura (HSP) is a common childhood systemic vascular inflammatory disease with clinical symptoms involving the skin, gastrointestinal tract, joints and kidneys. The severity of renal involvement often directly affects the course and prognosis of the disease (1,2). Currently, infection is regarded as a crucial inducing factor for HSP. Abnormal immune function may be observed at the acute phase of HSP; however, its underlying mechanism has not yet been fully elucidated.

Toll-like receptors (TLRs) are a class of cell surface receptors involved in transmembrane signal transduction. TLRs are able to directly identify and bind pathogen-associated molecular patterns and then prime the signal transduction pathways in the host cells, which may promote the synthesis of cytokines and the activation of T cells and thereby regulate the Th1/Th2 balance and immune status (3). TLRs serve a crucial function in the immune and inflammatory responses, and are reportedly involved in the onset of a variety of diseases, including bronchial asthma, systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis and Kawasaki disease (4-8). T helper (Th) cells, including Th1, Th2 and Th17, are involved in the pathogenesis of various types of vascular inflammation (9-11). Th cell subset functional imbalance and aberrant activation of Th2 and Th17 cells have been observed in patients with HSP (12-15). However, the association between the TLR signaling pathways and Th1, Th2 and Th17 in the immune pathogenesis of HSP remains unknown. Myeloid differentiation factor 88 (MYD88) is the key adaptor molecule in TLR signaling pathways and the key target molecule for downstream signal transduction (16,17).

Our previous study (18) indicated that the activation of TLR2 and TLR4 may mediate the pathogenesis of HSP by upregulating the Th2 immune response. However, the association between TLR6 and the Th1/Th2 balance and Th17 cells, and whether it has any involvement in the pathogenesis of HSP remains unclear. The present study was conducted to elucidate the association between the expression of TLR6 in the peripheral blood mononuclear cells (PBMCs) and the subpopulations of Th cells in children with HSP by determining the expression levels of TLR6 and MYD88 in the PBMCs and the interleukin (IL)-4, interferon (IFN)-γ and IL-17 levels in serum samples. This should provide further information concerning the pathogenesis of HSP in children

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and may provide novel methods for the treatment of this disease.

Materials and methods

Patients. A total of 42 children with acute HSP, hospitalized in the Affiliated Hospital of Qingdao University (Qingdao, China) were enrolled in the present study between June 2013 and November 2013. The subjects included 25 males and 17 females, with an age range of 4-13 years (mean age, 6.7 years). All patients met the 2006 diagnostic criteria for allergic purpura defined by EULAR/PReS (19). Patients were at the time of first onset and had received no related medications, such as glucocorticoids, immunodepressant or heparin, in the preceding 4 weeks. A further 30 children receiving physical examination in the child care health center of the Affiliated Hospital of Qingdao University during the same period were recruited as the healthy control group. The healthy control group included 20 males and 10 females, with an age range of 3-12 years (mean age, 6.5 years). There was no statistical difference in age or gender between the two groups. This study was conducted in accordance with the declaration of Helsinki and with the approval of the Ethics Committee of Qingdao University. Written informed consent was obtained from the guardians of all participants.

Sampling. A 3-ml peripheral venous blood sample was collected in a sterile heparin anticoagulant tube for flow cytometry and fluorescent reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. An additional 1 ml of blood was collected in a common tube in order to isolate the serum for the determination of cytokine levels. All serum samples were stored at -80˚C prior to use.

Flow cytometry. Two 100-μl blood samples from each patient treated with anticoagulant (Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, China) were added to two tubes. Subsequently, 10 μl monoclonal mouse anti-human fluorescein isothiocyanate (FITC)-labeled CD14 antibody (cat. no. 110419-42; eBioscience, Inc., San Diego, USA) and 10 μl monoclonal rat anti-human phycoerythrin (PE)-labeled TLR6 antibody (cat. no. 334708; BioLegend, Inc., San Diego, USA) were added into one of the two tubes for determining the expression levels of CD14 and TLR6. Next, 10 μl mouse FITC-labeled IgG2a isotype and 10 μl rat anti-human PE-labeled isotype (eBioscience, Inc.) were added to the second tube for each patient to serve as a blank control. Following incubation at room temperature for 15 min in the dark, 3 ml lysing solution was added to each tube to lyse the red blood cells, and the samples were stored at room temperature for a further 15 min. Then the tubes were centrifuged at 696 x g for 5 min. After discarding the supernatant, cells were washed with phosphate-buffered saline (PBS) twice and centrifuged at 696 x g for 5 min. Finally, the cells were resuspended in 500 μl PBS and analyzed using a flow cytometer (FC500; Beckman Coulter, Brea, CA, USA). The percentage of $CD14^+TLR6^+$ cells in each sample was calculated.

RNA extraction and RT-qPCR. PBMCs from each serum sample were isolated by Ficoll density-gradient centrifugation (Sigma-Aldrcih, St. Louis, MO, USA) and dissolved in RNAiso Plus reagent (Takara Biotechnology Co., Ltd., Dalian, China) for total RNA extraction, according to the manufacturer's instruction. The concentration and purity of the extracted total RNA were determined at a wavelength of 260 nm using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Next, 2.5 μl total RNA was used as a template to synthesize the first strand cDNA by reverse transcription (RT), using a One Step SYBR PrimeScript RT-PCR kit (Takara Biotechnology Co., Ltd.). The RT-qPCR reaction was performed using an ABI Prism 7000 sequence detection system (Life-Tech, Inc., Williston, VT, USA) using SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.), following the manufacturer's instructions. The following gene-specific primer pairs were used: MYD88, F 5'-GCACATGGGCACATACAGAC-3' and R 5'-TGGGTCCTTTCCAGAGTTTG-3'; and GAPDH, F 5'-AACAGCCTCAAGATCATCAGCAA-3' and R 5'-GAC TGTGGTCATGAGTCCTTCCA-3'. All samples were normalized against GAPDH reference gene levels, and the relative expression of MYD88 mRNA was calculated using the formula: $R = 2^{-\Delta\Delta CT}$ (20). All samples were run in duplicate.

ELISA. Serum levels of interferon (IFN)-γ, IL-4 and IL-17 were determined by ELISA using IFN-γ, IL-4 and IL-17 kits, following the manufacturer's instructions (Shanghai Yuan-Long Biotechnology Co., Ltd., Shanghai, China). The plasma was placed at room temperature for 20 min, until completely thawed. A 96-well plate consisted of blank control wells, standard wells, and sample wells. The wells were pre-coated with avidin (Shanghai Yuan-Long Biotechnology Co., Ltd.). A total of 50 μl standard solution (0, 3, 6, 12, 24 and 48 pg/ml) was added to the standard wells. A total of 10 μl serum sample diluted with 40 μl PBS was added to the sample wells. Subsequently, 100 μl peroxidase-labeled antibody was added to the wells containing sample or standard, and 100 μl non-peroxidase labeled antibody was added to the blank control wells (the antibodies were provided with the ELISA kits). The plates were incubated at 37˚C for 60 min. The liquid was removed, and the wells were washed three times with ELISA Wash Buffer (50 mM Tris-HCl, pH 7.4; 0.2% Tween 20; Shanghai Yuan-Long Biotechnology Co., Ltd.) and blotted dry with paper towels. A total of 50 μl substrate A (H_2O_2) and 50 μl substrate B (3,3',5,5'-tetramethylbenzidine; Shanghai Yuan-Long Biotechnology Co., Ltd.) were added to each well and incubated at 37˚C for 15 min in the dark. Following the addition of 50 μl stop buffer, the optical density was measured at 450 nm within 15 min using a Sp-MAX 1800 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data are expressed as the mean ± standard deviation and were processed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). Independent sample t-test was used to analyze the difference between the two groups with homogeneity of variance, while independent sample t'-test was used when the variance was heterogeneous. Pearson correlation analysis was performed to analyze the correlation between two parameters. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Expression of TLR6 protein and MyD88 mRNA in the PBMCs of the two groups.

Parameter	Cases	TLR6 MFI	TLR6 protein (%)	MYD88 mRNA
HSP	42	12.56±4.09	29.13±10.17	1.28±0.42
Control	30	2.97±1.83	6.86±4.05	0.99±0.25
t'-value	-	9.40	10.49	2.46
P-value	-	<0.01	<0.01	<0.01

TLR6, toll-like receptor 6; MYD88, myeloid differentiation primary response gene 88; PBMCs, peripheral blood mononuclear cells; MFI, mean fluorescence intensity; HSP, Henoch-Schönlein purpura.

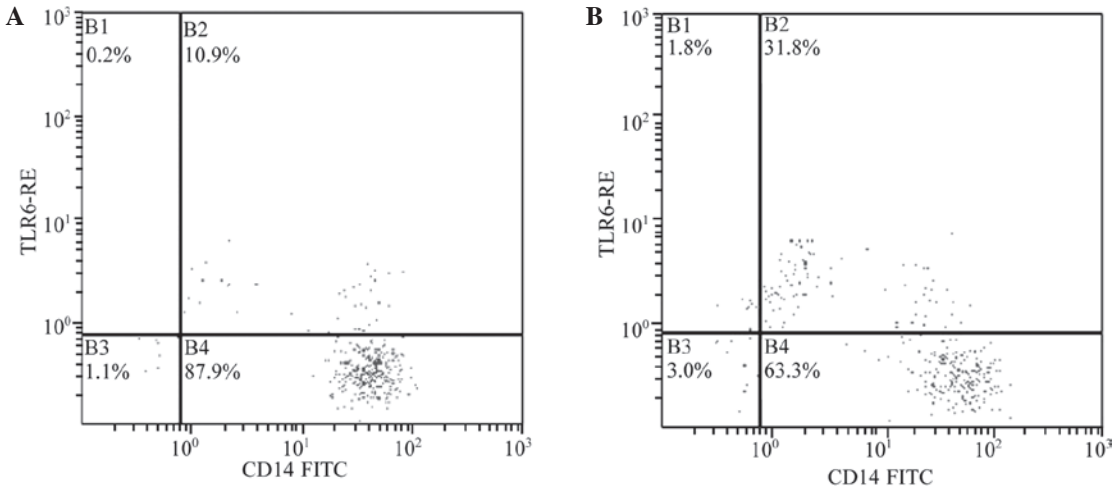

Figure 1. Expression of TLR6, determined by fluorescence-activated cell sorting, in the peripheral blood mononuclear cells of (A) healthy controls and (B) the patients with Henoch-Schönlein purpura. The expression of TLR6 was elevated markedly in the patients with Henoch-Schönlein purpura as compared with the healthy controls. TLR6-PE, toll-like receptor 6-phycoerythrin; FITC, fluorescein isothiocyanate.

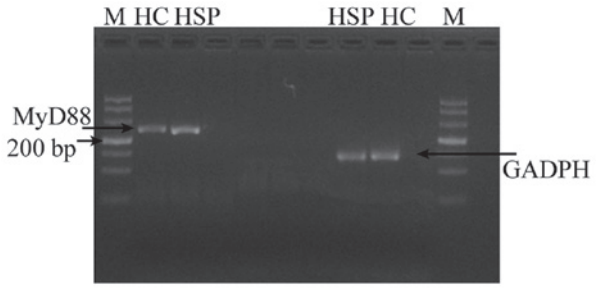

Figure 2. mRNA expression levels of MYD88 in the peripheral blood mononuclear cells of the two groups. M, DNA ladder; HC, healthy controls; HSP, Henoch-Schönlein purpura; MYD88, myeloid differentiation primary response 88.

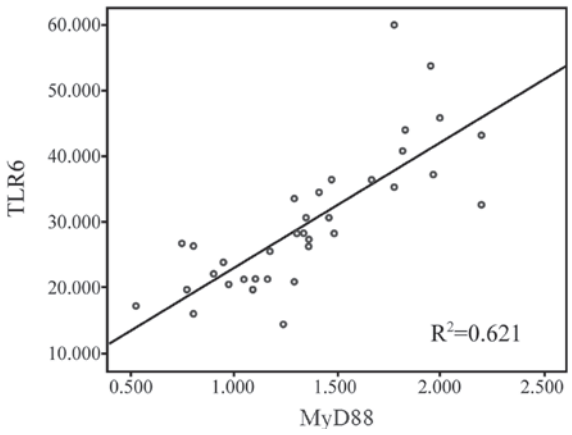

Figure 3. Mean fluorescence intensity of TLR6 protein in the peripheral blood mononuclear cells was positively correlated with the mRNA expression of MYD88 in patients with Henoch-Schönlein purpura. TLR6, toll-like receptor 6; MYD88, myeloid differentiation primary response 88.

Results

Positive percentage and mean fluorescence intensity (MFI) of TLR6 and the expression of MYD88. The positive percentage and MFI of TLR6 in the PBMCs was determined by fluorescence-activated cell sorting. These values increased significantly in the patients with HSP compared with those in the control group (P<0.01; Table I and Fig. 1). Furthermore, the mRNA expression levels of MYD88 in the PBMCs of the HSP group were markedly increased compared with those in the control group PBMCs (P<0.01; Table I and Fig. 2).

Correlation between TLR6 and MyD88 expression. Pearson correlation analysis indicated that the MFI of TLR6 protein in the PBMCs was positively correlated with the mRNA expression of MyD88 in the patients with HSP (r=0.79; P<0.01; Fig. 3).

Table II. Serum levels of IFN-γ, IL-4 and IL-17 and the IFN-γ/IL-4 ratio in the two groups.

Parameter	Cases	IFN-γ (pg/ml)	IL-4 (pg/ml)	IFN-γ/IL-4	IL-17 (pg/ml)
HSP	42	195.86±84.16	8.18±3.48	25.67±11.47	13.17±2.84
Control	30	140.83±77.78	5.05±1.88	47.90±10.06	11.09±1.77
t'-value	-	2.44	3.44	2.27	2.64
P-value	-	<0.05	<0.01	<0.05	<0.01

IFN-γ, interferon-γ; IL, interleukin; HSP, Henoch-Schönlein purpura.

Table III. Correlation between TLR6 MFI in patients with HSP and serum levels of IFN-γ, IL-4 and IL-17, and the IFN-γ/IL-4 ratio.

Parameter	IFN-γ	IL-4	IFN-γ/IL-4	IL-17
r	0.097	0.69	-0.38	0.36
P-value	>0.05	<0.01	<0.05	<0.05

TLR6, toll-like receptor 6; MFI, mean fluorescence intensity; HSP, Henoch-Schönlein purpura; IFN-γ, interferon-γ; IL, interleukin; r, Pearson product-moment correlation coefficient.

Serum levels of IFN-γ, IL-4 and IL-17, and the IFN-γ/IL-4 ratio. As determined by ELISA, the serum levels of IFN-γ, IL-4 and IL-17 in the HSP group were significantly higher compared with those in the normal control group. However, the ratio of IFN-γ to IL-4 in the HSP group was lower than that in the control group (Table II).

Correlation of TLR6 with IFN-γ, IL-4 and IL-17 levels and the IFN-γ/IL-4 ratio. The MFI of TLR6 in the PBMCs of the HSP group showed a marked positive correlation with serum levels of IL-4 (r=0.69; P<0.01) and IL-17 (r=0.36; P<0.05). However, the TLR6 MFI was negatively correlated with the ratio of IFN-γ to IL-4 (r=-0.38, P<0.05). Furthermore, no significant correlation was detected between the MFI of TLR6 in the PBMCs and serum IFN-γ levels (P>0.05; Table III).

Discussion

To date, the pathogenesis of allergic purpura remains unclear, although it has been proposed that the pathogenesis of the disease may be associated with humoral and cellular immune disorders, blood coagulation and fibrinolysis disorders and genetic susceptibility. No clear regulatory mechanism has been identified to explain the abnormal immune function observed in patients with allergic purpura. Previous studies indicate that cellular immune dysfunction, manifesting as a Th1/Th2 imbalance in the acute phase and dominant activation of Th2 cells, is present in a significant proportion of pediatric patients with HSP (12-15). Furthermore, Th17 cells are activated abnormally in these patients, and regulatory T (Treg) cells are reduced in number or exhibit reduced activity. In the present study, it was observed that Th1 cytokine IFN-γ and Th2 cytokine IL-4 increased significantly in the PBMCs of the patients with HSP at the acute phase. However, the IFN-γ/IL-4 ratio decreased markedly in the peripheral blood, suggesting that a Th1/Th2 imbalance is present in the patients with HSP, in particular a dominance of Th2. The upregulation of IFN-γ may be a compensatory response to the observed immunological imbalances *in vivo*. Th17 is a novel subset of $CD4^+$ helper T cells identified in recent years, and is characterized by the secretion of various cytokines, including IL-17, IL-21 and IL-22 (21,22). IL-17 induces monocytes/macrophages, smooth muscle cells, epithelial cells and endothelial cells to produce a variety of inflammatory cytokines and chemokines that are involved in the inflammatory response or autoimmune reaction. The present results suggest that the serum IL-17 levels in the patients with HSP were elevated markedly, indicating that Th17 cells serve a crucial function in the pathogenesis of HSP.

TLRs are a key variety of pattern recognition receptors, a bridge that link the innate immune response with the adaptive immune response. Following the activation of a TLR by its specific ligand, it may activate numerous transcription factors via MYD88-dependent or-independent pathways, including interferon regulatory factor 3 (IRF3), IRF7, activator protein 1 and nuclear factor-κB. In addition, a TLR may promote the gene expression of IL-6, IL-1β, IL-12, tumor necrosis factor-β and other inflammatory factors. TLRs may also upregulate the expression of co-stimulating molecules on the surface of antigen-presenting cells, leading to the production of a variety of cytokines and promoting the activation of Th cells, thereby priming the adaptive immune response (23-25). The excessive activation of TLRs may lead to the occurrence and development of a variety of autoimmune diseases (26,27). However, the function served by TLRs in the pathogenesis of HSP is not yet clear.

The upregulation of co-stimulating molecules and the formation of a microenvironment induced by the activated TLR signaling pathways may cause naïve T cells to transform into $CD4^+$, such as Th1, Th2, Th17 and $CD4^+CD25^+Foxp3^+$ Treg cells. Under certain conditions, the binding of specific ligands to TLRs may lead to the occurrence of Th2 or Th17 immune responses, although the majority of TLR ligands induce the Th1 immune response. Kim *et al* (28) observed that the overexpression of TLR4 and TLR9 may be involved in the immunopathogenesis of multiple dermatomyositis by activating Th1, Th2 and Th17 immune responses. Morgan *et al* (29) confirmed that TLR6 activation is able to promote Th1 and Th17 immune responses in gastrointestinal lymphoid tissue in inflammatory bowel disease. Zhao *et al* (30) observed that TLR2 and TLR4 levels are increased in the peripheral blood

and bronchoalveolar lavage fluid of mice that have inhaled various doses of fine particulate matter (PM2.5). Furthermore, Zhao *et al* demonstrated that the levels of IL-5 and IL-10 were elevated in the alveolar lavage fluid and peripheral blood of these mice, while IL-4 levels were increased in the peripheral blood only, suggesting that TLR2 and TLR4 may induce the Th2 immune response in the inflammatory reaction caused by the inhalation of PM2.5 particulates.

The results of this study suggest that TLR6 protein expression and MYD88 mRNA expression levels in the PBMCs of pediatric patients with HSP were significantly elevated compared with those in control subjects, and that the expression of TLR6 was significantly positively correlated with MYD88 mRNA expression ($P<0.01$). The observations suggest that TLR6 mediates the production of inflammatory cytokines and is involved in the pathogenesis of HSP, potentially via MYD88-dependent signal transduction pathways. Furthermore, the present results indicate that TLR6 protein expression is significantly positively correlated with serum IL-4 and IL-17 levels, but negatively correlated with the IFN-γ/IL-4 ratio in patients with HSP. These results suggest that TLR signal transduction pathways are activated by the binding of their specific ligands, which results in the production of a variety of inflammatory cytokines. However, this binding results predominantly in the activation of Th2 and Th17, through a variety of immunological mechanisms, thereby leading to the excessive production IL-4. This overproduction may result in a Th1/Th2 imbalance and the overexpression of IL-17, which induces the onset of HSP.

In conclusion, TLR6 protein and MYD88 mRNA expression levels are increased in the PBMCs of pediatric patients with HSP, and are significantly positively correlated. Th1/Th2 imbalance, excessive activation of Th17 and positive correlation of TLR6 protein expression with Th17 cells and the Th2 immune response were observed in the children with HSP. These findings suggest that the activation of TLR6 may mediate the immunopathogenesis of HSP by promoting Th2 and Th17 immune responses.

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