

Original Article

Relationship between atopy and mycoplasma pneumoniae infection in systemic juvenile idiopathic arthritis children and its influence on prognosis

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Abstract: Objective: To investigate the relationship between atopy and Mycoplasma pneumoniae (MP) infection in systemic juvenile idiopathic arthritis (SoJIA) children and its impact on the prognosis of the disease. Methods: The clinical data of 39 SoJIA children were collected including atopy, MP infection and course characteristics; RT-PCR method was performed to detect expression levels of IL-17, Foxp3, MDR-1 and MRP-1 mRNA in peripheral blood mononuclear cells; Flow cytometry was applied to analyze peripheral blood lymphocytes ratio discharging daunorubicin. Results: MP infection rate is significantly higher in SoJIA children with atopy ($X^2=11.7986$, $p=0.0006$); non-monophasic disease ratio in MP+Atopy+ group, MP+Atopy- group and MP-Atopy+ group were significantly higher than MP-Atopy- group ($X^2=14.4026$, $p=0.0024$). The mRNA expression levels of IL-17 and MDR-1 in Atopy+ group and MP+ group were significantly higher than MP-Atopy- group, Atopy- group, MP- group and the normal control group; moreover, levels of IL-17 and MDR-1 in MP+Atopy+ group were significantly higher than MP-Atopy- group and normal control group ($F=5.1016$, $P=0.0001$; $F=16.4463$, $P=0.0000$; respectively). In contrast, Foxp3 mRNA expression in Atopy+ group, MP+ group were significantly lower than MP-Atopy- group, MP- group, Atopy- group and the normal control group, also expression of Foxp3 in MP+Atopy+ group was significantly lower than MP-Atopy- group and the normal control group ($F=11.8421$, $P=0.0000$). The ratio of lymphocytes capable of discharging daunorubicin in MP+Atopy+ group is significantly higher than the MP-Atopy- group ($t=2.5399$, $p=0.0164$). Conclusion: There are close relations among SoJIA children complicated with atopy and MP infection and Th17/Treg cells imbalance. SoJIA children merged atopy or MP infection may promote Th17 cell proliferation and increase IL-17 expression resulting in the aggravation of an autoimmune reaction, subsequently induce SoJIA children resistance by upregulating the expression of the multidrug resistance gene MDR-1, maybe these two aspects eventually lead to SoJIA children with poor prognosis.

Keywords: Systemic juvenile idiopathic arthritis, IL-17, Foxp3, multidrug resistance gene, prognosis

Introduction

Systemic juvenile idiopathic arthritis (SoJIA) is a common autoimmune and systemic inflammatory disease [1, 2]. Although the strategy has been advanced in the long-term treatment [3-5], the etiology of SoJIA and the pathogenesis is still not fully known [6]. Most children with SoJIA symptoms were not completely relieved, and the prognosis was poor. In addition to immune system disorders can affect the prognosis of SoJIA [7, 8], the SoJIA was also associated with genetic [9], environmental [10], and infectious factors [11]. Many cytokines of the innate immune response have been suggested implicating with SoJIA [12]. A recent study dem-

onstrated that IL-17A was overexpressed in sera from patients with active systemic JIA [13]. Our experiment was designed to study the correlation of the expression of IL-17, Foxp3, MDR-1 in the mononuclear cells of patients with Mycoplasma pneumoniae (MP) infection and the atopy children of SoJIA, and the effect of this relationship on the prognosis in SoJIA.

Materials and methods

Collection of cases

Thirty-nine cases of patients with systemic juvenile idiopathic arthritis (the diagnostic criteria were referred to the diagnostic criteria

IL-17, FOXP3 and MDR-1 in SoJIA patients with atopy or MP infection

Table 1. List of primers

Genes	Primers	
	5R	3F
MDR-1	5'-TATAATGCGACAGGAGATAGG-3'	5'-TTGCCATTGACTGAAAGAAC-3'
GADPH	5'-GTGAAGGTCGGAGTCAACG-3'	5'-TGAGGTCAATGAAGGGGTC-3'
MRP-1	5'-GGACCTGGACTTCGTTCTCA-3'	5'-CGTCCAGACTTCTTCATCCG-3'
IL-17	5'-TGCTGGAGAAGATACTGGTG-3'	5'-TGAGTCCGAAATGAGGCTGT-3'
Foxp3	5'-CACTGCCCTAGTCATGGT-3'	5'-GGAGGAGTGCCTGTAACCTGG-3'

Table 2. Correlation between Atopy and MP infection in Children with SoJIA

	MP-	MP+	X ²	p
Atopy+	1	16	11.7986	0.0006
Atopy-	13	9		

Comparison between the two groups applied t-test, ANOVA was used for comparison among multiple groups, and the numeration data were statistically analyzed with X² test.

established by the International Association of Rheumatology in Edmonton in 2001) were collected in our hospital or clinic. 28 were males and 11 females with an average age of 9.54 ± 2.86 years old and followed up for at least 2 years. 30 healthy children at the same period were included for control group with 18 males and 12 females, with a median age of 8.61 ± 2.74 years old, and no personal and family history of allergic diseases, Mycoplasma pneumoniae (MP) infection test was negative. The patients were divided into 8 groups according to whether the patients suffering the MP infection and Atopy. The experimental group consisted of MP+Atopy+ group, MP-Atopy-group, MP+Atopy- group, MP-Atopy+ group, MP+ group, MP- group, Atopy+ group and Atopy- group. Mycoplasma pneumoniae infection diagnosis standard was blood MP-IgM $\geq 1:160$ by passive agglutination method and pharyngeal swab culture MP positive. The diagnostic criteria of atopy were as follows: 1) Skin prick test positive or specific IgE positive; 2) Any clinical manifestations of allergic rhinitis, allergic asthma and atopic dermatitis; 3) Having a personal/family history of allergies, food or drug allergy; patients were considered as Atopy if fulfill at least two of the above three conditions and the first one is essential.

According to the course of disease condition, the patients were divided into monophasic dis-

ease (single disease duration of no more than 24 months, and subsequently in a state of inactivity), persistent disease (in a state of active for more than 24 months), multiphasic disease (disease activity can occur at any time with an inactive state). Persistent disease and multiphasic were included in non-

monophasic course of disease [14]. Meanwhile, "The inactive state condition" must meet all the following criteria: (1) No active inflammation in the joints (assessment by ACR 0); (2) No fever, rash, serositis, hepatosplenomegaly or lymphadenopathy; (3) No active ophthalmic disease (Every ophthalmologist has the same opinion); (4) Normal erythrocyte sedimentation rate (ESR) and C-reactive protein level; (5) Physician overall assessment conclusion of inactivity condition [15].

RT-PCR

Peripheral blood mononuclear cells (PBMCs) isolation: 3 mL of EDTA blood were diluted with an equal volume of phosphate buffered saline (PBS). Diluted blood was deposited over Lympholyte in a tube. After centrifugation for 20 minutes at 2500 rpm, the lymphocyte layer was then extracted and suspended in 5 mL of PBS for cell counting (Cell viability was above 95%). Then cells were pelleted at room temperature for 10 minutes at 1500 rpm.

Total RNA was extracted with TRIzol reagent. Preparation of cDNA: The total 10 μ L cDNA synthesis reaction contained 4 μ L total RNA, 2.0 μ L 5 \times primerScriptTM Buffer, 0.5 μ L Random 6 mers (100 μ M), 0.5 μ L PrimerScriptTM RT Enzyme Mix 1, 0.5 μ L Oligo dT Primer (50 μ M) and 2.5 μ L RNase Free water. The reverse transcription PCR reaction was 37°C for 15 min, followed by 85°C for 5 second.

The RT-PCR reaction mixture (25 μ L volume) contained 2 μ L of template cDNA, 0.5 μ L (10 μ M) of each primer set, 12.5 μ L SYBR Premix Ex Taq TM, and 9.5 μ L of ultrapure millipore water. The PCR amplification conditions were as follows: 35 cycles at 95°C for 1 min, 52°C for 1 min and 72°C for 1 min. Final extension step was carried out at 95°C for 15 s. The prim-

IL-17, FOXP3 and MDR-1 in SoJIA patients with atopy or MP infection

Table 3. Comparison of Course characteristics in each group

	MP-Atopy-	MP+Atopy+	MP+Atopy-	MP-Atopy+	X ²	p
Monophase course	12	4	4	0	14.4026	0.0024
Non monophase course	1	12	5	1		

Comparison between the two groups applied t-test, ANOVA was used for comparison among multiple groups, and the numeration data were statistically analyzed with X² test.

Table 4. The expression of *IL-17* and *Foxp3* in different group

	n	IL-17 mRNA	Foxp3 mRNA
MP+Atopy+	16	13.81 ± 1.94	6.55 ± 1.78
MP-Atopy-	13	9.81 ± 0.64	10.02 ± 1.52
Atopy+	17	13.41 ± 2.78	7.33 ± 1.83
Atopy-	22	10.46 ± 4.59	9.15 ± 2.83
MP+	25	12.99 ± 3.44	7.04 ± 2.01
MP-	14	9.83 ± 3.75	9.94 ± 3.06
Control	30	9.67 ± 4.45	10.07 ± 0.98
F		5.1016	11.8421
p		0.0001	0.0000

Comparison between the two groups applied t-test, ANOVA was used for comparison among multiple groups, and the numeration data were statistically analyzed with X² test.

ers information used for RT-PCR as shown in **Table 1**.

Flow cytometry analysis

Mononuclear cells were isolated from 3 ml peripheral blood using EDTA anticoagulant negative pressure vacuum vessel and suspended in PBS with concentration of 1×10^6 /ml. Three test tubes were injected with 1ml single cell suspension liquid. Tube 1 and 2 added with 20 µl Daunorubicin (400 µM), well-mixed respectively. Tube 1 was keep at 37°C in the dark for 30 min by using water bath, Tube 2 and 3 were hold in the dark on ice for 30 min, then flow cytometry tests were performed.

Statistical analysis

SAS8.2 software was used for statistical analysis, and the measurement data were presented as mean ± standard deviation. Comparison between the two groups applied t-test, ANOVA was used for comparison among multiple groups, and the numeration data were statistically analyzed with X² test. $p < 0.05$ means the difference was statistically significant.

Result

Correlation between atopy and MP infection in patients with SoJIA

In the patient group, there were 17 patients suffering from Atopy, 25 patients with MP infection, and 16 patients both with atopic and MP infection (**Table 2**). Statistics show that the SoJIA patients with Atopy were more likely to be infected by MP. Meanwhile, the number of patients with both MP infection and Atopy were greater than patients with MP infected only ($p < 0.05$).

Disease course characteristics of atopy and MP infection in patients with SoJIA

In MP+Atopy+ group, MP+Atopy- group and MP-Atopy+ group, the rates of non-monophasic course were significantly higher as compared with MP-Atopy- group ($P < 0.05$) (**Table 3**).

The expression of *IL-17* and *Foxp3*

The expression of *IL-17* in different groups was determined by RT-PCR. The results showed that expression of *IL-17* of Atopy+ group was significantly higher than that of MP-Atopy- group, Atopy- group and normal control group; MP+ group was significantly higher than MP-Atopy- group, MP- group and normal control group; MP+Atopy+ group was significantly higher than the MP-Atopy- group and the control group (**Table 4**).

Comparison of *Foxp3* expression in different groups as follow: Atopy+ group was significantly lower than MP-Atopy- group, Atopy- group and normal control group; MP+ group was significantly lower than MP-Atopy- group, MP- group and normal control group; MP+Atopy+ group was significantly lower than the MP-Atopy- group and the normal control group (**Table 4**).

Differences between the individual groups has statistically significant ($p < 0.05$).

Table 5. The expression of *MDR-1* and *MRP-1* in different group

	n	MDR-1 mRNA	MRP-1 mRNA
MP+Atopy+	16	9.20 ± 1.47	4.82 ± 1.67
MP-Atopy-	13	7.39 ± 0.69	4.02 ± 1.27
Atopy+	17	9.06 ± 1.51	4.76 ± 1.64
Atopy-	22	7.53 ± 0.96	4.10 ± 1.75
MP+	25	8.70 ± 1.55	4.61 ± 1.94
MP-	14	7.37 ± 0.67	4.00 ± 1.23
Control	30	6.03 ± 1.41	3.08 ± 1.96
F		16.4463	1.3043
P		p=0.0000	p=0.4287

Comparison between the two groups applied t-test, ANOVA was used for comparison among multiple groups, and the numeration data were statistically analyzed with X² test.

The expression of MDR-1 and MRP-1

The expression of *MDR-1* in Atopy+ group was significantly higher than that in MP-Atopy- group, Atopy- group and normal control group; also *MDR-1* expression in MP+ group was significantly higher than that in MP-Atopy- group, MP- group and normal control group; moreover, *MDR-1* level in MP+Atopy+ group was significantly higher than that of MP-Atopy- group and normal control group, the differences were statistically significant (p<0.05) (**Table 5**). However, there was no significant difference in *MRP-1* expression in different groups.

Comparison of ratios of lymphocytes capable of discharging daunorubicin

The lymphocyte ratio of MP+Atopy+ group was significantly higher than that of group MP-Atopy- (41.18 ± 17.28% vs 27.89 ± 8.28%, t=2.5399, p=0.0164) (**Figure 1**). In addition, there was no significant difference among other groups.

Discussion

SoJIA is an autoimmune disease with irregular fever, arthritis, skin rash, hepatosplenomegaly and lymphadenopathy, serositis with systemic involvement [1, 2]. According to statistics, the incidence of SoJIA is about 1/10000, SoJIA accounted for only 10%~15% of juvenile idiopathic arthritis (JIA), but the morbidity and mortality occupied more than 2/3 in JIA [16]. The SoJIA disease is dangerous, repeatedly, and difficultly to control. At present, people often

use glucocorticoid combined with drug treatment to change the condition [17]. Although the treatment plan has been improved in the long-term treatment, most of the children with SoJIA have not been completely relieved. And some of them even have fatal complications [8, 18, 19], and the prognosis was poor [20]. In several studies, the immune system disorder, genetic, environmental, and infectious factors were found to be associated with the prognosis of SoJIA.

Both atopic and autoimmune diseases are disorders of the immune system, which is caused by the tissue injuries and chronic inflammation induced by the excessive immune response to autologous or external antigens. With further understanding of the etiology and pathogenesis of those diseases, the relationship between them has become a concern for medical researchers and clinicians. Simpson and colleagues [21] conducted a cross-sectional study of the disease information of 252538 patients in a Scotland population disease database, they found that patients with a history of allergic diseases showed increased incidence of autoimmune diseases mediated by Th1 cells, while the prevalence of Th2 mediated eczema and allergic rhinitis in patients with autoimmune diseases was significantly higher than that in the general population [21]. The researchers also suggest that there was a positive correlation between psoriasis and eczema with the two typical Th1 and Th2 mediated diseases. In our clinical work, we also found a considerable part of the SoJIA children with atopy also accompanied by allergic asthma, allergic rhinitis and atopic dermatitis or family history of allergy, and an increased content of specific IgE by laboratory examination. Therefore, we also investigated the effects and mechanisms of atopy on the severity and prognosis of children with SoJIA.

In the present study, our results showed that children with SoJIA and Atopy were more likely to be infected by MP. Moreover, the number of patients with MP and Atopy were more than MP infected only. In MP+Atopy+ group, MP+Atopy- group and MP-Atopy+ group, the rate of non-monophasic course was significantly higher than that of MP-Atopy- group (P<0.05). Correspondingly, we speculated that MP infection in children with SoJIA was significantly correlat-

IL-17, FOXP3 and MDR-1 in SoJIA patients with atopy or MP infection

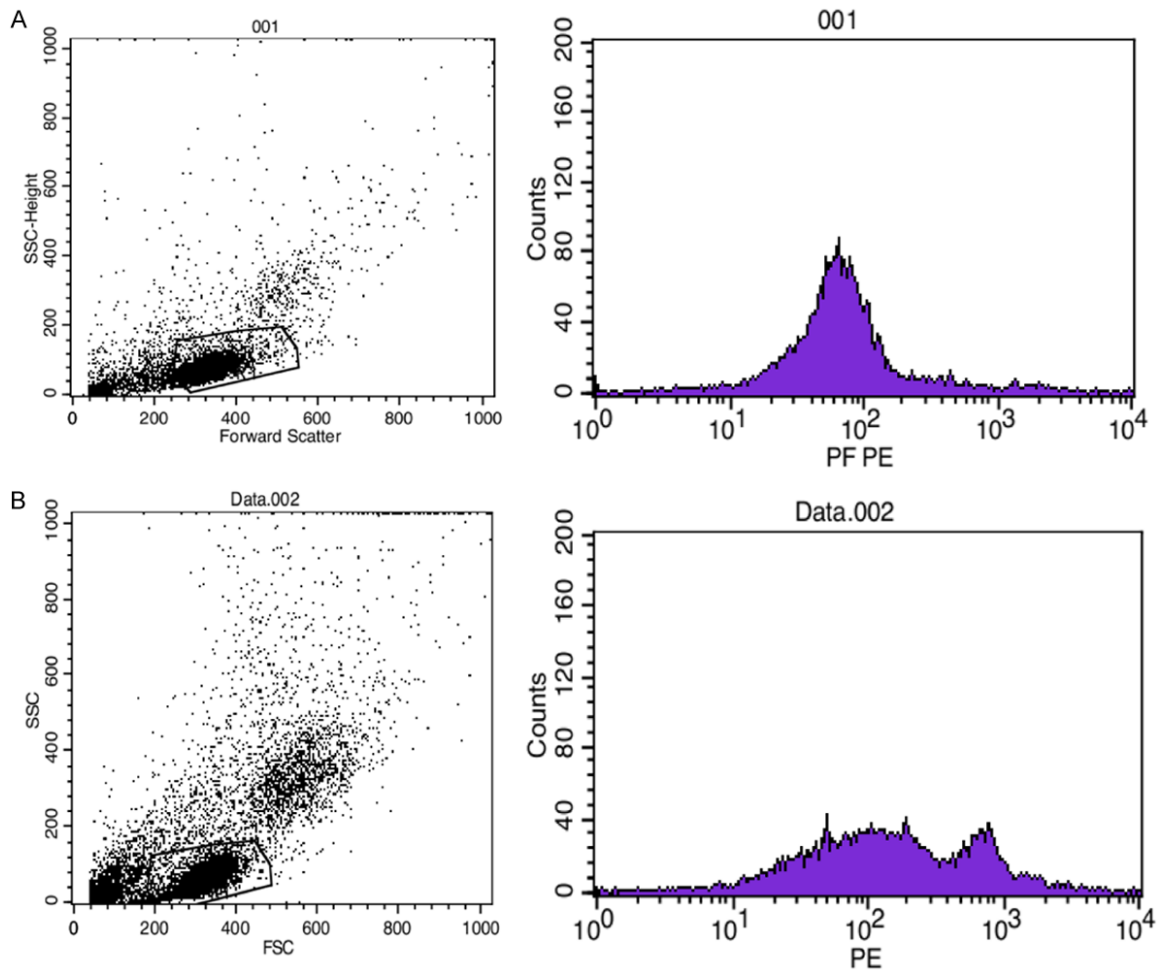


Figure 1. Ratios of lymphocytes capable of discharging daunorubicin. A. MP+Atopy+ group; B. MP-Atopy- group.

ed with the Atopy. SoJIA patients with atopy may be more susceptible to complicate with MP infection. while Atopy and MP infected SoJIA patients had a higher ratio of non-monophasic course, that is disease lasted longer, repeated more times and worse prognosis. Thus, Atopy and MP infection play an important role in the prognosis of the disease. Furthermore, what are the possible mechanisms and target site about the effects of MP and atopy on the prognosis of SoJIA?

From the immunological point of view that autoimmune diseases were mediated by Th1 cells, Atopy was mediated by Th2 cells [20], the traditional view was that the imbalance of proliferation and differentiation of Th1/Th2 cells plays a crucial role in the pathogenesis of autoimmune diseases and allergic diseases, the differentiation processes of IFN- γ producing Th1 cells and IL-4 producing Th2 cells are mutually

restricted and antagonistic [22]. However, in recent years, more and more studies have found that it is difficult to explain the relationship between autoimmune diseases and allergic diseases only simply with the imbalance of Th1/Th2 cell differentiation. This may contain a complex immune regulatory network which interconnected and interrelated. For example, the balance of Th17 and regulatory T (Treg) cells might play an important role in the networks.

Th17 and Treg cells are recently identified CD4+ T cell subsets. Th17 cells were characterized by the secretion of IL-17A (commonly known as IL-17), IL-17F, IL-6 and TNF- α [23]. And IL-17 was involved in various inflammatory and autoimmune diseases [24]. Simultaneously, Treg cells can specifically express the transcription factor Foxp3 [25], and play the role of anti-inflammatory and maintenance of immune tolerance

through cell-to-cell contact and secretion of inhibitory cytokines (such as IL-10, TGF- β and IL-35) [26]. A recent study indicated that CD4+ CD25^{high} FOXP3+ T (Treg) cells in peripheral blood of patients with JIA was significantly higher as compared to the normal controls [27]. *Foxp3* and *Bcl-xL* can be cooperatively expressed by CD4+ T cells, which were capable to differentiate into functional Tregs, with the capacity to treat arthritis [28]. In differentiation and function, Th17 and Treg inhibited each other to maintain the immune balance. Under the stable state of the immune system without activation, *Foxp3* can inhibit ROR γ (the signature transcription factor for Th17 cells) function [29], CD4+ Th0 induce themselves to differentiate into Treg cells to prevent the occurrence of autoimmune diseases. However, if in the case of external stimuli, innate immune system can produce a large number of cytokines such as IL-1 β and IL-6. IL-6 together with TGF- β and IL-1 β can induce the production of IL-17, meanwhile, it also inhibits *Foxp3* function and the differentiation of CD4+ Th0 cells into Treg cells [29], which makes the disruption of Th17/Treg balance, leading to the formation of autoimmune diseases [30].

It has been reported that IL-17 can stimulate synovial cell to secrete the keratinocyte growth factor, hepatocyte growth factor and heparin binding epidermal growth factor stimulated which can promote the formation of pannus in patients with rheumatoid arthritis, and occurrence of joint damage in early disease [31]. Another study has found that IL-17 can also combine with local inflammatory factors like IL-6, IL-8, matrix metalloproteinase 1 (MMP-1) and MMP-3, which further aggravate joint damage [32]. Generally, the number of Th17 cells and the secretion of cytokine IL-17 levels in children with juvenile idiopathic arthritis were increased [33], and they were positively correlated with the disease progress [34, 35]. IL-17A was reportedly overexpressed in sera from patients with active systemic JIA [13]. IL-17/IL-17 receptor (IL-17R) family is implicated in the pathogenesis of psoriasis arthritis and psoriasis [36]. IL-17 levels strongly correlated with CXCL4 levels in synovial fluid from psoriatic arthritis patients [37]. Bullens et al found that IL-17 plays an important role in airway allergic reactions through recruiting neutrophils and inducing the production of chemokine CXCL8

(IL-8), to promote the secretion of mucus in airway mucus glands and increase airway hyper-responsiveness, it was very important in the process of airway remodeling of asthma patients [38]. At present, there is no literature research about the effect of SoJIA children complicated with atopy and MP infection on Th17/Treg cells imbalance or whether it affects the prognostic mechanism remains unknown.

In our work, we observed that the expression of *IL-17* in MP+Atopy+ group, MP+ group and Atopy+ group were higher than other groups and the difference was statistical significance ($p < 0.05$). The expression of *Foxp3* in the patient group was all lower than that in the control group. Especially in the MP+Atopy+ group and MP+ group, the expression of *Foxp3* was significantly less than the control group ($p < 0.05$). These results suggest that the proliferation and differentiation of Th17 cells in SoJIA Children with atopy or MP infection was elevated, but the proliferation and differentiation of Treg cells were decreased, and all these triggered Th17/Treg cell being further imbalanced than before. On the other hand, we concluded that SoJIA children with atopy or MP infection might promote the proliferation of Th17 cells, the increased expression of IL-17 resulted in aggravated immune response and further enhanced inflammatory response, which makes the disease difficult to control. Especially, the effect was more obvious in patients complicated with MP infection and Atopy, which may be one of the factors lead to adverse prognosis of SoJIA.

Multidrug resistance mechanism was one of the mechanisms of self-protection which had been extensive researched. ABC transporter superfamily is closely related to multidrug resistance, it is a group of transmembrane proteins which can complete the active transmembrane transport of molecules and lead to drug resistance [39]. The members of ABC transporters mainly include P-glycoprotein (P-gp) and the multidrug resistance related proteins (MRP) [40]. Moreover, MRP is classified into many subtypes. MRP1, a subtype of MRP, was a focus. The P-gp and MRP1 were encoded by *MDR-1* and *MRP-1*, respectively [41, 42]. Our research found that the expression of *MDR-1* in MP+Atopy+ group, MP+ group and Atopy+ group were higher than other groups. The ratio of Daunorubicin releasing lymphocyte in

MP+Atopy+ group was obviously higher than MP-Atopy- group. These results indicated that the expression of drug resistance gene *MDR-1* was up-regulated in MP+Atopy+, MP+ and Atopy+ groups. These data suggest that the up-regulation of *MDR-1* can induce drug resistance in patients and influence the efficacy of drug therapy. This may be another possible contributor to poor prognosis in children with SoJIA.

In conclusion, this study explored the expression of *IL-17*, *Foxp3*, *MDR-1* and *MRP-1* in the peripheral blood mononuclear cells of SoJIA patients through RT-PCR method and laboratory examination correspondingly and the ratios of peripheral blood lymphocytes that can discharge daunorubicin were analyzed by flow cytometry. The results showed that SoJIA children with atopy or MP infection were more protracted repeated, poor prognosis, and SoJIA accompanied by atopy were more likely to complicate with MP infection. Moreover, SoJIA combined with both atopy and MP infection have a significant impact on patient's prognosis. The expression of *IL-17* and *MDR-1* was up-regulated, but the expression of *Foxp3* was down-regulated in SoJIA children with Atopy or MP infection. Furthermore, the ratio of Daunorubicin release lymphocyte of SoJIA patients with atopy and MP was significantly higher than other patients. The results suggest that the advance of proliferation and differentiation of Th17 cells in SoJIA patients with atopy or MP can increase the expression of IL-17 resulting in increased immune response and inflammatory response. Meanwhile, the drug resistance gene was up-regulated to induce drug resistance in children with SoJIA. This is the main cause of poor prognosis in children with SoJIA. Therefore, in clinical treatment, we need to consider the influence of Atopy and MP infection on persistent disease and treatment resistance of SoJIA patients. We proposed to actively control the quality, prevention and treatment of MP infection, in order to prevent persistent deterioration of SoJIA and disease duration in children.

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Disclosure of conflict of interest

None.

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