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Analysis of the changes in Th9 cells and related cytokines in the peripheral blood of spontaneous urticaria patients

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Abstract. Spontaneous urticaria (SU) is characterized by immune deregulation of mast cells and T helper (Th) cells. Th9 cells, a subset of Th cells, serve a key role in initiating mast cell accumulation and activation. To understand the role of Th9 cells in the pathogenesis of SU, the authors conducted a control study of 28 patients with acute SU (ASU) and chronic SU (CSU) and 28 healthy controls. The percentage of Th9 cells in peripheral blood was assessed using flow cytometry and levels of Th9 related serum cytokines including interleukin (IL)-4, IL-9, IL-17A, IL-33, IL-1β and transforming growth factor-β1 (TGF-β1) using Luminex 200. ASU patients exhibited higher percentages of Th9 cells and increased serum levels of cytokines IL-9, IL-4 and TGF-β1 compared to healthy controls. In addition, high mRNA expression of the PU.1 transcription factor was observed in ASU patients. However, the percentage of Th9 cells was similar between patients with CSU and healthy controls. Furthermore, the percentage of Th9 cells demonstrated a positive correlation with IL-4 and IL-9 levels in the peripheral blood of ASU patients, but not with disease severity. The current findings suggested that the numbers of Th9 cells increased in ASU patients and indicated its novel role in the pathogenesis of ASU.

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Abbreviations: SU, spontaneous urticaria; PBMCs, peripheral blood mononuclear cells; TGF-β, transforming growth factor-β; IL, interleukin; PBS, phosphate-buffered saline; Th, T helper cells; UAS, urticarial activity score

Key words: spontaneous urticarial, Th9 cells, interleukin-9, interleukin-4, transforming growth factor-β1

Introduction

Spontaneous urticaria (SU) is a common skin condition characterized by the recurrent appearance of pruritic wheals, occasionally accompanied by angioedema (1). Analysis of skin biopsies from SU patients has demonstrated that most inflammatory cells surrounding the small venules include $CD4^+$ T cells, neutrophils, mast cells, basophils and eosinophils (2,3). In particular, the release of histamine and other proinflammatory mediators, due to aberrant activation of mast cells, is the key pathophysiological event for urticaria (4). However, the mechanistic insight into mast cells degranulation remains poorly understood (5).

Recent studies have indicated that Th9 cells, a subset of T-helper cells, serve a key role in mast cell accumulation and activation during allergic inflammation (6,7), and are a major source of interleukin-9 (IL-9). PU.1 (also known as SPI1) is the key transcription factor through which Th9 cells regulate IL-9 production. More importantly, the study by Schlapbach *et al* (8) revealed that most memory Th9 cells were skin-tropic and appeared to possess both autocrine and paracrine pro-inflammatory abilities (8). Moreover, gene expression analysis of SU skin lesions had also demonstrated significant upregulation of cytokine IL-9 signaling pathways (9). These data overall indicate that Th9 cells serve an important role in skin inflammatory diseases.

Cytokines have generally been known to be critical to skin inflammation and regulation of naive T cell differentiation into distinct effector T cells subsets. For example, IL-4 stimulation leads to Th2 cell polarization, while TGF-β induce regulatory T cell differentiation (10). However, it has been observed that, in the absence of IL-6, TGF-β and IL-4 induce Th9 cell generation (11). Additionally, IL-1β and IL-33 stimulation can induce Th9 cell differentiation by the activation of nuclear factor-κB (12,13). Despite considerable attention paid to the research of Th9 cells and related cytokines, their expression levels and roles in SU patients remain largely unclear.

Thus, in the present study, the authors have made an effort to understand the contribution of Th9 cells in the pathogenesis of SU, by comparing Th9 cell populations in the peripheral blood of patients with SU and healthy controls, along with analyzing the expression of the transcription factor PU.1. Moreover, as cytokines have been linked with the regulation of Th9 cell differentiation and function, the authors assessed the

plasma concentrations of cytokines, IL-4, IL-9, IL-17A, IL-33, IL-1β and transforming growth factor-β1 (TGF-β1). Finally, the correlation between Th9 cells population and levels of cytokines was explored.

Materials and methods

Study design. The present study is a case-control study (EC/2015/005) approved by the Ethics Committee of Binhai County Hospital (Yancheng, China). The study was conducted in the Dermatology Clinic at Binhai County Hospital (Yancheng, China) between November 2015 and March 2016, following obtaining written informed consent from all patients.

Study participants. The current study comprised 28 healthy volunteers and 56 patients diagnosed with acute SU (ASU, hives lasting <6 weeks; n=28) or chronic SU (CSU, hives lasting >6 weeks; n=28), who were all 12 years or older. SU patients were interviewed regarding the duration and type of urticaria by trained dermatologists, and the average urticaria activity score was measured to assess disease severity (typically, 7 days) based on EAACI/GA(2)LEN/EDF/WAO guidelines (14). Treatment was suspended for at least 2 weeks prior to enrolling patients in the study. Patients with physical, cholinergic, aquagenic, contact and exercise-induced urticaria were excluded from the study.

Healthy volunteers (n=28) who were blood donors in the control group were age and sex matched. Any patients receiving immunosuppressive medication or with any immune system disorder were excluded. Clinical characteristics of the subjects are summarized in Table I.

Blood sample preparation. At the initial visit, 8 ml peripheral blood was drawn from each subject in a tube with heparin sodium. Out of the 8 ml blood, 1 ml was used for flow cytometric detection of the Th9 cell population within 24 h according to manufacturer's instructions. Another 2 ml was used for preparation of serum and the remaining 5 ml was used for the isolation of peripheral blood mononuclear cells (PBMCs). All blood samples were obtained before patients received any treatment.

Detection of Th9 cell population in peripheral blood. For Th9 cell detection, peripheral blood cells (100 μl) were activated with phorbol-12-myristate-13-acetate (50 ng/ml) and ionomycin (1 μg/ml) in the presence of 1 μg/ml brefeldin (all from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 5 h at 37˚C. Following activation, the samples were stained with fluorescein isothiocyanate-labeled anti-CD3 (cat. no. 555339), and phycoerythrin-labeled anti-CD4 (cat. no. 555347) monoclonal antibodies for 30 min (BD Biosciences, San Jose, CA, USA) at room temperature according to the manufacturer's instructions. This was followed by the lysis of red blood cells (BD Biosciences) and fixation with BD Cytofix™ fixing buffer (BD Biosciences). Next, cells were permeabilized by adding BD Perm/Wash™ buffer (BD Biosciences) and incubated with PerCP-cy5.5 labeled anti-IL-9 (cat. no. 561461; 1:10; BD Biosciences) and PEcy7 labeled anti-IL-17 antibodies (cat. no. 25717942; 1:10; eBioscience, Inc.; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 30 min at room temperature. Finally, the labeled cells were suspended in 200 μl phosphate-buffered saline and immediately analyzed with CellQuest Pro (BD Biosciences). $CD4^+$ T cells with the $CD4^+IL\text{-}9^+IL\text{-}4^-IL\text{-}17^-$ phenotype represented the Th9 cell population.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). PBMCs purified by Ficoll-Hypaque density gradient centrifugation method (Shanghai Westang Bio-Tech Co., Ltd., Shanghai, China) were used to isolate total RNA using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA synthesis and amplification was done using isolated RNA with the cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and QuantiTect SYBR-Green PCR kit (Invitrogen; Thermo Fisher Scientific, Inc.), respectively. The following primer sequences were used for gene specific amplification: PU.1 sense, 5'-TGAGAAGGACAGGGAGCCAA-3' and antisense, 5'-GAGAAGCTGAGTGCCATGCA-3'; β-actin sense, 5'-TGGCACCCAGCACAATGAA-3' and antisense, 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'. The reaction mix was run with each 20 μl reaction containing ~50 ng cDNA, 0.3 μM sense and antisense primers on a thermal cycler [7500 PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.)] with following conditions: 1 min at 95˚C, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. The relative mRNA levels of the PU.1 gene were calculated by the $2^{-\Delta\Delta Cq}$ method (15).

Cytokine analysis. To detect cytokines, serum samples were collected from 2 ml peripheral blood and immediately stored at -80˚C, until cytokine analysis. The detection of cytokines IL-4, IL-17A, IL-9, TGF-β1, IL-1β and IL-33 was performed according to the manufacturer's protocol (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and their concentrations were determined using Luminex 200 (Luminex Corporation, Austin, TX, USA). Each sample was measured twice and the mean value was used for statistical analysis. The minimum detectable concentrations of cytokines IL-9, IL4, IL-17A, TGF-β1, IL-1β and IL-33 in the present assay were 6.17, 13.10, 9.21, 6.12, 3.12 and 2.16 pg/ml, respectively.

Statistical analysis. Statistical analysis was performed using Stata (version, 7.0; (StataCorp LLC, College Station, TX, USA) software. The skewed data were expressed as median (M, 25-75 percentiles). Statistical significance between three and two groups was determined by the Kruskai-Wallis test and a two-tailed Mann-Whitney U test, respectively. The normal distribution of data was presented as mean ± standard deviation and statistical significance in two groups was compared using the Student's t-test. Linear correlations were calculated by Spearman coefficients. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ASU patients displayed increased Th9 cell population. Changes in Th9 cell population have been reported in patients with allergic skin diseases (16). Here, the authors assessed the percentage of Th9 cells (Fig. 1A-C) in peripheral blood isolated from ASU, CSU and healthy control subjects. The

Table I. The clinical characteristics of patients and healthy controls.

Parameters	ASU	CSU	Controls
Age (years)	36.5±5.6	35.6±6.1	35.8±7.9
Gender (female/male)	16/12	17/11	16/12
Serum total IgE (IU/ml)	226.8±63.9[a,b]	82.4±31.2[a]	60.7±28.3
Disease severity			
Mild (0-2)/day	8/28	7/28	
Moderate (3-4)/day	9/28	9/28	
Severe (5-6)/day	11/28	12/28	

[a]P<0.05 vs. healthy controls; [b]P<0.05 vs. CSU. ASU, acute spontaneous urticaria; CSU, chronic spontaneous urticaria.

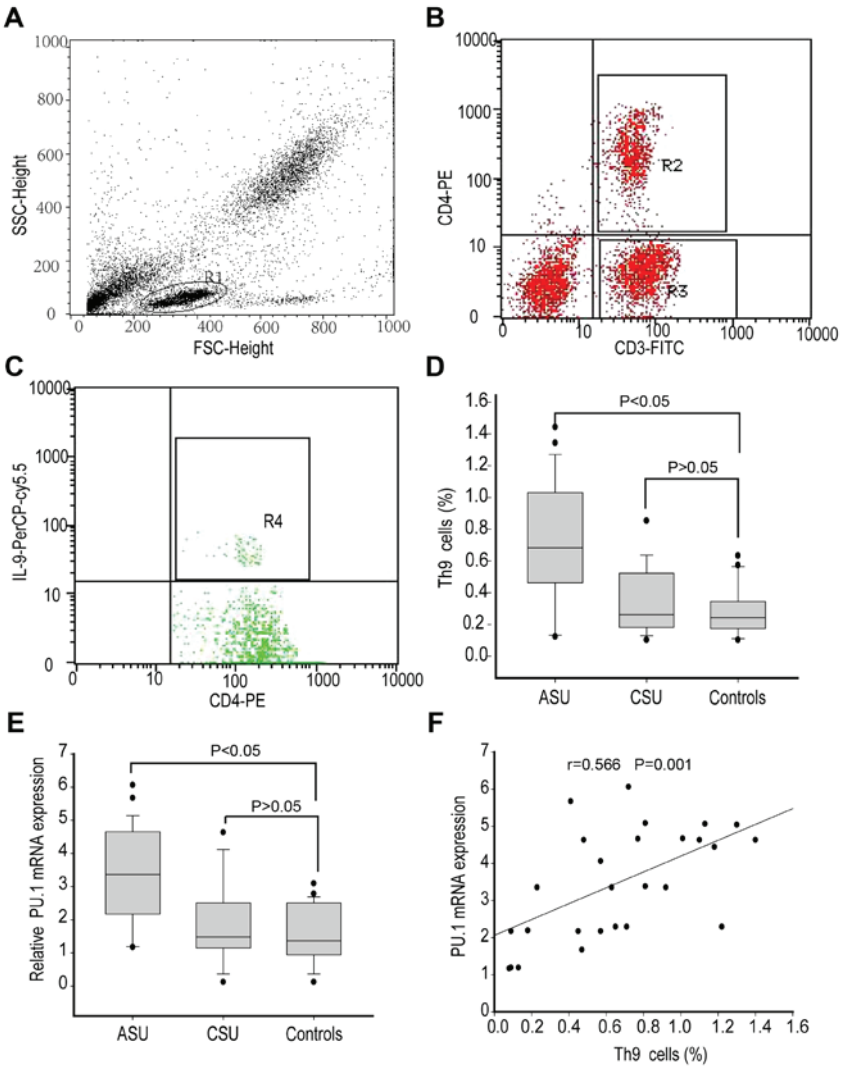

Figure 1. Analysis of Th9 cell percentage in SU patients. Peripheral blood samples were stained and analyzed by flow cytometry. (A) Lymphocytes were initially gated based on light forward and side scatter. (B) Plots presenting CD4+ lymphocytes (R2) on gated CD3+ lymphocytes. (C) Plots demonstrating IL-9 expression on gated CD4+ lymphocytes (R4). (D) Percentages of Th9 cells among CD4+ T cells from healthy controls and patients with SU. (E) PU.1 mRNA expression in PBMCs from SU patients and healthy controls was measured by reverse transcription-quantitative polymerase chain reaction. (F) Correlation analysis between the percentage of Th9 cells and the PU.1 mRNA expression in PBMCs from ASU patients. Box plots represent the median (25-75 percentile) and dots represents outliers. Statistical significance was determined by Kruskai-Wallis test. Th9, T helper cell 9; SU, spontaneous urticaria; IL, interleukin; ASU, acute spontaneous urticaria; FITC, fluorescein isothiocyanate; PE, phycoerythrin; CSU, chronic spontaneous urticaria.

data demonstrated that ASU patients had a higher percentage of Th9 cells (median 0.65%, range 0.43-0.97%; P<0.05) when compared to CSU patients (median 0.21%, range 0.13-0.44%) and healthy controls (median 0.20%, range 0.13-0.32%) as

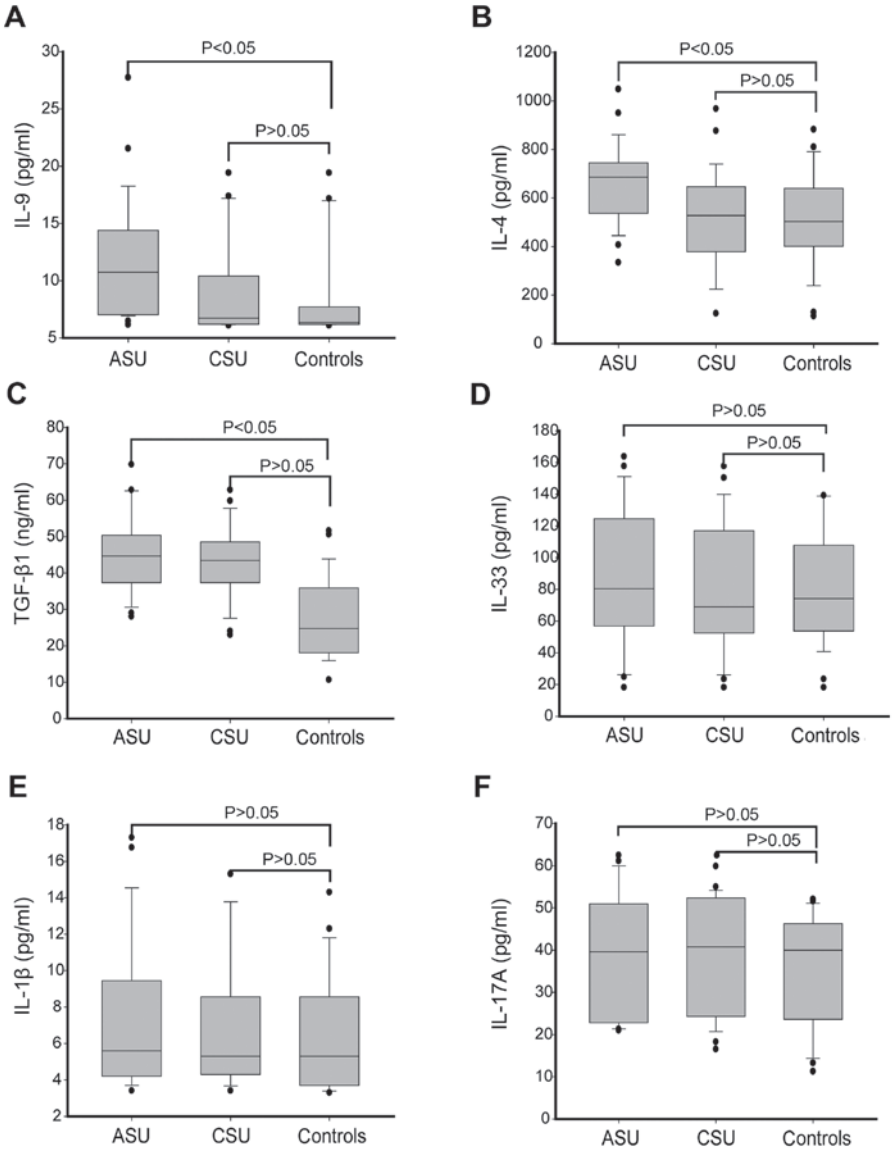

Figure 2. Analysis of Th9 related cytokines in SU patients and control groups. Plasma levels of Th9 related cytokines (A) IL-9, (B) IL-4, (C) TGF-β1, (D) IL-33, (E) IL-1β and (F) IL-17A. Box plots represent the median (25-75 percentile) and dots represents outliers. Statistical significance was determined by Kruskai-Wallis test. Th9, T helper cell 9; SU, spontaneous urticaria; IL, interleukin; TGF-β1, transforming growth factor-β1; ASU, acute spontaneous urticaria; CSU, chronic spontaneous urticaria.

indicated in Fig. 1D. However, the percentage of Th9 cells indicated similarities between CSU patients and healthy controls (P>0.05; Fig. 1D).

Next, the authors assessed the mRNA levels of PU.1, which is the primary transcription factor involved in Th9-mediated function. RT-qPCR analysis suggested that the expression of PU.1 mRNA was significantly increased in the PBMCs isolated from ASU patients (median 3.44, range 2.23-4.85; P<0.05) in comparison to CSU patients (median 1.41, range 1.13-2.52) and healthy controls (median 1.32, range 0.98-2.36), as presented in Fig. 1E. However, CSU patients and healthy controls did not present any significant difference. Moreover, the percentage of Th9 cells also demonstrated a positive correlation with PU.1 mRNA levels (r=0.566, P<0.05) in ASU patients (Fig. 1F).

Comparison of Th9 related cytokines in SU patients. As cytokines can affect Th9 cells differentiation and function, serum concentrations of cytokines IL-9, IL-33, IL-4, TGF-β1, IL-1β and IL-17A were measured. It was observed that ASU patients had higher levels of IL-9 and IL-4 (P<0.05) compared to CSU patients and healthy controls, although there was no significant difference between them (P>0.05), as identified in Fig. 2A and B. However, TGF-β1 presented higher levels in both ASU and CSU patients as compared to healthy controls (P<0.05; Fig. 2C), whereas no significant differences were observed in IL-33, IL-1β and IL-17A levels between these three groups (P>0.05; Fig. 2D-F).

Th9 cell percentage and serum cytokines IL-4 and IL-9 levels demonstrated a positive correlation in ASU patients. Next, the authors examined whether there was any correlation between the percentage of Th9 cells and the concentrations of cytokines TGF-β1, IL-4 IL-9, IL-1β, IL-33 and IL-17A in the serum of ASU patients. Interestingly, a positive correlation was observed between the percentage of Th9 cells and the concentration of IL-9 (r=0.644, P<0.05) and IL-4 (r=0.444,

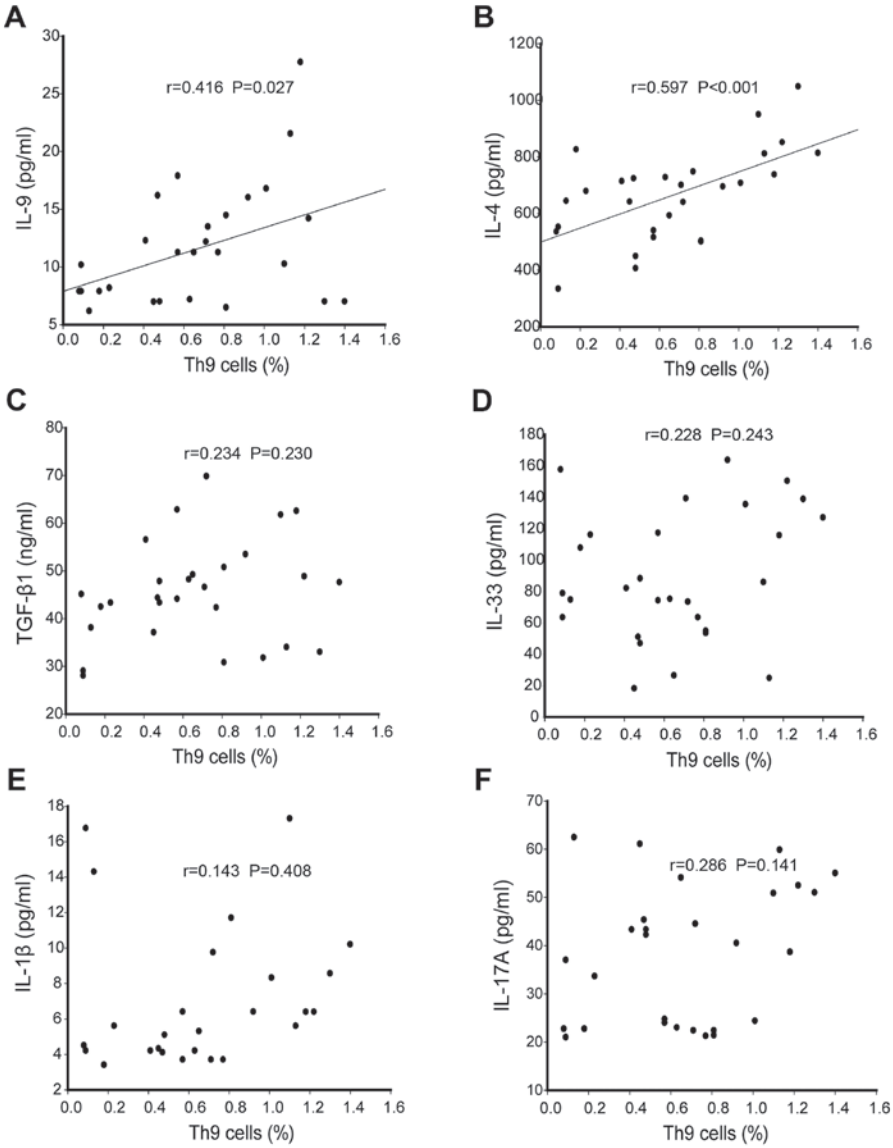

Figure 3. Relationship between serum cytokines and Th9 cells in SU patients. Correlation between Th9 cells and the serum levels of (A) IL-9, (B) IL-4, (C) IL-33, (D) IL-10, (E) IL-6 and (F) TGF-β1 in ASU patients. Th9, T helper cell 9; SU, spontaneous urticaria; ASU, acute spontaneous urticaria; IL, interleukin; TGF-β1, transforming growth factor-β1.

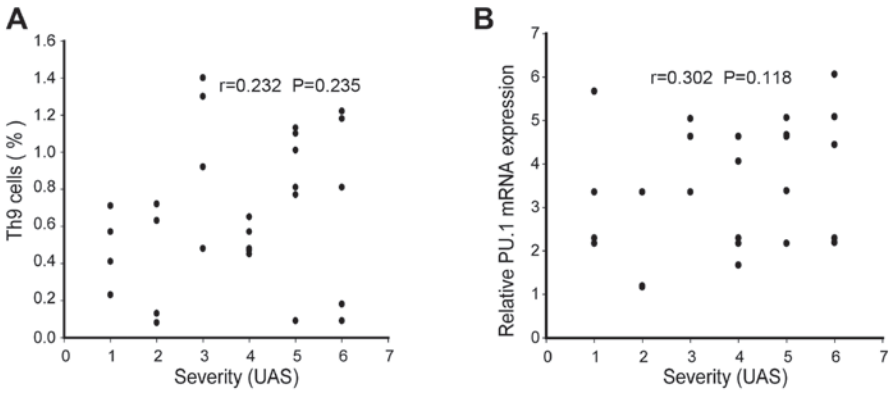

Figure 4. Correlation between disease severity and Th9 cells in ASU patients. Correlation between UAS and the levels of (A) Th9 cells and (B) PU.1 mRNA expression in ASU patients. Th9, T helper cell 9; ASU, acute spontaneous urticaria; UAS, urticaria activity score.

P<0.05) in serum, as presented in Fig. 3A and B. However, Th9 cell percentage did not demonstrate any correlation with the concentrations of TGF-β1, IL-1β, IL-17A and IL-33 cytokines in the serum of ASU patients (P>0.05; Fig. 3C-F).

Th9 cell percentage and PU.1 mRNA expression did not present correlation with disease severity in ASU patients. Finally, the correlation between urticaria activity score and the levels of Th9 cells and PU.1 mRNA expression was tested in ASU patients. The current analysis revealed that disease severity indicated no correlation with the percentage of Th9 cells (r=0.232, P>0.05,) and PU.1 mRNA expression (r=0.302, P>0.05) in the peripheral blood (Fig. 4A and B).

Discussion

The present study identified that ASU patients displayed significantly increased frequency of Th9 cells in peripheral blood than CSU patients and healthy controls. This observation was consistent with RT-qPCR results, which demonstrated higher mRNA expression of the PU.1 transcription factor, primarily responsible for Th9 cell-mediated regulation of IL-9 production. In addition, there were increased levels of Th9-related cytokines, such as TGF-β1, IL-9 and IL-4 in the peripheral blood of ASU patients, which has been suggested to serve an important role in Th9 development (11).

PU.1 is a transcription factor implicated in the regulation of Th9 cells function by directly binding to IL-9 loci (17). The observation of an increased percentage of Th9 cells along with higher expression of PU.1 mRNA and IL-9 cytokine in the peripheral blood of ASU patients suggested that, Th9 is functionally important in ASU. The study by Ma *et al* (16) also confirmed the pathogenic role of Th9 cells in atopic dermatitis based on similar findings. Furthermore, Th9 cell percentage positively correlated with IL-9 levels. As it has been suggested previously that Th9 cells appear to be important cellular sources of IL-9, which contributes to mast cell proliferation (6), it seems logical that Th9 cells may serve an important role in SU, which involves mast cell regulation. However, surprisingly, a correlation between an increased proportion of Th9 cells and disease severity in ASU patients was not observed. The tentative explanation may be that Th9 cells were skin-tropic or skin-resident, as described previously (8).

In addition, the association between the plasma TGF-β1 levels and the risk of SU has been reported in a previous study (18). Patients with CSU displayed TGF-β genetic variability, which leads to increased production of TGF-β (19,20). In agreement with these findings, the present data indicated that ASU patients had significantly high plasma levels of TGF-β1 and IL-4, which may account for increased Th9 development (11).

IL-33, the other epithelial cytokine, and a newly recognized member of the IL-1 family, is a multifunctional protein. It has been reported to bind to the cell membrane receptor ST2 and promote Th2 responses in T cells, mast cells, eosinophils, basophils and innate lymphoid cell populations (21). Consistent with a previous report (22), the present study confirmed that all three groups had similar levels of IL-33, and thus indicated that a Th2 response may not be important in SU pathogenesis. However, IL-1β has been presented to induce Th9 differentiation (13), but similar levels between SU patients and controls were also observed.

Another cytokine, IL-9, following stimulation by Jak1, can also induce Th17 cell proliferation (23). Th17 cytokines, such as IL-17A-F, are believed to be crucially involved in the pathogenesis of some autoimmune diseases (24). However, there are some discrepancies between the results of the levels of IL-17 in ASU and CSU patients reported in different studies (19,25,26). These data, however, demonstrated no significant difference in IL-17A levels between ASU, CSU patients and healthy controls.

Importantly, the current study has a few limitations. Firstly, due to the limitation of the study protocol, it could not be determined whether the increase in Th9 cell population was primary or secondary to other changes, such as TGF-β1 and IL-4 in the peripheral blood. Secondly, the sample size of patients with SU and healthy controls was very small. Thirdly, it has been shown that ~30-50% of patients with CSU produce circulating antibodies, while most cases of ASU are associated with viral infections or allergens (5,27). Thus, in future studies, the authors intend to investigate the effect of Th9 cells underlying each of these precipitating factors.

In conclusion, the present study demonstrated that the percentage of Th9 cells in the peripheral blood of ASU patients was markedly increased when compared to healthy controls. Furthermore, ASU patients exhibited an increased Th9 related cytokines, such as TGF-β1 and IL-4. These results indicated that the increased levels of Th9 cells may serve a role in the pathogenesis of SU.

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