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Identify differential inflammatory cellular and serology pathways between children and adult patients in the lupus registry

Chung-Yuan Hsu, MD, Wen-Chan Chiu, MD, Yi-Ling Huang, MS, Yu-Jih Su, MD, PhD*

Abstract

Background: Age variances in systemic lupus erythematosus (SLE) may reflect different patterns and consequences. Monocyte differentiation is critical, and cytokine and chemokine milieu may be associated with long term outcome and treatment responses. This study aims to evaluate the inflammatory cellular and serology pathways associated with age in our lupus registry.

Methods: We included patients with SLE and divided them into 2 groups according to age, ≤18 or >18 years old. We performed flow cytometry analysis to define the peripheral blood monocyte differentiation pattern and phenotypes and used the multiplex method to detect cytokine and chemokine panels. The results were then compared between the 2 subgroups.

Results: In total, 47 SLE patients were included in this study. Of those, 23 patients were 18 years old or younger, and 24 patients were over the age of 18 years old. An increased distribution of circulating Type 2b macrophage (M2b) subsets was found in patients over 18 years old ($P < 0.01$), and we found the Type 1 macrophage (M1) to demonstrate a marked increase in those patients ≤18 years old ($P = .05$). Eotaxin values were significantly higher in patients >18 years old ($P = .03$), and Macrophage Inflammatory Protein (MIP)-1alpha, MIP-1beta, Interleukine (IL)-1Ra, Interferon (IFN)-alpha2, IL-12, IL-13, IL-17A, IL-1beta, IL-2, IL-4, IL-5, IL-7, IL-9, Monocyte Chemoattractant Protein (MCP)-3, Transforming Growth Factor (TGF)-alpha, and Tumor necrosis factor (TNF)-beta were significantly higher in patients ≤18 years old (all $P < .05$).

Conclusions: We found significant M2b polarization in adult SLE patients, and several cytokines and chemokines were significantly higher in SLE patients ≤ 18 years old. Peripheral blood mononuclear cell differentiation and cytokine milieu could represent composite harm from both Type 2 helper T cells (Th2) and Type 17 helper T cells (Th17) pathways and may thus be a potential therapeutic target in younger SLE patients.

Abbreviations: APC = Allophycocyanin fluorescent dye, CCR = CC chemokine receptor, CD = Cluster of differentiation, CXCR = CXC chemokine receptor, Cy7 = Sulfo-Cyanine7 fluorescent dye, ELISA = Enzyme-linked immunosorbent assay, FITC = Fluorescein isothiocyanate fluorescent dye, IFN = Interferon, IL = Interleukine, IQR = Interquartile range, LN = Lupus nephritis, LTA = Lymphotoxin-alpha; tumor necrosis factor-beta, M1 = Type 1 macrophage, M2 = Type 2 macrophage, MCP = Monocyte Chemoattractant Protein, MHC = Major histocompatibility complex, MIP = Macrophage Inflammatory Protein, MNCs = Mononuclear cells, PBS = Phosphate buffered saline, PE = Phycoerythrin fluorescent dye, PerCP = Peridinin-Chlorophyll-Protein fluorescent dye, RBC = Red blood cells, SLE = Systemic Lupus Erythematosus, SLEDAI-2K = Systemic Lupus Erythematosus Disease Activity Index-2000, TGF = Transforming Growth Factor, Th17 = Type 17 helper T cells, Th2 = Type 2 helper T cells, TNF = Tumor necrosis factor.

Keywords: age, macrophages, pathway, systemic lupus erythematosus, Th2, Th17

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that causes widespread inflammation and can ultimately lead to permanent organ damage. This disease primarily affects young women and encompasses a wide range of severities and manifestations. Lupus nephritis (LN) is one of the most severe manifestations of SLE and occurs in 60% of afflicted patients.[1] Juvenile

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The authors declare that they have no conflict of interest.

Data availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval: The study has been approved by the Institutional Review Boards of the Chang Gung Medical Foundation (No. 201601734B0, 103-7505B, 104-7089C, 105-4874C, and 1612150063).

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Departments of Rheumatology, Allergy and Immunology, Kaohsiung Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Kaohsiung, Taiwan.

**Correspondence: Yu-Jih Su, Departments of Rheumatology, Allergy and Immunology, Kaohsiung Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Kaohsiung, Taiwan, No. 123, Ta Pei Road, Niao Sung Hsiang, Kaohsiung City 833, Taiwan (e-mail: ym6154@cgmh.org.tw).*

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Key Point

In patients less than or equal to 18 years old with systemic lupus erythematosus, M1 macrophage polarization in peripheral blood is increased, and several cytokines are elevated when compared to those similar clinical situation SLE patients >18 years old.

and adult-onset SLE differed in genetic,[2] clinical,[3] and outcome[4] characteristics. Children with SLE have been presented with small controlled trials,[5] but with higher disease activity,[6] thus requiring more attention. Age-related differences in SLE were identified before patients' adulthood,[7] and childhood and adolescent lupus nephritis may be more severe than adult lupus nephritis.[8] Transitional care of young SLE patients is an often neglected problem.[9] Very few clinical studies have compared age-difference SLE characteristics,[10] but have obtained a similar result, which is that the younger the age of SLE patients, the more severe the disease is. A national-wide healthy insurance registry study analyzed the characteristics of avascular necrosis in childhood SLE[11] and found 1472 children with newly-diagnosed SLE between 2005 and 2013 in Taiwan. This finding may reflect a significant financial burden if the disease activity is not well-controlled among these SLE children. Although new medication for treating children with SLE is on the horizon,[12] and the efficacy is similar to treating adults with SLE, the mechanism behind why the disease severity differs between adult and childhood SLE remains unknown. In this study, we aimed to evaluate the inflammatory cellular and serology pathways associated with the effect of age in our lupus registry.

The development of LN is related to the differentiation, accumulation, and activation of monocytes, which can result in abnormal immune function.[13] Peripheral monocytes can differentiate into macrophages or dendritic cells, migrate to the inflammation site, and participate in either the inflammatory process or tissue remodeling.[14] The heterogeneity of monocytes results in different subsets of macrophages with varying roles in inflammation.[15] In recent years, monocyte differentiation has been categorized into Type 1 macrophage (M1), Type 2 macrophage (M2)a, M2b, and M2c subtypes based on the apparent activity and expression of cell surface markers.[16] Furthermore, both the differentiation and the polarization of monocytes play vital roles in the pathogenesis of LN.[17] Although the differentiation of monocytes into the M1 subtype contributes to inflammation, monocyte differentiation into the M2b subtype may have a more direct and important role in SLE development.[18] Reduced differentiation of monocytes into the M2a and M2c subtypes may lead to a lack of antiinflammatory activity, which is evident in SLE. The activated type II macrophage population is a key marker of proteinuria onset.[19] Overall, M2b polarization may play a key role in the pathogenesis of SLE and LN progression, thus providing potential therapeutic options.[20] According to all the previous evidence, we have focused on clarifying macrophage differentiation differences between SLE patients >18 years old and those equal to/less than 18 years old.

Regarding the serology study, no definite cytokines or chemokines were mentioned as being specifically associated with young or old SLE patients, especially in childhood SLE because clinical trials of SLE children[21] may encounter more ethical issue than those of adult SLE. We decided to detect a whole panel of cytokines or chemokines to clarify our question using multiplex assay. Our study will provide evidence in either the cellular or serological aspect that compares the pathogenesis between younger and older SLE patients.

2. Materials and Methods

2.1. Study population

This study was conducted at Kaohsiung Chang Gung Memorial Hospital, a tertiary hospital in southern Taiwan. First, we included patients diagnosed with SLE between August 1, 2014 and July 31, 2017 in our lupus registry (Phase 1), and then we obtained consent from each patient to collect their blood for the peripheral blood mononuclear cell study (Phase 2) and another consent for multiplex assay cytokines and chemokines examination (Phase 3) (Table 1). All patients with SLE met the SLE classification criteria revised by the American College of Rheumatology in 1997.[22] During these 3 years, we sought consent from all consecutive patients who were followed up in the hospital for participation in the study. We recorded disease activity according to the Systemic Lupus Erythematosus Disease Activity Index-2000 (SLEDAI-2K).[23]

In the registry, phase 1 in this study, we recorded such demographic data as age, gender, white blood cell counts, erythrocyte sedimentation rate, and levels of hemoglobin, platelets, serum creatinine, creatinine clearance, and albumin. We further analyzed the immune profiles, including serum complement and anti-dsDNA and SLE disease activity index, of each patient, as well as clinical symptoms. The setting of 18 years old as adulthood is based on Taiwan's law.

During the phase 2 study, we collected peripheral blood and separated peripheral blood mononuclear cells for macrophage differentiation analysis and then analyzed the plasma for selected cytokines and chemokines after obtaining further consent from the patients aged over 18 years old.

For patients less than 18 years old, in the phase 3 study, we obtained their parents' consent before each phase. We analyzed each parameter between those patients >18 years old and those equal to/less than 18 years old.

All participants provided written informed consent, either from themselves (>18 year-old) or from their guardians (≤18 years old). This study was approved by the Institutional Review Board of the Chang Gung Medical Foundation (No. 201601734B0, 103–7505B, 104–7089C, 105–4874C, and 1612150063).

3. Flow cytometry analysis

In this study, the flow cytometry acquisition process starting from white blood cells is shown in Supplement Figure 1 and

Table 1

Patient enrollment flow-chart in this study (N = 47).

Timetable of study period	Age ≤ 18 year-old	Age > 18 year-old
Initiation of study, registry study (phase 1, Aug 1, 2014)		
Phase 2 of study: chart review, clinical serology tests, monocyte subgroup analysis, Luminex cytokines and chemokines examination for adult patients (N = 24).	0	24
Protocol modification, clinical observational study (Aug 1, 2015)		
Phase 3 of study: chart review, clinical serology tests, monocyte subgroup analysis, Luminex cytokines and chemokines examination for children (N = 23).	23	0
End of study (July 31, 2017)		

Figure 2, http://links.lww.com/MD/G965. Whole blood was immediately separated into plasma and blood cells via centrifugation at 3000 rpm (about 1700 g) for 10 minutes. Leukocytes were separated from erythrocytes at a ratio of 1:5 by 4.5% dextran precipitation, i.e., dextran was used to further isolate red blood cells (RBC) following the RBC lysis buffer. Leukocytes were then separated into polymorphonuclear cells and mononuclear cells (MNCs) by density gradient centrifugation using Ficoll-Paque (Amersham Pharmacia Biotech) at a ratio of 2:1 for 30 minutes at 1700 rpm. After washing several times with phosphate buffered saline (PBS), MNCs were collected for flow staining. We added MNCs to 100 μL blocking buffer and 10 μL Fc blocking agent at 4°C for 15 minutes, then mixed it with primary antibody PM2K for 30 minutes at 4°C and added the secondary antibody with antimouse IgG-FITC for 30 minutes at 4°C. Afterwards, we washed the cells with PBS prior to performing the cell surface staining by mixing them with fluorescence-conjugated mouse antibodies (Cluster of differentiation (CD)3- Fluorescein isothiocyanate fluorescent dye (FITC), CD19-Pacific blue, CD14- Phycoerythrin fluorescent dye (PE)/ Sulfo-Cyanine7 fluorescent dye (Cy7), CD3-pacific blue, CCR7-APC, CCR2-PE, CXCR1-Alexa 700, and CD86-Peridinin-Chlorophyll-Protein fluorescent dye (PerCP)) and corresponding isotype control antibodies for 30 minutes at 4°C. After washed the cells in PBS twice, they were resuspended in 300 μL PBS containing 1% paraformaldehyde and were ready for flow cytometry analysis (BD LSR II, BD Biosciences, San Jose, CA). Under standard culture conditions of human macrophages, PM2K is considered an established marker for identifying mature tissue macrophages and distinguishing macrophages and fibroblasts from monocyte-derived cell populations.[24] We focused on MNCs and stained them with a PM2K surface marker to identify macrophage-like monocyte differentiation cells.[24] Based on previous studies,[25] we chose the higher PM2K and CD14 in gate 3 (Supplement Figure 2, upper right quadrant, http://links.lww.com/MD/G965) for further research. Elevated CD86 expression can be found in M1 and M2b.[26] However, the percentage of monocytes expressing the homing chemokine receptor CCR7 was increased by M1 polarization and decreased by M2 polarization.[27] Therefore, we defined all subgroups of macrophage-like monocytes as follows: M1 polarization as PM2K + CCR7 + CD86 + cells; M2a polarization as PM2K + CCR7-CXCR1 + cells; M2c polarization as PM2K + CCR7 + CCR2 + cells; and M2b polarization as PM2K + CCR7-CD86 + cells. Antibodies came from the following reagents: PM2K (AbD Serotec- a Bio-Rad Company, Hercules, CA), CD19-Pacific blue (HIB19,eBioscience, San Diego, CA), CD14-PE/Cy7 (61D3,eBioscience), CD3-pacific blue (UCHL1, BD Biosciences, San Jose, CA), CCR7-APC (Miltenyi Biotec, Bergisch Gladbach, Germany), CCR2-PE, (BioLegend, cat#357205), CXCR1-Alexa 700 (R & D Systems, Minneapolis, MN), and CD86-PerCP (BioLegend, San Diego, CA). We used Flowcytometry Express 4 Plus Research Edition to obtain flow cytometry analysis data.

4. M2b polarization among patients with or without proteinuria

Gate 3 and gate 4 (Supplement Figure 2, upper and lower right quadrants, http://links.lww.com/MD/G965), in which mononuclear cells presented with either a high or low surface amount of CD14 and persistently high levels of PM2K, were gated for further study. The percentage of each mononuclear cell subgroup in the peripheral blood of each patient was then determined.

5. Selected cytokines and chemokines determined by multiplex assay among SLE patients

A standard capture sandwich assay by multiplex assay (MAGPIX, USA) was used to determine the levels of cytokines and chemokines in plasma. Each captured antibody was coupled to a different bead set (MILLIPLEX, USA). Standards (recombinant cytokines) diluted in pooled blank plasma collected from healthy adults and test plasma from SLE patients were examined using multiplex assays.

6. Pathway analysis with STRING[28–33]

The determined cytokines and chemokines were then compared between the 2 subgroups, and we performed pathway analysis according to the test results with the STRING.[34] Prior to analysis, we confirmed the result by detecting 5 different irrelevant proteins with ELISA kits in each sample from phase 3 of the multiplex assay study. If the 5 protein levels were the same between the 2 subgroups of the older and younger SLE patients, we would be able to confirm that all of the samples were kept in good quality in the freezer throughout the entire study period.

7. Statistics

For the analysis of characteristics and results, the median (25–75% interquartile range (IQR)) was used to describe nonnormally distributed variables. We adopted Pearson chi-square test to analyze categorical variables. For other parameters, we compared them between the age over 18 years and that equal to or less than 18 years old. P values <0.05 were considered statistically significant. All analyses were performed using SPSS Statistics for Windows, Version 17.0. Chicago: SPSS Inc.

8. Results

8.1. Patient demographics

This study included 47 SLE patients. Among them, 23 patients were equal to or less than 18 years old, and 24 patients were >18 years old. The clinical data, including disease activity index, ferritin, leukocytes, lymphocytes, monocytes, hemoglobulin, platelets, erythrocyte sedimentation rate, creatinine, albumin, complement levels and anti-dsDNA titers, were all comparable (all $P > .05$). Only neutrophil percentage differed significantly between the 2 different age groups ($P = .04$) (Table 2). The disease activity of patients from each subgroup are demonstrated in Table 2. Those patients' disease activities under or equal to 18-year-old were 8.5 (4,10.25) and those patients over 18-year-old were 5 (2,8.75), and there is no significant difference between these 2 groups ($P > .05$). The treatment of immunosuppressants and glucocorticoids were comparable between the 2 subgroups. (all $P > .05$, Table 2)

In addition to the laboratory data, we also collected clinical symptoms for evaluation and compared their occurrence rate between each age group (Table 3). According to our records, the clinical manifestations of each age group were similar. Although hematuria, proteinuria, or pyuria occurred more frequently in patients ≤18 years old compared to the older group, none of the clinical manifestations reached statistical significance (all $P > .05$).

9. Identification of circulating subtypes of monocytes in peripheral blood

The PM2K + CD14 + cells were gated, and then cells were selected for further M1, M2a, M2b, and M2c percentage analysis. We identified each population with different cell surface markers in the SLE peripheral blood (Table 4). Increased distribution of circulating M2b subsets was found in patients >18 years old ($P < .01$). The M2b number was higher in the peripheral blood of the patients aged over 18 years old. Furthermore, the M1 (P value = 0.05) was found to have a marked increase in those patients equal to/less than 18 years

Table 2

Comparison age, disease activity, and laboratory data between age ≤ 18 year-old and age > 18 year-old systemic lupus erythematosus patients (N = 47).

N = 47	Age ≤ 18 year-old	Age > 18 year-old	*P* value
n	23	24	x
Age	16 (13, 17)	36 (24, 45.5)	0.00*
SLE disease activity index-2k	8.5 (4, 10.25)	5 (2, 8.75)	0.10
Ferritin	71.4 (8.5,)	119.9 (119.9119.9)	0.65
Leukocyte (1000/L)	5.1 (4, 6.7)	4.8 (3.9, 6.45)	0.55
Neutrophil (%)	54.6 (48.2, 65.5)	63.65 (56.95, 68.675)	0.04*
Lymphocyte (%)	35.9 (27.7, 41.9)	28.4 (23.025, 33.45)	0.06
Monocyte (%)	7.9 (6.6, 10.2)	7.5 (5.1, 9.825)	0.42
Hemoglobin	12 (11.4, 12.3)	12.2 (11.2, 13.65)	0.28
Platelets	237 (190, 278)	230 (198.5317.5)	0.62
Erythrocyte sedimentation rate	14 (8.5, 23)	18 (11, 34)	0.40
Creatine	0.6 (0.49, 0.68)	0.6 (0.57, 0.725)	0.53
Albumin	4.5 (4.4, 4.7)	4 (2.7, 4.3)	0.08
C3	83.35 (71.925, 116.25)	79.8 (71.1107.85)	0.66
C4	13.45 (11.475, 22.225)	14.6 (11.45, 20.7)	0.88
Anti-dsDNA	54.40 (212.90, 338.10)	65.10 (177.00, 270.45)	0.43
Prednisolone (mg/d)	7.5 (5.0, 10.0)	5.0 (5.0, 15.0)	0.72
Hydroxychloroquine (mg/d)	200.0 (200.0, 225.0)	200.0 (200.0, 400.0)	0.22
Azathioprine (mg/d)	37.5 (25.0, 75.0)	50.0 (25.0, 100.0)	0.50
Mycophenolic acid (mg/d)	x	x	x

old, although this finding does not reach statistical significance ($P = .05$).

Compare cytokine and chemokine profiles between patients >18 years old and patients equal to/less than 18 years old.

We determined dozens of cytokines and chemokines among the SLE patients. However 3 patients >18 years old and 9 patients equal to/less than 18 years old withdrew their consent, either by themselves or through their guardians, prior to the phase 3 study. As a result, only 14 patients in the younger group and 21 patients in the older group completed the multiplex assay study (Table 5). We found several cytokines and chemokines that were significantly different between the 2 subgroups. Eotaxin was significantly higher in the patients >18 years old ($P = .03$). Meanwhile, all of the following cytokines and chemokines, including MIP-1alpha, MIP-1beta, IL-1Ra, IFN-alpha2, IL-12, IL-13, IL-17A, IL-1beta, IL-2, IL-4, IL-5, IL-7, IL-9, MCP-3, TGF-alpha, and TNF-beta, were significantly higher in patients ≤ 18 years old (all $P < .05$, Table 5). For quality control, we picked up 5 other irrelevant plasma proteins and tested the concentrations of each protein among these SLE patients, finding comparable protein concentrations between the 2 subgroups (all $P > .05$, Table 5).

10. Pathway analysis with STRING[34–37]

With the data from the multiplex assay, we demonstrated that MIP-1alpha, MIP-1beta, IL-1Ra, IFN-alpha2, IL-12, IL-13, IL-17A, IL-1beta, IL-2, IL-4, IL-5, IL-7, IL-9, MCP-3, TGF-alpha, and TNF-beta were significantly higher in patients ≤ 18 years old (all $P < .05$) (Table 5). We used the STRING database to calculate and determine the protein interaction in the SLE patients equal to/less than 18 years old, and the simulation shows that those patients have a single dominant protein-protein interaction (Fig. 1). The activation of both Th2 (IL-4 and IL-13) and Th17 (IL-17A) in adaptive immunity and the activation of monocyte with Major histocompatibility complex (MHC) class II stimulation with profound elevation of IL-1beta and TNF-beta were demonstrated in the analysis.

Table 3

Compare clinical manifestations between age ≤ 18 year-old and age > 18 year-old systemic lupus erythematosus patients (N = 47).

	Age ≤ 18 year-old	Age > 18 year-old	*P* value
n	23	24	x
Male	1	3	0.61
Neurologic disorders:			
Seizure	1	0	0.49
Psychosis	0	0	x
Organic brain lesion	0	0	x
Visual disturbance	0	0	x
Cranial neuropathy	0	0	x
Lupus headache	0	1	0.61
Cerebrovascular event	2	1	0.61
Any CNS involvement	3	2	0.67
Musculoskeletal disorders:			
Arthritis	3	4	1.00
Myositis	0	0	x
Genital-urinary disorders:			
Urinary casts	0	0	x
Hematuria	12	7	0.14
Proteinuria	4	2	0.42
Pyuria	9	8	0.61
Any kidney involvement	14	10	0.25
Mucocutaneous disorders:			
Vasculitis	0	0	x
Rash	1	3	0.61
Alopecia	0	1	0.23
Mucosal ulcers	0	3	0.23
Serositis disorders:			
Pleurisy	0	1	1.00
Pericarditis	1	0	0.49
Immunological disorders:			
Low complement	10	11	0.46
Constitutional disorders:			
Fever	2	2	0.23
Hematological involvement:			
Thrombocytopenia	2	0	0.23
Leukopenia	1	2	1.00

P value is determined with Fisher exact test.

11. Discussion

Our current study has several interesting findings. First, although the clinical manifestations between patients >18 years old and those equal to/less than 18 years old were similar, the background cellular and serological composition differed. Second, we observed the increased distribution of circulating M2b subsets in patients >18 years old ($P < .01$) and a markedly increased M1 in patients equal to/less than 18 years old, although this finding does not reach statistical significance ($P = .05$). Third, we demonstrated that Eotaxin was significantly higher in the patients >18 years old ($P = .03$), while MIP-1alpha, MIP-1beta, IL-1Ra, IFN-alpha2, IL-12, IL-13, IL-17A, IL-1beta, IL-2, IL-4, IL-5, IL-7, IL-9, MCP-3, TGF-alpha, and TNF-beta, were significantly higher in patients equal to/less than 18 years old (all $P < .05$). Eotaxins help recruitment to sites of inflammation in response to parasitic infections and induce such allergic and autoimmune diseases as asthma and atopic dermatitis. The role of eotaxin in autoimmunity has been shown in previous studies[38]; for example, high levels of eotaxin have been described in several chronic inflammatory diseases, including allergic rhinitis,[39] atopic dermatitis,[40] and asthma.[41] Eotaxin was shown to recruit neutrophils[40] to the inflammatory site in different tissues, which may be associated with SLE pathogenesis[38] and target organ damage. In adult patients, the average neutrophil to lymphocyte ratio was around 2.29 (1.75, 2.93), but in adolescent patients, the average neutrophil to lymphocyte ratio (N to L ratio) was

Table 4

Compare PM2K positive cells, percentage of M1, M2b, M2a, and M2c cells between age ≤ 18 year-old and age > 18 year-old systemic lupus erythematosus patients (N = 47).

N = 47	Age ≤ 18	Age > 18	*P* value
n	23	24	x
PM2K + CD14-_Gate4	0.99 (0.71, 3.2)	0.93 (0.2625, 2.0125)	0.32
CCR7 + CD86+_M1 (%)	6.3 (2.27, 22.43)	2.06 (0, 4.415)	0.05
CCR7-CD86+_M2b (%)	3.94 (0, 13.59)	15.97 (4.145, 32.015)	0.01*
CCR7-CXCR1+_M2a (%)	8.74 (0, 21.18)	5.825 (1.13, 25.37)	0.81
CCR7 + CCR2+_M2c (%)	16.67 (6.15, 33.33)	24.37 (6.3875, 51.9475)	0.35

*indicates *P* value <0.05.

Table 5

Compare cytokines and chemokines levels between age ≤ 18-year-old and age > 18-year-old systemic lupus erythematosus patients (N' = 35).

N' = 35	Age ≤ 18-year-old	Age > 18-year-old	*P* value
n	14	21	x
Eotaxin (pg/mL)	309.61 (169.775, 412.5375)	415.38 (288.825, 1073.5)	0.03*
TGF-alpha (pg/mL)	4.455 (2.02, 11.8475)	1 (0.58, 3.01)	0.00*
IFN-alpha2 (pg/mL)	5.835 (1.985, 65.755)	0.29 (0.04, 1.51)	0.00*
IFN-gamma (pg/mL)	19.355 (5.79, 58.63)	7.69 (2.87, 25.96)	0.12
IL-1Ra (pg/mL)	110.825 (19.8775, 433.04)	1.31 (0.145, 22.985)	0.00*
IL-1alpha (pg/mL)	2.675 (0.05, 68.8)	0.23 (0.005, 1.485)	0.09
IL-1beta (pg/mL)	1.925 (0.7975, 9.375)	0.72 (0.4, 1)	0.00*
IL-2 (pg/mL)	1.83 (0.905, 4.9775)	0.61 (0.44, 0.855)	0.00*
IL-3 (pg/mL)	0.505 (0.32, 1.015)	0.32 (0.2, 0.55)	0.08
IL-4 (pg/mL)	0.675 (0.0275, 17.765)	0.01 (0, 0.085)	0.00*
IL-5 (pg/mL)	11.42 (2.375, 46.42)	0.45 (0.375, 1.31)	0.00*
IL-6 (pg/mL)	3.315 (1.4225, 14.99)	2.1 (0.975, 5.555)	0.25
IL-7 (pg/mL)	1.585 (1.03, 3.53)	0.81 (0.73, 1.055)	0.00*
IL-8 (pg/mL)	21.805 (6.425, 92.2325)	5.79 (3.025, 15.005)	0.05
IL-9 (pg/mL)	2.935 (1.3125, 6.635)	0.89 (0.48, 1.325)	0.00*
IL-10 (pg/mL)	5.975 (1.5475, 13.3275)	1.75 (0.79, 9.735)	0.28
IL-12 (p40) (pg/mL)	12.94 (0.105, 73.175)	0.01 (0, 0.485)	0.00*
IL-13 (pg/mL)	38.69 (1.0325, 121.5775)	0 (0, 0.105)	0.00*
IL-15 (pg/mL)	2.8 (1.5825, 4.775)	1.86 (1.235, 2.72)	0.16
IL-17A (pg/mL)	5.635 (3.89, 18.31)	2.73 (1.495, 7.055)	0.03*
IP-10 (pg/mL)	1314.5 (465.5775, 2022.75)	917.21 (652.79, 1205)	0.63
MCP-1 (pg/mL)	474.405 (244.115, 1048.4075)	669.81 (601.925, 1122)	0.17
MCP-3 (pg/mL)	119.715 (13.2675, 238.0125)	0 (0, 0)	0.00*
MIP-1alpha (pg/mL)	28.005 (8.975, 60.9675)	3.62 (0.97, 20.315)	0.00*
MIP-1beta (pg/mL)	88.88 (31.175, 159.9)	18.14 (6.23, 49.675)	0.00*
TNF-alpha (pg/mL)	8.635 (6.2475, 29.8325)	14.81 (7.31, 25.8)	0.45
TNF-beta (pg/mL)	102.565 (0.6175, 232.675)	0 (0, 0)	0.00*
ATP1beta4 (ng/uL)	6.66 (3.26, 9.31)	5.26 (4.28, 7.72)	0.77
REST (pg/uL)	177.78 (133.51, 275.03)	212.81 (140.69, 274.82)	0.55
MAP6 (ng/uL)	15.37 (7.98, 36.75)	18.62 (8.75, 26.65)	0.66
PXC1 (ng/uL)	0.66 (0.48, 1.34)	0.93 (0.61, 1.104)	0.72
BMF (ng/uL)	1.08 (0.83, 1.30)	1.08 (1.01, 1.46)	0.58

*indicates *P* value <0.05.

around 1.5 (1.09, 2.36). (P = .049) The condition might be due to relatively immature adaptive immunity (low lymphocytes) in adolescents and the predominant innate immunity (high neutrophils, IL-17A) and type II immunity (high IL-4, IL-13, IL-1b, LTA) amount adolescents, which is delineated in Figure 1.

Few human studies have examined the relationship between monocyte differentiation and patient age in SLE, but only the target organ damage, and such studies have suggested that the differentiation of M2 monocytes is dominant in kidney biopsies and urine samples of LN.[42] However, no studies have determined early macrophage differentiation between SLE patients >18 years old and patients ≤ 18 years old. Our study is based on previously published research protocols[25] and focuses on this aim. Our research on patients with initial proteinuria in SLE shows that patients with proteinuria have a significantly higher M2b value in the blood than the control group, which may play a direct role in early renal involvement in SLE (data not shown). On the other hand, the M1, M2a, and M2c cell subsets demonstrated no significant differences between patients with and without proteinuria (data not shown). Increased distribution of circulating M2b subsets was found in patients >18 years old (P < .01) and the M1 was found to have a marked increase in those patients ≤ 18 years old, despite not reaching statistical significance (P = .05).

Traditionally, immune complex deposition in the glomeruli has been known to induce the accumulation of M1-polarized monocyte differentiation and inflammatory reaction, thereby augmenting LN pathogenesis,[43] which may partially explain that the LN manifestation in younger patients could lead to a more devastating consequence as the M1 was found to be markedly increased in those patients equal to/less than 18 years old

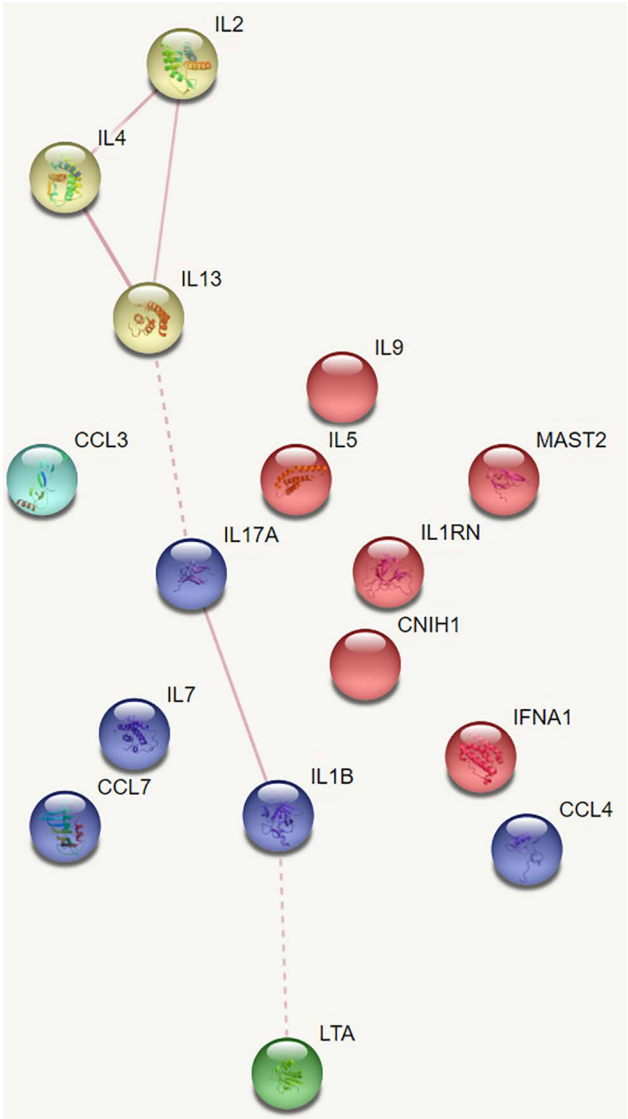

Figure 1. The protein-protein interaction and pathway analysis by STRING from the result of this study.

(Table 4). In contrast, M2-polarized monocyte differentiation is thought to provide a protective antiinflammatory environment. In fact, the subtypes of M2 (M2a, M2b, and M2c) have different characteristics. For example, M2a monocyte differentiation is referred to as profibrotic, whereas M2c as deactivated and remodeling.[44] On the other hand, differentiation and polarization of M2b monocytes may actually lead to SLE[20] and may also become a key mediator of LN initiation and progression.[45] Furthermore, LN may be inhibited by switching monocyte differentiation and polarization from the pro-inflammatory M2b to the antiinflammatory M2a phenotype.[28] Previous studies have proposed the use of peroxisome proliferator-activated receptor-γ agonists to switch the differentiation of monocytes from the M2b to the M2a subtype,[29] which has had therapeutic efficacy in mouse LN.[30] Therefore, although M2b is significantly higher in patients >18 years old,[42] the laboratory markers of LN, such as albumin and creatinine levels, and the anti-dsDNA, complement levels, and ferritin levels were similar between the 2 subgroups (all $P > .05$).

The disease durations are median 1.99 (0.5, 3.04) years in average in adolescent subgroup, but median 4.69 (1.39, 15.44) years in average in adult subgroup. There is significant different between the 2 subgroups in adolescent and in adult group, $P = .03$. We understand the chronicity of lupus affects the clinical manifestations, and the disease duration of adult lupus patients should be longer than those adolescent lupus patients, which is affected by the age itself mainly. We believe disease duration is not a major concern while physicians make decision in treating a SLE patient. Considering target organ damage, proteinuria is caused by direct or indirect podocyte damage, which is the most specific feature of the disease and is associated with the activity of LN patients. Infiltration of differentiated monocytes in glomeruli may be a crucial mechanism for podocyte injury.[31] Furthermore, several markers expressed by M2b monocyte differentiation correlated with the proteinuria status in the LN animal model.[32] In a study by Li et al, which included renal tissue samples from patients with glomerulonephritis, the results indicated that M2 monocyte differentiation may be involved in the acute renal injury of glomerulonephritis with crescents.[42] Monocytes proliferate in the bone marrow, migrate into the circulatory system, tilt towards inflammation, and then reach the kidneys, ultimately causing macrophages to accumulate in the kidney.[13] In the current study, the monocyte levels in peripheral blood did not differ between the younger and older groups. No significant differences were observed in the total macrophage counts between the 2 groups. However, further analysis of the monocyte number of different phenotypes revealed a significantly increased M2b count in circulation in the over-18-years-old group. However, in human studies, the role of the differentiation of monocytes into certain macrophage subtypes in the pathogenesis of SLE has not yet been determined. Recent studies have shown that M2 macrophages are the major subpopulation of human LN and dominate crescent glomerulonephritis.[42] Since the differentiation of M2 monocytes comes primarily from the blood to the kidneys, the next step is to determine whether the differentiation of M2 monocytes can be detected earlier, prior to the occurrence of LN.

In the current study, we have demonstrated the significant differences of several cytokines and chemokines with only limited SLE patients, and we confirmed our study results by detecting 5 other irrelevant proteins to assure the quality of the sample during the stock period. The results showed that MIP-1alpha, MIP-1beta, IL-1Ra, IFN-alpha2, IL-12, IL-13, IL-17A, IL-1beta, IL-2, IL-4, IL-5, IL-7, IL-9, MCP-3, TGF-alpha, and TNF-beta were significantly higher in patients equal to/less than 18 years old (all $P < .05$). These cytokines and chemokines link type II immunity and the Th17 pathway. The Th2 pathway (represented by IL4, IL13, and the level of eotaxin) could be associated with the impaired clearance of viruses from the host with deviated activation of Th17 (IL-17 and IL-23),[33] which was noted in the young SLE cytokine milieu. The activation of monocytes with MHC class II stimulation[46] or some atypical infections[47] with profound elevations of IL-1beta and TNF-beta were demonstrated in the analysis, as shown in Figure 1. The M1 activation demonstrated by flowcytometry in younger SLE patients may have been a result of concurrent elevated M1 associated cytokines and chemokines concentration; for example, MCP3 was demonstrated to increase M1 and M2a chemotaxis.[48] Furthermore, MIP-1alpha and MIP-1beta[49] were found to be associated with SLE, as well as with M1 polarization[50] during inflammation, just like the condition we found in the younger SLE patients (Table 5).

In general, despite improved medical care in SLE, the prognosis for SLE remains unsatisfactory. In addition to exploring more effective treatments, early detection of SLE activity and its underlying immunopathogenesis can help reduce target organ damage. Therefore, the discovery of significant peripheral M2b polarization in SLE patients with older age and the predominant M1 polarization in the child/adolescent SLE may provide direction for further translational studies to develop predictive biomarkers for SLE. In the near future, using high-throughput multicolor flow cytometry immunophenotyping technology,[51]

we can easily access macrophage differentiation patterns and other immune cells in patients with SLE.

This study has some limitations. First, this exploratory study has a small sample size. We found that disease activity, general laboratory data, and immune profiles were compatible between the 2 different age groups, but that kidney injuries might be under-estimated in the subgroup of patients less than/equal to 18 years old. Furthermore, due to the case number limitation, the association between M1 and the younger age did not reach statistical significance like M2b did in the older age group.[45] These 2 limitations were primarily due to the limited case number. Nevertheless, several of our parameters compared between the 2 subgroups reached statistically significant differences, which means that even if we expand our case number, the differences will not be affected, and the pathway that we identified (Fig. 1) will remain.

We found M2b-polarization in the peripheral blood of SLE patients aged over 18 years old. Furthermore, the M1 cells were borderline outnumbered in the younger patients than in the older patients, while some of the M1-associated cytokines and chemokines were statistically higher in the patients 18 years old and younger than in the patients >18 years old. These findings suggest that the key immunological players, including cells and cytokines, differed significantly between younger and older patients and could potentially serve as a therapeutic target in the future.

Author contributions

CYH and YJS conceived and designed research. YLH and WCC conducted experiments. YLH contributed new reagents or analytical tools. WCC and YJS analyzed data. CYH and YJS wrote the manuscript. All authors read and approved the manuscript.

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