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Evaluation of Monocyte Subpopulations in Patients with Systemic Sclerosis and its Association with Clinical Manifestations of the Disease: a Cross-sectional Controlled Study

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ABSTRACT

Background: Systemic sclerosis (SSc) is a chronic autoimmune disorder characterized not only by fibrosis and vasculopathy but also by inflammation. Previous studies have demonstrated monocyte involvement in SSc development, suggesting a role for immune dysfunction in SSc pathogenesis.

Objective: To investigate the relationship between SSc's clinical manifestations and altered levels of monocyte subpopulations.

Methods: Twenty-six patients meeting the ACR/EULAR SSc criteria along with twenty healthy individuals as the control group, were enrolled in the study. Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood samples of both the SSc patients and the control group. Subpopulations of monocytes were assessed based on HLA-DR, CD14, and CD16 expression using multi-color flow cytometry. The one-way ANOVA, Student's t-test, and Mann-Whitney U test were employed for normally and non-normally distributed data. The Spearman correlation test was utilized to identify correlations between the variables.

Results: The SSc patients showed a significant increase in the number of circulating peripheral blood monocytes ($p<0.001$). The percentage of $CD16^+$ monocyte subpopulations was higher in the SSc cases compared to the control group. A significant decrease in the ratio of classic to non-classic monocytes was observed in SSc cases (7.43%) compared to the control group (52.09%, $p<0.001$). No association was observed between monocyte subpopulations and clinical characteristics of SSC.

Conclusion: Our results showed an increase in the level of $CD16^+$ monocytes in patients with SSc compared to healthy individuals. Further investigation is required to determine the clinical significance of this alteration.

Keywords: Fibrosis, Flow Cytometry, Inflammation, Monocytes, Systemic Sclerosis

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INTRODUCTION

Systemic sclerosis (SSc), or rather scleroderma, is a multisystem autoimmune disorder characterized by chronic inflammation production of autoantibodies with a significant rate of mortality and morbidity (1). Although the exact mechanisms underlying the pathogenesis of SSc have not been determined, three features are proposed as the main factors for the clinical and pathologic manifestations of the disorder: (1) aberrant accumulation of collagen and other biomolecules connecting tissues in the skin and different internal organs; (2) microvascular damage; (3) and changes in innate and acquired immune response (4). SSc is principally associated with aberrant accumulation of extracellular matrix (ECM) proteins, particularly elastin, primarily collagen types, fibronectin, and fibrin, that consequently increase mechanical tension and decrease plasticity of ECM and result in tissue fibrosis (2). The infiltration of inflammatory cells, especially macrophages and T cells in early disease stages, is considered the histopathological hallmarks of SSc (3, 5-7). The association of classical and non-classical monocyte activation in the pathogenesis of SSc is widely documented (8-10). Monocytes are heterogeneous and multifunctional cells of innate immunity that possess great plasticity. Pan-monocytes were characterized as non-T, B, NK, and non-neutrophil cells expressing HLA-DR and further subdivided into three main subpopulations classical, intermediate, and non-classical phenotypically with different functions (11). Classical monocytes are about 80% of all monocytes expressing $CD14^{++}$ $CD16^{-}$ with phagocytic functions capable of the producing reactive oxygen species and the secretion of pro and anti-inflammatory cytokines through toll-like receptor (TLR)-4 (12).

In contrast, non-classical monocytes are characterized by the expression of $CD16^{+}$ and represent higher activity against viruses with the ability to produce pro-inflammatory cytokines as compensation for agonist stimulation of TLR-8 and TLR-9 (13, 14). The third group of monocytes, known as intermediate monocytes expressing $CD14^{++}$ $CD16^{+}$, mainly participated in angiogenesis and tissue repair (15). Activation of intermediate monocytes appears to be associated with M2 macrophages leading to T helper (Th) 2 response (11). The critical role of $CD14^{-}$ $CD16^{+}$ non-classical monocytes in the pathogenesis of several autoimmune diseases such as Systemic Lupus Erythematosus, Multiple Sclerosis, Type 1 Diabetes, and Rheumatoid Arthritis has been identified (16). Higashi-Kuwata et al. reported a distinct upregulation in the macrophages/monocytes pathways in the SSc patients and introduced the CD163+ or CD204+ activated macrophages as a potential fibrogenic regulators in the SSc skin (17). The high percentage of peripheral monocytes such as $CD14^{+}$ in the SSc was correlated with poor prognosis of visceral disorders (18). Moreover, increased levels of circulating monocytes, particularly in the $CD16^{+}$ subpopulation, have been reported to be associated with the severity of skin fibrosis, pulmonary fibrosis, function impairment, and restrictive ventilatory defect (19). Evidence suggests a positive correlation between an elevated count of total circulating monocytes and the pathogenesis of SSc. However, further studies are required to provide detailed insights into the role of peripheral blood monocyte heterogeneity in the pathophysiology of the SSc. Therefore, in the present study, we aimed to assess the frequency of monocyte subpopulations in the SSc patients and the relationship between clinical manifestations and altered monocyte subpopulation levels. We also assessed the correlation between the ratio of classical and non-classical monocytes and the clinical manifestations of SSc.

MATERIALS AND METHODS

Patients

Twenty-six consecutive patients fulfilling

SSc criteria (20) were recruited with informed consent from Imam Khomeini Hospital, Ardabil, Iran. In the current study, limited cutaneous systemic sclerosis (lcSSc) patients who were on corticosteroid therapy were included. Because of the effect of immunosuppressive drugs on monocyte count and function, the subjects who used immunosuppressive therapies like mycophenolic acid, cyclophosphamide, azathioprine and etc., at the time of inclusion, were excluded. The disease duration was determined as an interval since the occurrence of skin symptoms or Raynaud's phenomenon, which started first. The severity of skin symptoms was evaluated by the altered Rodnan skin score (21). Laboratory tests, comprising serological and autoantibodies analysis, as well as pulmonary function assessment such as lung diffusion capacity for carbon monoxide (DLCO) and forced vital capacity (FVC) testing previously conducted for three months, were acquired in medical records.

The Controls

Twenty healthy subjects with the same average gender and age as the SSc patients attended as the control group with informed consent. The inclusion and exclusion criteria for the control group were similar to those used for blood donation. Individuals without any medical illnesses, such as a history of hematological or autoimmune disorders, and not taking any medication were included. Those who had undergone recent surgery or had a history of blood transfusion within the past six months were excluded.

Study Design

The current study was a cross-sectional examination conducted from 2020 to 2021. The Research Ethics Committee approved the compliance of this study with the ethical standards of Ardabil University of Medical Sciences (IR.ARUMS.REC.1399.217), Ardabil, Iran, regarding the guidelines. The sample size was determined based on the existing studies' results regarding the sample size calculation formula (22). According to the study by Schneider L. et al., with power=80%, confidence interval 95%, α=0.05, and β=0.02, the sample size was estimated to be about 30 using the OpenEpi software.

Sample Collection and PBMC Isolation

Human peripheral blood monocytes (PBMCs) were collected from the heparinized peripheral blood samples of both the SSc patients and the control group using density gradient centrifugation utilizing Ficoll with a density of 1.077 (Histopaque, Germany). The blood samples were diluted with the same volumes of RPMI 1640 medium and were slowly added to Ficoll in the ratio of 2:1. The samples were centrifuged at 800 g and 4 °C for 25 min. PBMCs were isolated from the middle phase and used for further experiments. The viability and number of the cells were measured via Trypan blue dye exclusion method.

Flow Cytometry

Phenotypes of the isolated circulating monocytes were investigated by multi-color flow cytometry. About 1,000,000 cells were initially rinsed with phosphate-buffered saline (PBS), next, surface staining was carried out by incubating cells with titrations of fluorochrome-conjugated monoclonal antibodies (MoAbs) against CD16-Fluorescein-5-isothiocyanate (FITC), CD14-Allophycocyanin (APC), HLA-DR-PE-Cyanine7 dye for 30 min at 4 °C and darkness. All MoAbs were purchased from BioLegend (BioLegend, USA). The stained samples along with respective isotype controls for each one were incubated in PBS containing 0.5% bovine serum albumin (BSA) to reduce nonspecific binding before specific antibodies were added to the cells. After incubation, the cells were washed twice with 1-2 ml of FACS buffer for 5 min at 4 °C at 300g. The results were recorded on a MACSQuant 10 Analyser (Miltenyi Biotec, Germany) and analyzed with FlowJo software version 7.6.

Statistical Analysis

All the results were assessed with SPSS 16.0 software. The results were reported as the mean±standard deviation (SD). The One-way ANOVA and Student's t-test were used for non-parametric and parametric analysis of normally distributed variables, while Mann–Whitney test was used for not normally distributed results. The Spearman correlation test was applied to identify the correlation between the variables. A *p*-value of 0.05 or lower was considered statistically significant.

RESULTS

Clinical and Demographic Features of Participants

Demographic features, laboratory results, and clinical information of the SSc cases and the healthy subjects are comprehensively listed in Table 1. The SSc patients' mean age was 49.85 years, and 92.3% were female, while the average age of the control group was 49.42 years, and 90% female. According to the chi-square test, the cases and the control group had no significant difference in gender (p=0.78) and age (p=0.25). Systolic pulmonary arterial pressure (SPAP) was abnormal in 26.8% of the SSc cases, and 50% of the patients had pulmonary fibrosis. Modified Rodnan Skin Score was 1-14 in 96.2% of the SSc cases. 35.8% of the patients had upper gastrointestinal (GI) symptoms, including dysphagia, nausea, and vomiting, and 19.2% had lower GI symptoms, including diarrhea and constipation. All the patients had Raynaud's phenomenon observed in all cases, while Electrocardiography (ECG), Finger palm distance, and Creatinine level were normal. No muscle weakness and renal symptoms were observed in the SSc cases. FVC was above 80% in 53.8% of the SSc cases compared with the control group (Table 2).

Subpopulations of Circulating Monocytes

The results of the monocytes evaluation are represented in Figs. 1 and 2. The frequencies and phenotype of monocytes in peripheral blood samples of the SSc patients and the healthy donors were measured using multi-color flow cytometry. Monocytes were marked as the HLA-DR$^+$ CD14$^+$ population and quantitated as the percentage of gated cells by acquiring a minimum of 10,000 live events per sample. Percentages of monocyte subpopulations were presented as follows: CD14$^+$ CD16$^-$ (classical monocyte), CD14$^+$ CD16$^+$ (intermediate monocyte), CD14$^-$ CD16$^+$ (non-classic monocyte). Accordingly, the SSc patients had considerably higher counts of circulating monocytes compared with the control group (482.38±41.95 cells/µl vs. 265.65±293.51 cells/µl, p<0.001).

Table 1. Demographic data and general information

Variable		Patient %(N)	Control %(N)	*p*-value
Sex	Male	%7.7 (2)	%10 (2)	0.78
	Female	%92.3 (24)	%90 (18)	
Age		49.85±14.62	49.42±11.35	0.25
Occupation	Housewife	%88.5 (23)	%66.7 (14)	-
	Employed	%11.5 (3)	%33.3 (7)	-
Marital status	Single	%7.7 (2)	%19 (4)	-
	Married	%92.3 (24)	%81 (17)	-
Number of children	0	%19.2 (5)	%52.4 (11)	-
	1	%7.7 (2)	%14.3 (3)	-
	2	%42.3 (11)	%23.8 (5)	-
	3	%19.2 (5)	%9.5 (2)	-
	6	%7.7 (2)	-	-
	7	%3.8 (1)	-	-

Table 2. Frequency distribution of the clinical symptoms in the patients

		Number	Percent
Kidney	Yes	1	%3.8
	No	25	%96.2
Pulmonary fibrosis	Yes	13	%50
	No	13	%50
sPAP	Normal	19	%73.1
	Mild	3	%11.5
	Moderate	3	%11.5
	Severe	1	%3.8
Raynoud	Yes	26	%100
	No	0	%0
MRSS	1-14	25	%96.2
	15-29	1	%3.8
	30<	0	%0
FTP	Normal	23	%88.5
	Mild	3	%11.5
Creatine	Normal	26	%100
	High	0	%0
ECG	Normal	26	%100
	Pathologic	3	%0
LVEF	%45-49	3	%11.5
	%50<	23	%88.5
Muscle weakness	Yes	0	%0
	No	26	%100
FVC	%50>	4	%15.4
	%50-69	4	%15.4
	%70-79	4	%15.4
	%80<	14	%53.8
Upper-intestine	Yes	10	%35.8
	No	16	%61.5
Lower-intestine	Yes	5	%19.2
	No	21	%80.8
Loss weight	%5>	23	%88.5
	%5-9.9	2	%7.7
	%10-14.9	0	%0
	%15-19.9	1	%3.8
SRC	Yes	0	%0
	No	26	%100

sPAP: Systolic pulmonary arterial pressure; MRSS: Modified rodnan skin score; FTP: Finger to palm distance; ECG: Electrocardiography; LVEF: Left ventricle ejection fraction; FVC: Forced vital capacity; SRC: Scleroderma renal crisis

Among the monocyte subpopulations, the percentage of classical monocytes was higher in the control group compared with the SSc cases (80.74% vs 74.99%, p=0.18), however these differences were not significant. This increase was also observed in intermediate monocytes, with their percentage appearing higher in the control group (15.5% vs 11.25%, p=0.12). The SSc patients were found to have significantly higher expression of non-classical monocytes compared with the control group (1.55% vs 10.08%, p<0.0001).

The Ratio of Classical Monocytes to Non-classical Monocytes

Analyzing the ratio of classical to non-classical monocytes, we identified a significant decrease in this ratio in the SSc cases compared with the control group (7.43% vs. 52.09%, p<0.001).

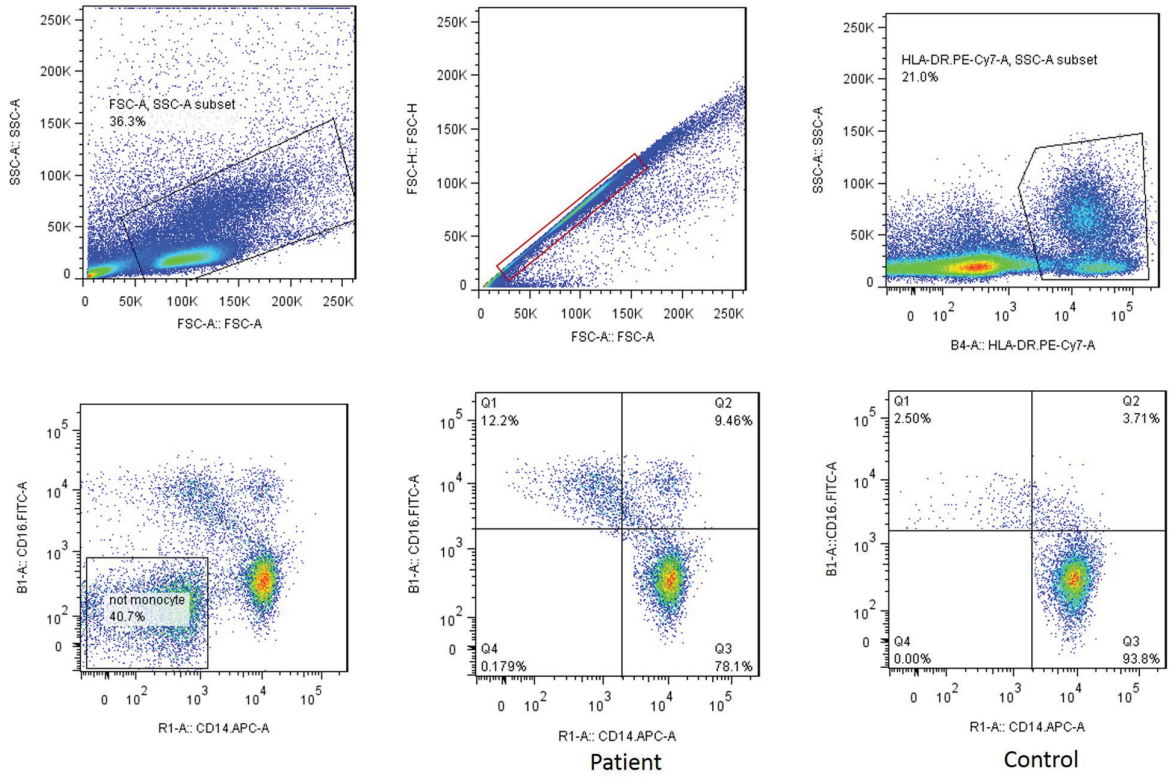

Fig. 1: Phenotypic examinations of the circulating monocytes in SSc patients and the control group. The frequencies and phenotype of monocytes in SSc patients' peripheral blood and healthy subjects were assessed via multi-color flow cytometry. Monocytes were marked as the HLA-DR^{+} CD14^{+} populations and quantitated as a percentage of gated cells by the acquisition of a minimum of 10,000 live events in each sample. The percentages of monocytes subpopulations were presented as follow; CD14^{++} CD16^{-} (classical monocyte), CD14^{++} CD16^{+} (intermediate monocyte), CD14^{-} CD16^{+} (non-classic monocyte)

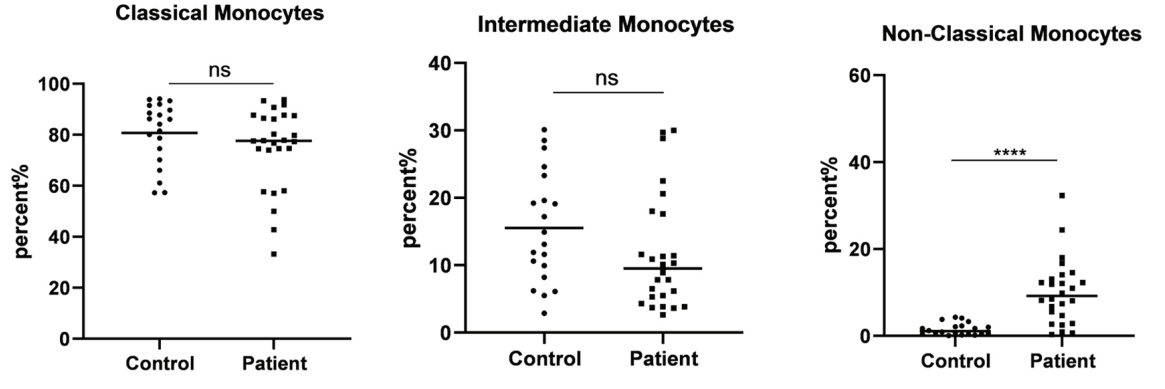

Fig. 2: Subpopulations of circulating monocytes. The total count of circulating monocytes was determined using flow cytometry. The percentages of HLA-DR^{+} (excluding monocytes) in different monocyte subpopulations were presented as follows: CD14^{++} CD16^{-} (classical monocyte), CD14^{++} CD16^{+} (intermediate monocyte), CD14^{-} CD16^{+} (non-classic monocyte) in peripheral blood of the study groups.

Monocyte Subpopulations and Clinical Manifestations

Our findings demonstrated no significant correlation between the monocyte subtypes, the ratio of classical to non-classical monocyte, and the total count of monocytes with clinical manifestations of the SSc, including GI symptoms, pulmonary and skin fibrosis, increased pulmonary artery blood pressure and forced vital capacity.

In addition, all the patients had a normal pattern of ECG with no significant change in the LVEF compared with the control group (p=0.92). The results are listed in Table 3.

Monocyte Subpopulations and the Duration of the Disease

According to the chi-square test, there was no significant correlation between monocyte subtypes, the ratio of classical to non-classical monocyte, and the total count of monocytes with the disease duration (p=0.69, p=0.73, p=0.78, p=0.8,and p=0.88 respectively).

Distribution of Autoantibodies in the SSc Cases

Identifying particular antibodies is a key factor in predicting probable organ involvement and could influence the prognosis, detection, and treatment of a disease (23). Specific autoantibodies associated with SSc are mainly linked to the distinct clinical characteristics of the SSc (24). The presence of circulating anti-nuclear antibodies (ANA) is an important diagnostic feature for immunological abnormalities in 90–95% of SSc cases (25, 26). As shown in Fig. 3,

Table 3. Correlation between monocytes subpopulation and clinical symptoms in the SSc patients

		Classic	Non-classic	Intermediate	Total monocyte	Classic/Non-classic ratio
Upper-intestine	Correlation	-0.10	-0.19	-0.31	-0.18	-0.20
	p-value	0.62	0.35	0.12	0.36	0.10
Lower-intestine	Correlation	-0.31	-0.10	-0.18	-0.37	-0.16
	p-value	0.11	0.61	0.37	0.0.6	0.23
Pulmonary fibrosis	Correlation	0.29	-0.0.2	-0.0.7	-0.0.3	-0.15
	p-value	0.14	0.92	0.70	0.85	0.0.6
Skin fibrosis	Correlation	0.25	0.25	0.28	0.28	-0.0.9
	p-value	0.21	0.21	0.16	0.16	0.65
sPAP	Correlation	0.21	0.0.5	-0.0.5	0.23	0.0
	p-value	0.30	0.78	0.80	0.25	0.10
LVEF	Correlation	-0.12	-0.16	0.13	-0.0.8	0.90
	p-value	0.55	0.41	0.50	0.66	0.0.2
FVC	Correlation	-0.14	-0.0.9	-0.0.2	-0.12	0.32
	p-value	0.48	0.65	0.91	0.55	0.10
Disease duration	Correlation	-0.08	-0.07	0.05	-0.03	-0.05
	p-value	0.69	0.73	0.78	0.88	0.80

FVC: Forced vital capacity; sPAP: Systolic pulmonary arterial pressure; LVEF: Left ventricle ejection fraction

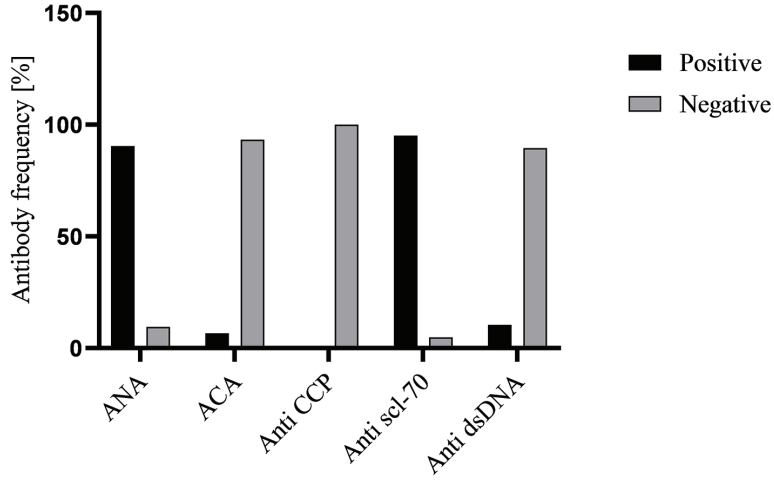

Fig. 3: Distribution of autoantibodies in SSc patients.

Table 4. Correlation between autoantibodies and monocytes subpopulation in the SSc patients

		Classic	Non-classic	Intermediate	Total monocyte	Classic/Non-classic ratio
ANA	Correlation	-0.007	-0.05	-0.02	-0.02	0.04
	p-value	0.97	0.79	0.90	0.91	0.81
ACA	Correlation	0.09	0.07	0.003	0.09	0.9
	p-value	0.64	0.70	0.98	0.64	0.35
Anti CCP	Correlation	0.04	0.007	0.01	0.03	-0.15
	p-value	0.81	0.97	0.92	0.85	0.44
Anti scl-70	Correlation	-0.13	-0.06	-0.19	-0.16	-0.26
	p-value	0.51	0.76	0.33	0.42	0.19
Anti dsDNA	Correlation	-0.35	-0.15	-0.21	-0.34	0.09
	p-value	0.07	0.45	0.30	0.08	0.64

ANA: Anti-nuclear antibody; Anti-CCP: Anti-cyclic citrullinated peptide; anti-dsDNA: Anti-double stranded DNA; ACA: Anti-centromere antibody; anti-Scl-70: Anti-topoisomerase 1

the presence of ANA was detected in 90.5% of the patients. The presence of anti-centromere antibody (ACA) was detected in 6.7% of the patients attributed to limited skin involvement and rejected pulmonary involvement. In addition, the presence of anti-topoisomerase 1 (anti-Scl-70) and anti-double stranded DNA (anti-dsDNA) was respectively detected in 95.2% and 10.5%, while no anti-cyclic citrullinated peptide (anti-CCP) was detected in the examined patients. According to the results, a non-significant relation was observed between monocyte subtypes, the ratio of classical to non-classical monocyte, and the total count of monocytes with any subtypes of autoantibodies, including ANA, ACA, anti-CCP, anti-Scl-70, anti-dsDNA. The findings are listed in Table 4.

DISCUSSION

The accumulation of monocytes at the site of inflammation activates the pro-fibrotic properties of these cells, which play a critical role in the pathogenesis of SCC (27). Herein, we investigated the correlation between the frequencies of monocyte subtypes with the clinical manifestation of the SSc through colorimetric flow cytometry.

The findings here show that the percentage and the total number of monocytes were considerably high in the SSc patients than in the control group. In addition, the ratio of classical to non-classical monocytes in the SSc cases significantly decreased compared with the healthy subjects. The evaluation of the expression of CD14 and CD16 molecules permitted the classification of monocyte subtypes, including classical, non-classical, and intermediate monocytes (28-30). Recent studies have demonstrated an increase in the total count of $CD16^+$ monocytes in the peripheral blood of SSc patients. However, the difference between non-classical and intermediate monocytes was not accurately distinguished. Our findings were in accordance with a study performed by Higashi et al. Flow cytometry analysis of circulating monocytes on 51 SSC cases, including 33 cases of lSSc and 18 cases of dSSc, showed that $CD14^+$ cells significantly elevated in the SSc patients in comparison with the controls (17, 19). Previous investigations have reported a significant association between the $CD16^+$ monocyte subtypes and the severity of pulmonary and skin fibrosis (19, 31).

Accordingly, Lescoat et al. reported the correlation between the clinical features of SSc with the amount of $CD16^+$ monocyte subtypes in 48 SSc patients. The amount of circulating monocytes in SSc increased compared with the healthy subjects representing a positive correlation with the fibrosis severity of skin and lungs (19). According to our findings, no significant relation was observed between

the monocyte subtypes and ratios between the clinical manifestations of the SSc. Our findings might be attributed to the contribution of non-classical and intermediate monocyte subtypes, classified together as $CD16^+$ monocytes. Despite the significant increase in the total count of monocytes, there was a slight difference between the intermediate and non-classical monocyte subtypes (32, 33), which suggests a positive correlation with increased expression of $CD14^+$ and $CD16^+$ monocytes could induce monocytes maturation (10, 34). In another study, the frequency of circulating monocytes in SSc patients increased compared with the control group. Our results indicated the frequency of monocytes is significantly related to skin fibrosis. In addition, classical monocytes in patients with Scleroderma-related interstitial lung disease have been reported to be enhanced compared with the control group. Yet, an inverse correlation was observed between the total number of circulating monocytes and DLCO (35). Our findings are in accordance with the results reported by Trombetta et al. and Lescoat et al. about the increased levels of circulating $CD16^+$ monocytes as major monocytes presented in SSc patients (36, 37).

Nonetheless, our findings differ regarding clinical manifestations correlated with $CD16^+$ monocyte enhancement. A possible explanation for this outcome could be related to the ability of different tissues to induce and exacerbate inflammatory responses, which reinforces the hypothesis of cellular activation and modulation in loco (38). Hence, the results of monocyte activation in the bloodstream may not be observed in the tissue affected by SSc. Likewise, in a study by Schneider et al., the expression of CD64, CD14, and CD16 monocytes was examined in 50 SSc cases, in which 72% were lSSc. Accordingly, the expression of the analyzed monocytes increased in SSc patients in comparison with the control group. In addition, classical and intermediate monocytes expressing CD206 were much higher in SSc patients compared with the healthy subjects. The clinical symptoms of the SSc in the investigation had no significant relation with monocyte subgroups, as previously documented (39). Monocyte subtypes have also been studied in rheumatic diseases. Kawanaka et al. reported an increased infiltration of non-classical and intermediate $CD16^+$ monocytes from the peripheral blood to the synovial tissue of rheumatoid arthritis patients (40).

On the contrary, Barrera García et al. reported decreased levels of non-classical monocytes in PBMC samples of patients with severe lupus nephritis. Despite increased infiltration of $CD16^+$ monocytes in the glomerulus, this outcome indicated the accumulation of $CD16^+$ monocytes in renal tissue (41). Regarding the controversial data, further studies are required to precisely illuminate the effects of circulating monocytes as well as the activation and proliferation of these cells in chronic inflammatory disease. There were some limitations in our study. First, the population of patients was relatively low. Furthermore, due to the uncommon dSSc, we only examined patients with the lSSc form. Examining more samples and various severe and fatal disease symptoms will be useful.

CONCLUSION

Altogether, our findings demonstrated that SSc patients had higher non-classical monocytes compared with the control cases. In addition, the results of this study indicated that the ratio of classical to non-classical monocytes considerably decreased in SSc cases compared with the control group, indicating their effects on the SSc pathogenesis.

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AUTHORS' CONTRIBUTION

ES and AEM conceptualized and supervised the study; VA and SHP conducted the experiments and managed the data; AF designed the study and prepared the initial draft; AEM and ES analyzed the statistical data, created visualizations, and verified the accuracy of the tests.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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