Investigation of the change in CD4⁺ T cell subset in children with Henoch–Schonlein purpura

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Abstract Helper T (Th) cells comprising Th1, Th2, Th17, and Treg are involved in the pathogenesis of various vascular inflammations, and information about Th cells in Henoch–Schonlein purpura (HSP) is still controversial. The aim of our study was to investigate the changes in CD4⁺ T cell subsets and their roles in the pathogenesis of HSP. Thirty children with diagnosis of HSP and thirty age-matched healthy controls were enrolled in this study. Real-time PCR was used to evaluate the mRNA expression levels of transcriptional factors and cytokines of CD4⁺ T cells. Proportions of Th1, Th2, Th17, and Treg cells in peripheral blood were detected by flow cytometry. Plasma cytokine concentrations were measured by ELISA. The proportions of Th2 and Th17 cells increased significantly in children with acute HSP (P < 0.05), while there were no significant differences between HSP and healthy controls regarding the proportions of Treg cells and Th1 cells (P > 0.05). mRNA levels of transcriptional factors and cytokines of Th2 and Th17 cells were significantly up-regulated (P < 0.05), while the differences were not significant as to those of Th1 and Treg cells (P > 0.05). Plasma concentrations of IL-17A, IL-4, and IL-6 in patients with HSP were found to be much higher than those of the control group (P < 0.05), and no differences between IFN-γ, IL-12, and TGF-β were detected between the two groups (P > 0.05). Presence of higher proportions of Th2 and Th17 cells in patients with HSP could be closely correlated with aberrant creation of antibody and development of vessel vasculitis. The changes in cytokine milieu in peripheral blood may play an important role in the derangement of CD4⁺ T cell subset.

Keywords Vasculitis · CD4⁺ T cell · Cytokines

Introduction

Henoch–Schonlein purpura (HSP) is the most systemic form of small vessel vasculitis that primarily affects children and is characterized by cutaneous purpura, arthritis or arthralgia, abdominal pain, gastrointestinal bleeding, and renal involvement [1]. Its etiology and pathogenesis has not been completely elucidated. Elevated serum IgA levels, vascular deposition of IgA-contained immune complexes, and increased pro-inflammatory cytokines during the acute stage suggest the possibility of immunemediated mechanisms [2]. However, the mechanisms resulting in increased IgA or pro-inflammatory cytokines are not clear. The role of adaptive immune response in HSP, especially CD4⁺ helper T (Th) cell, is required to be systemically investigated.

Previous studies shown that high-soluble IL-2 receptor value and a shift to Th1 lymphocytes existed in children with HSP [3, 4]. In contrast, Davin et al. [5] reported that plasma IgE levels were significantly higher in HSP patients, and there was a shift to Th2 lymphocytes. However, there is no clear notion of Th1/Th2 balance of HSP. As the T cell phenotype of cells in HSP has not been systemically studied, we investigated the proportions of Th1, Th2, Th17, and CD4⁺CD25⁺Foxp3⁺ Treg cells in peripheral blood using flow cytometry. Transcriptional factors and cytokines involved in Th1, Th2, Th17, and Treg differentiation and maintenance were also evaluated in this study.
Materials and methods

Study population

Thirty children (17 boys and 13 girls) with a clinical diagnosis of acute HSP, aged from 3.02 to 10.35 years (mean 5.93 ± 1.48 years), and 30 age-matched normal controls (NC, 15 boys and 15 girls; mean age: 6.21 ± 1.71 years; age range: 3.5–10.8 years) without allergic history were enrolled in this study. All patients fulfilled the American College of Rheumatology 1990 criteria for the classification of HSP and were confirmed as the first onset of HSP and without treatment of glucocorticoid or cytotoxic drugs in the last 4 weeks before admission in hospital. Of 30 children with HSP, all the children had typical skin purpura and/or maculopapular rash, 14 children (46.67%) had arthritis or arthralgia, 18 children (60.00%) suffered from abdominal pain, and 17 children (56.67%) had renal complications (renal complications including: hematuria ≥10 red blood cell/high power field and/or proteinuria ≥150 mg/24 h, and/or serum creatinine concentration ≥133 μmmol/L). Two children (6.67%) still remained with renal sequelae after clinical therapy. The principal clinical characteristics of these patients with HSP are listed in Table 1. The study was approved by local Ethical Committee, and informed consent was obtained from all the participants.

Blood samples

Using EDTA-Na₂ as an anticoagulant, venous blood (5 ml) was collected from healthy controls and patients with HSP during the acute course of 3–7 days and was immediately analyzed without any stimulation of mitogens or culture in vitro, except for special indication. Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll density gradient for analysis of flow cytometry. Plasma was obtained after centrifugation and stored at −80°C for the measurement of the enzyme-linked immunosorbent assay (ELISA). According to the manufacturer’s instructions, CD4⁺ T cells were immediately isolated from peripheral blood by micro-bead (Dynal 111.49D, US). Cell purity was identified as >97% with flow cytometry (FCM) while results of cells activity were >95% by 0.05% trypan blue staining.

Total RNA extraction and cDNA synthesis

Total RNA from CD4⁺ T cells was prepared using Versa gene RNA Kit (Genta 0050C, US) according to the manufacture’s instruction. DNase I (Genta 0050D, US) was used to eliminate the trace DNA during extraction. Isolated total RNA integrity was verified by the average optical density (OD) OD_260/OD_280 absorption. Using RevertAid™ H Minus MMLV reverse transcriptase (Fermentas K1632, Lithuania), cDNA was synthesized with oligo-dT primer. Negative control samples (no first strand synthesis) were prepared by performing reverse transcription reaction in the absence of reverse transcriptase.

LightCycler real-time PCR

The cDNA levels of transcription factors and cytokines were quantitated by real-time PCR, using Quantitect™ SYBR green PCR Kit (Qiagen 204143, Germany) and a LightCycler® 2.0 Instrument (Roche Molecular Biochemicals, Switzerland). The primers used for real-time PCR are listed in Table 2. The second derivate maximum method was performed for CP determination using LightCycler Software V3.5.30 (Roche Molecular Biochemicals). After normalization with Relative Quantification Software V1.0 (Roche Molecular Biochemicals), the final results were calculated as ratios of the relative transcript levels of the target genes to the relative amount of β-actin.

Flow cytometry analysis of Th1, Th2, Th17, and Treg

PBMC were cultured for 4 h at 37°C in humidified atmosphere containing 5% CO₂, in RPMI1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 1 μg/ml PMA, 50 μg/ml ionomycin, and 0.1 mg/ml menadine. After incubation, the suspended cells were washed twice in PBS. For Th1, Th2, and Th17 analysis, the cells were incubated with fluorescein isothiocyanate (FITC) anti-human CD4 (eBioscience, USA) for 20 min at 4°C. After surface staining, the cells were stained with phycoerythrin (PE)-anti-human IL-17A antibody, PE-anti-human IFN-γ,
and PE-anti-human IL-4 antibody after fixation and permeabilization according to the supplier’s protocol. For the identification of Treg, cells were surface-stained with FITC-anti-human CD4 and PE-Cy5-anti-human CD25, followed by staining for Foxp3 using the Foxp3 staining kit, according to the manufacturer’s instruction. Isotype controls were given to enable correct compensation and confirm antibody specificity. All the antibodies were purchased from eBioscience. Stained cells were analyzed by flow cytometry analysis, using an Epics-XL4 cytometer (Beckman Coulter, USA). Th1, Th2, Th17, and Treg were defined as CD4+ IFN-γ+IL-4− cells, CD4+ IFN-γ−IL-4+ cells, CD4+IL-17A+ cells, and CD4+CD25+ Foxp3high cells, respectively.

ELISA detection of plasma IFN-γ, IL-12, IL-4, TGF-β, IL-17A, and IL-6

The plasma levels of IFN-γ, IL-12, IL-4, TGF-β, IL-17A, and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA), using ELX-800 microplate reader (BioTek Corporation, USA) in accordance with the manufacturer’s instructions (Bender MedSystems, Austria). All samples were measured in duplicate.

Statistical analysis

To determine the statistical significance, expression levels of those genes were compared between healthy controls

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-bet</td>
<td>Sense: 5′-TGA CCC AGA TGA TTG TGC TC-3′&lt;br&gt;Antisense: 5′-TAT GCG TGT TGG AAG CTT TG-3′</td>
<td>56</td>
<td>111</td>
</tr>
<tr>
<td>GATA-3</td>
<td>Sense: 5′-CAG CAC AGA AGG CAG GGA GT-3′&lt;br&gt;Antisense: 5′-AGG CTT TGA ACA GGT AGT GTC-3′</td>
<td>58</td>
<td>92</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Sense: 5′-GTG GCA TCA TCC GAC AAG G-3′&lt;br&gt;Antisense: 5′-TGT GGA GGA ACT CGT GGA AT-3′</td>
<td>58</td>
<td>166</td>
</tr>
<tr>
<td>ROR-γt</td>
<td>Sense: 5′-GTG CTG GTT AGG ATG TGC CG-3′&lt;br&gt;Antisense: 5′-GTG GGA GAA GTC AAA GAT GGA-3′</td>
<td>58</td>
<td>135</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Sense: 5′-GGA GAC CAT CAA GGA AGA CA-3′&lt;br&gt;Antisense: 5′-GGC GAC AGT TCA GCC ATC AC-3′</td>
<td>58</td>
<td>156</td>
</tr>
<tr>
<td>IL-4</td>
<td>Sense: 5′-TCA TTT TCC CTC GGT TTC AG-3′&lt;br&gt;Antisense: 5′-ATA GGT GTC GAT TTG CAG TG-3′</td>
<td>56</td>
<td>122</td>
</tr>
<tr>
<td>IL-5</td>
<td>Sense: 5′-TCA GGG AAT AGG CAC ACT GGT-3′&lt;br&gt;Antisense: 5′-ACT CTT GCA GGT AGT CTA GG-3′</td>
<td>56</td>
<td>161</td>
</tr>
<tr>
<td>IL-13</td>
<td>Sense: 5′-GCATGGTATGGAGCATCAACC-3′&lt;br&gt;Antisense: 5′-CTTTACAAACTGGGCCACCTC-3′</td>
<td>59</td>
<td>197</td>
</tr>
<tr>
<td>IL-17A</td>
<td>Sense: 5′-CAG ATT ACT ACA ACC GAT CC-3′&lt;br&gt;Antisense: 5′-CAT GTG GTA GTG CAC ATC CC-3′</td>
<td>57</td>
<td>140</td>
</tr>
<tr>
<td>IL-17F</td>
<td>Sense: 5′-CCG TTC CCA TCC AGC AAG AG-3′&lt;br&gt;Antisense: 5′-ACA GTC ACC AGC ACC TTT TC-3′</td>
<td>58</td>
<td>191</td>
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<tr>
<td>IL-21</td>
<td>Sense: 5′-AATCAAGCTCCCAAGGTCAAG-3′&lt;br&gt;Antisense: 5′-CAGAAATGACCCAGCAACTC-3′</td>
<td>59</td>
<td>107</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Sense: 5′-CAG CAA CAA TTC CTG GGC ATA C-3′&lt;br&gt;Antisense: 5′-TCA ACC ACT GCC GCA CAA CT-3′</td>
<td>56</td>
<td>104</td>
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<tr>
<td>CTLA-4</td>
<td>Sense: 5′-GTC CGG GTG ACA GTG CTT CG-3′&lt;br&gt;Antisense: 5′-CCA GGT AGT ATG GCG GTG GG-3′</td>
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<td>220</td>
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<tr>
<td>GITR</td>
<td>Sense: 5′-ACA CGC ACT TCA CCT GGG TCG-3′&lt;br&gt;Antisense: 5′-TGT GCC ATG CTC GGG TTT CA-3′</td>
<td>58</td>
<td>129</td>
</tr>
<tr>
<td>IL-10</td>
<td>Sense: 5′-GGG GCT TCC TAA CTG CTA CA-3′&lt;br&gt;Antisense: 5′-GGG GAA TTC CCC CGA GAC AC-3′</td>
<td>56</td>
<td>95</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Sense: 5′-GAG CTA CGA GCT GCC TGA CG-3′&lt;br&gt;Antisense: 5′-GTA GTT TCG TGG ATG CCA CAG-3′</td>
<td>56−59</td>
<td>120</td>
</tr>
</tbody>
</table>
and patients with HSP using unpaired Student t test. Level of significance assumed in these comparisons was a P value of less than 0.05. Data were analyzed using Statistical Package for the Social Sciences for Windows (SPSS, Chicago, IL, USA) software, and the results are presented as mean value ± standard deviation as appropriate.

Results

Circulating Th1, Th2, Th17, and Treg frequencies

To investigate the changes in Th cells in patients with HSP, Th1, Th2, Th17, and Treg frequencies were detected by flow cytometry. As shown in Fig. 1, the proportions of peripheral Th2 cells (1.32 ± 0.41% vs. 0.76 ± 0.24%) and Th17 cells (2.30 ± 0.55% vs. 1.20 ± 0.23%) in CD4+ cells in patients with HSP were significantly higher than those in NC (P < 0.05), while the proportions of Th1 cells (13.87 ± 3.52% vs. 15.79 ± 3.45%) and Treg cells (9.41 ± 1.43% vs. 12.20 ± 3.95%) in CD4+ cells did not differ significantly between patients with HSP and NC (P > 0.05).

Expression of transcriptional factors in CD4+ T cells from patients with HSP

As transcription factors have been proven to control the differentiation and function of Th cell subsets, real-time PCR was used to evaluate the expression levels of them to further observe the changes in Th cells in HSP. The data showed that the expression levels of GATA-3 (5.43 ± 1.33 vs. 3.19 ± 0.75) and ROR-γt (6.56 ± 1.95 vs. 1.89 ± 0.86) mRNA were much higher in the HSP group than NC group (P < 0.05), while there were no significant differences in the expression levels of T-bet (3.32 ± 0.86 vs. 3.02 ± 0.81) and Foxp3 (11.81 ± 3.76 vs. 8.48 ± 3.11) between the HSP group and NC group (P > 0.05) (Fig. 2).

Expression of cytokines in CD4+ T cells from patients with HSP

Different Th cells are characterized by their mutually exclusive expression patterns of cytokines. So expression of cytokines was detected by real-time PCR in order to deeply observe the changes in Th cells in HSP. It was shown that the IL-4, IL-5, IL-13, IL-17A, IL-17F, and IL-21 mRNA transcription levels in patients with HSP (IL-4: 11.19 ± 1.97 vs. 3.06 ± 1.06; IL-5: 37.06 ± 8.64 vs. 8.58 ± 1.74; IL-13: 29.66 ± 7.78 vs. 11.82 ± 4.10; IL-17A: 19.51 ± 7.92 vs. 9.75 ± 2.08; IL-17F: 6.41 ± 1.92 vs. 1.19 ± 0.57; IL-21: 7.37 ± 2.52 vs. 1.83 ± 0.67) were significantly higher than those in NC group (P < 0.05), while no significant differences of IFN-γ, TGF-β, CTLA-4, GITR, and IL-10 were detected between the HSP group (IFN-γ: 16.90 ± 4.43 vs. 19.08 ± 4.54; TGF-β: 6.67 ± 2.60 vs. 4.51 ± 0.90; CTLA-4: 9.01 ± 3.58 vs. 7.37 ± 2.40; GITR: 3.93 ± 1.87 vs. 2.75 ± 1.15; IL-10: 26.8 ± 9.66 vs. 20.9 ± 9.10) and the NC group (P > 0.05) (Fig. 3).

Plasma cytokine concentrations in patients with HSP

Cytokine milieu in which naïve Th cells encounter their specific antigens has long been considered to be the predominant factor that drives Th subset differentiation. Therefore, we measured plasma concentration of the cytokines that affecting Th subset differentiation in HSP. Compared to the NC group, increased IL-4, IL-17A, and IL-6 concentrations were observed among patients with HSP (P < 0.05), while the differences were not significant as to IFN-γ, IL-12, and TGF-β (P > 0.05) (Table 3).

Discussion

Previous studies had proved that Th cells differentiated into at least four subsets, Th1, Th2, Th17, and Treg cells, based on their pattern of cytokine production. Th1 cells produce large amounts of IFN-γ and direct cell-mediated immunity against intracellular pathogens, while Th2 cells involve in the humoral immunity and allergic reactions, respectively. Recently identified Th17 cells are crucial in certain autoimmune diseases and allergic diseases. Treg cells are known to suppress various immune responses including autoimmune responses [6, 7]. In this study, the proportions of Th2 and Th17 cells, and expression levels of their master transcription factor (GATA-3 and ROR-γt) in patients with HSP increased significantly, while no differences of Th1 and Treg, or their transcription factors, were found between the two groups. It suggested that aberrant activation of Th2 and Th17 cells may be involved in onset and development of HSP.

Th2 cells, which secrete IL-4, IL-5, and IL-13, are associated with antibody production and suppress the production of inflammatory cytokines. IL-4 acts as a B-cell stimulatory humoral factor through inducing cell surface antigen CD23 and promotes the shift of resting B cells to the early phase of the stimulating stage [8, 9]. Similarly, IL-13 induces IgE class switch in human cells in vitro and triggers IgG and IgM synthesis, which is closely related to IL-4 in many biological activities [10–13]. IL-5 can switch the B-cell immunoglobulin to IgA and activate eosinophils [14]. Previous study had proved that murine B cells exposed to IL-4 and IL-5 produced IgA, which plays a pivotal role in the immunopathogenesis of HSP [15]. There were also plenty of evidences that showed that increased production of IgA and IgE, circulating IgA-containing
Fig. 1 The proportions of Th1, Th2, Th17, and Treg cells in patients with HSP. Dot plots shown T cell phenotypes in peripheral blood from normal controls and patients with HSP. Th1, Th2, Th17, and Treg were defined as CD4+ IFN-γ+ cells (a), CD4+ IL-4+ cells (b), CD4+IL-17A+ cells (c), and CD4+CD25+Foxp3high cells (d), respectively.
immune complexes, and cryoglobulins existed in patients with HSP [5, 16, 17]. Consistent with the results mentioned above, transcription levels of IL-4, IL-5, and IL-13 in patients with HSP were found to be much higher than those in NC group. Therefore, it implied that the irregularities of the immune response to aberrant production of antibody might be closely correlated with the high expression of IL-4, IL-5, and IL-13 from Th2.

Th17 cells are a recently described subset of CD4+ T cells that are protective against extracellular microbes and play an important role in the pathogenesis of experimental autoimmune diseases [18–21]. IL-17A (IL-17) produced by Th17 cells enhances the expression of chemokines and inflammatory cytokines such as IL-1, IL-6, cell-adhesion factors, and other inflammatory factors and promotes leukocyte migration to inflammatory sites [22]. HSP is a systemic inflammatory disease. Lot of studies in the past revealed that expression levels of proinflammatory cytokines such as IL-6, IL-8, and TNF-α were elevated in patients with acute HSP [23–25]. High expression of ICAM-1, ICAM-2, ICAM-3, and P-selection has been reported in the patients with HSP [26, 27]. Considering increased proportions of Th17 cells in patients with HSP, it is not surprised to found that mRNA levels of IL-17A/F and IL-21 were significantly up-regulated in the patients with acute HSP, which suggested that aberrant activation of
Table 3  Plasma cytokine concentrations in patients with HSP

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>NC group (pg/ml)</th>
<th>HSP group (pg/ml)</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>9.61 ± 3.83</td>
<td>7.87 ± 2.71</td>
<td>0.978</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-12</td>
<td>10.90 ± 1.94</td>
<td>11.48 ± 5.79</td>
<td>0.267</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-4</td>
<td>18.49 ± 2.98</td>
<td>36.52 ± 16.20</td>
<td>2.90</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IL-17A</td>
<td>15.20 ± 2.49</td>
<td>23.36 ± 6.04</td>
<td>3.02</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IL-6</td>
<td>8.42 ± 1.71</td>
<td>21.87 ± 6.46</td>
<td>5.32</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TGF-β</td>
<td>20.16 ± 4.36</td>
<td>24.32 ± 6.59</td>
<td>1.49</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

Th17 cells may be responsible for the chemokines and inflammatory cytokines cascade activation, and play an important role in the development of vessel vasculitis in HSP.

Generation of different types of T cells from naïve T cells depends on the cytokine milieu. T cells exposed to TGF-β up-regulate Foxp3 and differentiate into Treg cells. However, when cultured with TGF-β and IL-6, naïve T cells generate Th17 cells with high pathogenic activities [28, 29]. In this study, increased IL-6 concentration was observed in patients with HSP, suggesting that high expression of IL-6 might play an important role in the aberrant activation of Th17 cells. Similarly, IL-12 is important for Th1 development and IL-4 for Th2 development. Hence, our data of higher concentration of IL-4 could suggest its role in the abnormal differentiation of Th2 in HSP.

In conclusion, our data demonstrate that Th cell subset functional imbalance and aberrant activation of Th2 and Th17 cells exist in patients with HSP. High expression of Th2 and Th17 cells might be closely correlated with aberrant production of antibody and the development of vessel vasculitis. The strong polarizing cytokine milieu in peripheral blood may play an important role in the Th cell subset imbalance.

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References


