

# Increased levels of VCAM-1 in sera and VLA-4 expression on neutrophils in dermatomyositis with interstitial lung disease

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## Research article

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# Abstract

**Background:** Vascular cell adhesion molecule-1 (VCAM-1) and its ligand very late antigen (VLA-4) play important roles in many autoimmune diseases. Our study aimed to investigate serum VCAM-1 level and VLA-4 expression on peripheral blood neutrophil surface in patients with dermatomyositis (DM), especially focusing on patients with interstitial lung disease (ILD).

**Methods:** Blood specimens of 30 patients with DM and 30 healthy controls matched for age and gender were recruited. Total serum VCAM-1 level was measured using commercial enzyme-linked immunosorbent assay (ELISA) and the percentages of VLA-4 expression on the surface of neutrophils were analyzed by flow cytometry. We divided patients into subgroups according to whether they had ILD and whether they exhibited diffuse alveolar damage (DAD) via high-resolution computed tomography (HRCT).

**Results:** Serum VCAM-1 levels were increased in DM patients compared with healthy controls ( $p < 0.001$ ). Patients with DM-ILD had higher serum VCAM-1 levels than those with none-ILD ( $p = 0.015$ ). The VCAM-1 levels were significantly increased in the DM-DAD group compared to the none-DAD group ( $p = 0.002$ ). The percentages of VLA-4 expression on neutrophils surface in DM patients were significantly elevated than that in healthy controls ( $p < 0.001$ ). The percentage of VLA-4 expression on neutrophils in DM patients with ILD were higher than none-ILD group ( $p = 0.013$ ). In the patients with ILD, DAD group had higher percentage of VLA-4 expression on neutrophils than none-DAD group ( $p = 0.008$ ).

**Conclusions:** Our findings indicated that serum VCAM-1 level could be used as a potential serological biomarker for DM-ILD.

## Background

Dermatomyositis (DM) is characterized by myositis and rash with complications in other vital organs such as the lung and heart. Interstitial lung disease (ILD) is one of the most common and life-threatening complications of DM, with a prevalence up to 86%[1]. The one-year survival rate for patients with DM-ILD is 56.7%, and even lower in patients with acute ILD[2]. However, the pathogenesis of DM-ILD remains unclear.

VCAM-1 (CD106) is a 90-kDa glycoprotein predominantly expressing on endothelial cells. Its main ligand is VLA-4 ( $\alpha 4\beta 1$  integrin) which plays a major role in mediating rolling and firm adhesion of leukocytes to the endothelium, as well as leukocyte transmigration[3]. The soluble ectodomain of VCAM-1 (sVCAM-1) is released from the cell surface into the circulation by proteolysis, a process that is upregulated in inflammatory diseases[4]. The expression of VCAM-1 is closely related to tumor angiogenesis and metastasis in gastric carcinoma and breast cancer[5, 6]. In addition, VCAM-1 and VLA-4 are major factors in promoting survival of endothelial and mural cell during angiogenesis[7]. VLA-4 is expressed mainly on lymphocyte, monocytes, eosinophils, and neutrophils[8]. VCAM-1/VLA-4 pathway has been proved to be associated with inflammatory and autoimmune diseases by recruiting leukocytes to tissue[3, 9]. For

example, VCAM-1/VLA-4 pathway seems to be critical for the infiltration in rheumatoid arthritis (RA)[10, 11]. In addition, VLA-4/VCAM-1 pathway had been implicated in mediating leukocyte adherence to the inflamed endothelium in the central nervous system of multiple sclerosis (MS) patients[12].

In DM, it had been found increased expression of VCAM-1 on blood vessels and muscle fibers[13, 14]. In addition, circulating levels of sVCAM-1 were significantly higher in juvenile dermatomyositis (JDM)[15]. However, for adult DM, the serum level of VCAM-1 and its ligand expression are still not clear. Bronchoalveolar lavage had revealed elevated levels of lymphocytes and neutrophils in patients with DM accompanied by rapidly progressive interstitial lung disease (RP-ILD)[16]. It has been reported that increased VLA-4 expression on lymphocytes may promote lymphocyte transmigration, leading to the onset of ILD[17]. However, the mechanism of pathological recruitment of neutrophils was unclear. In this study, we investigated VCAM-1 levels in sera and VLA-4 expression on neutrophils in DM, especially focusing on the patients with ILD.

## Methods And Materials

### 2.1. Patients and controls

Blood specimens of 30 untreated patients with DM, 20 classic DM and 10 clinical amyopathic dermatomyositis (CADM), were recruited from the Department of Rheumatology of the First Affiliated Hospital of China Medical University during May 2019 to Jan 2020, as well as 30 healthy blood donors. Other autoimmune diseases, current or chronic infections, and other severe concomitant diseases were excluded. The study was supported by the Ethics Committee of the First Affiliated Hospital of China Medical University (No. 2018-214-3), and was conducted according to the principles expressed in the Declaration of Helsinki. All participants signed an informed consent prior to the start of the study. The diagnosis of DM was based on the Bohan and Peter criteria for PM(polymyositis)/DM[18, 19]. DM patients with ILD were diagnosed by two trained rheumatologists based on high-resolution computed tomography (HRCT) and pulmonary function tests. All blood samples were centrifuged to obtain serum immediately, and stored at -80°C. The anticoagulated blood samples with ethylenediamine tetra acetic acid (EDTA) were disposed with polymorphonuclear leucocytes separation medium (Polymorphprep™, Axis-Shield) to get granulocytes.

### 2.2 Radiological patterns for DM-ILD

The most frequently described patterns in DM are non-specific interstitial pneumonia (NSIP, ground-glass opacity with little honeycombing) and organizing pneumonia (OP, irregular consolidation with a predominantly basal/ peripheral or peri-bronchovascular distribution). Usual interstitial pneumonia (UIP) pattern can also be observed (basal, subpleural reticulation and honeycombing), but at a lower frequency. Another pattern is diffuse alveolar damage (DAD, diffuse ground-glass opacity and extensive consolidation) which is associated with a poor prognosis and rapidly developing dyspnoea.[20–22].

### 2.3 Laboratory parameters

Data of laboratory tests such as pulmonary function tests, whole blood counts, fibrinogen (Fg), D-dimer (D-D), creatine kinase (CK), immunoglobulin (IgG, IgA, IgM), complements (C3, C4) were measured at the time of obtaining blood sample.

## 2.4 Measurement of serum VCAM-1 level by ELISA

Total serum VCAM-1 level was measured using ELISA kits according to the manufacturer's instructions (Cat. BMS232, eBioscience, San Diego, CA, USA). All assays were performed in duplicate, and the data are presented as ng/ml.

## 2.5 Detection of VLA-4 expression on the surface of neutrophils by flow cytometry

Neutrophils were incubated with antibody coupled to fluorescent dyes by a 30 min incubation at 4°C. Then the samples were analyzed by FACS Aria  $\times$  Flow cytometer (BD Biosciences), and the results were analyzed using the FlowJo v10 software (Tree Star, Ashland, OR, USA). The antibodies include fluorescein isothiocyanate (FITC)-conjugated CD16 antibody (clone eBioCB16(CB16); eBioscience); isotype for CD16(clone P3.6.2.8.1; eBioscience); allophycocyanin (APC)-conjugated VLA-4 antibody (clone 9F10; BD Biosciences) and isotype for VLA-4 (Mouse IgG1  $\kappa$ ; clone MOPC-21; BD Biosciences).

## 2.6 Statistical analysis

Continuous variables were expressed as mean  $\pm$  standard deviation or median (interquartile range). Differences between two groups were compared using student's t test. One-way analysis of variance (ANOVA) followed by post hoc analysis with the least significant difference (LSD) test were performed to compare differences between multiple groups. The correlation of serum VCAM-1 level and VLA-4 expression on neutrophils with laboratory parameters was performed by Pearson's or Spearman's rank correlation coefficient. Analysis was conducted by the SPSS 20.0 software and GraphPad Prism for Windows version 8.00 (Graph Pad Software, La Jolla, CA, USA). Two-tailed p values less than 0.05 were statistically significant.

## Results

### 3.1 Participants characteristics

30 patients with DM were enrolled, while 30 healthy controls were also recruited matched for age and gender. Demographic and clinical characteristics of DM patients were shown in Table 1.

Table 1

Demographic and clinical characteristics of individuals with dermatomyositis.

Features	DM (n = 30)	HC (n = 30)
Age, years	55.27±11.3	50.13±13.1
Sex, women/men	22:8	18:12
Duration, years	0.33 (0.92)	-
LY ( $\cdot 10^9/L$ )	1.02 (1.03)	2(0.6)
NE ( $\cdot 10^9/L$ )	4.19±2.17	4.71±1.12
Albumin (g/L)	34.02±4.21	-
CRP (mg/L)	6.05 (7.4)	-
CK (U/L)	175.5 (777)	-
LDH (U/L)	344.5 (149)	-
D-D (ug/mL)	0.88 (1.26)	-
Fg (g/L)	3.86±0.84	-
IgG (g/L)	13.86±2.85	-
IgA (g/L)	2.72±1.15	-
IgM (g/L)	1.32 (1.13)	-
C3 (g/L)	1.01±0.18	-
C4 (g/L)	0.2±0.05	-
Ferritin (ug/L)	438.85 (348.6)	-
VC (% predicted)	61.62±18.47	-
FVC (% predicted)	64.83±16.42	-
DLCO (% predicted)	61.17±24.82	-
PaO2 (mmHg)	77.47±15.48	-

HC: healthy controls; VC: vital capacity; FVC: forced vital capacity; DLCO: carbon monoxide diffusing capacity; PaO2: partial pressure of arterial oxygen.

### 3.2 Serum VCAM-1 level

Serum VCAM-1 level in DM patients was significantly increased when compared with healthy controls ( $654.78 \pm 196.29$  ng/ml vs.  $454.68 \pm 131.4$  ng/ml,  $p < 0.001$ , Fig. 1A). Patients with ILD had higher serum VCAM-1 levels than those with none-ILD ( $699.1 \pm 197.72$  ng/ml vs.  $532.89 \pm 138.55$  ng/ml,  $p = 0.015$ , Fig. 1B). The VCAM-1 levels were significantly increased in the DAD group compared to the none-DAD group ( $889.15 \pm 145.31$  ng/ml vs.  $627.83 \pm 166.81$  ng/ml,  $p = 0.002$ , Fig. 1C).

### 3.3 VLA-4 expression on the surface of neutrophils

The percentage of VLA-4 expression on the surface of neutrophils was described as CD16 + VLA-4+. Figure 2 showed an example of FACS dot plot of CD16 + VLA-4+. Statistical analysis showed that the percentage of VLA-4 expression on the surface of neutrophils was significantly increased in patients with DM compared with controls ( $7.96\% \pm 3.94\%$  vs.  $3.22\% \pm 1.85\%$ ,  $p < 0.001$ , Fig. 3A). The percentage of VLA-4 expression on neutrophils in patients with ILD was higher than none-ILD group ( $8.88\% \pm 4.1\%$  vs.  $5.41\% \pm 1.98\%$ ,  $p = 0.014$ , Fig. 3B). Patients with DAD had increased percentage of VLA-4 expression on neutrophils than none-DAD group ( $12.43\% \pm 3.88\%$  vs.  $7.55\% \pm 3.4\%$ ,  $p = 0.004$ , Fig. 3C). The percentage of VLA-4 on the surface of neutrophils was positively correlated with corresponding serum VCAM-1 levels ( $r = 0.655$ ,  $p < 0.001$ , Fig. 3D).

3.4 The clinical significance of serum VCAM-1 level and VLA-4 expression on neutrophils with laboratory parameters in DM patients

Table 2 showed the relationship of serum VCAM-1 levels and VLA-4 expression on neutrophils with laboratory parameters in DM patients. Serum VCAM-1 levels were significantly negatively correlated with FVC, DLCO, PaO<sub>2</sub>, and significantly positively correlated with D-D, Fg, IgG and IgM. VLA-4 expression on neutrophils was also significantly negatively correlated with PaO<sub>2</sub> and significantly positively correlated with D-D, IgG and IgM.

Table 2

Correlation of serum VCAM-1 level and VLA-4 expression on neutrophils with clinical indicators in DM patients.

Parameter	VCAM-1		VLA-4	
	r	p	r	p
LY	0.031	0.871	-0.056	0.77
NE	-0.016	0.932	-0.037	0.845
CK	-0.154	0.416	-0.167	0.379
LDH	0.403*	0.037	0.141	0.456
D-D	0.439*	0.032	0.457*	0.025
Fg	0.447*	0.032	0.185	0.355
IgG	0.441*	0.024	0.438*	0.025
IgA	-0.009	0.963	-0.133	0.492
IgM	0.39*	0.036	0.394*	0.034
C3	-0.41*	0.037	-0.446*	0.022
C4	-0.482*	0.013	-0.398*	0.044
CRP	-0.117	0.537	0.067	0.724
FVC	-0.623*	0.023	-0.316	0.251
DLCO	-0.587*	0.013	-0.439	0.069
PaO2	-0.561**	0.007	-0.607**	0.003

\*: p < 0.05. \*\*: p < 0.01. r: Pearson or spearman regression.

## Discussion

In our study, DM patients exhibited increased sVCAM-1 level in sera and VLA-4 expression on neutrophils surface compared with healthy controls. To our knowledge, this is the first time to detect VLA-4 expression on the surface of neutrophils in DM patients. In addition, these two indicators were elevated in patients with ILD compared to non-ILD. Patients with DAD patterns had higher VCAM-1 levels in sera and VLA-4 expression on neutrophils than those without DAD.

The migration of leukocytes to sites of injury or infection is tightly regulated by the leukocyte adhesion cascade. In the beginning, rolling of leukocytes is mediated by selectins[23]. Then, leukocyte activation and slow rolling are induced by chemokines which activated the extracellular domains of integrins including LFA-1, Mac-1 and VLA-4[24–26]. Activated integrins binding adhesion molecules contribute to firm adhesion of leukocytes to endothelial cells and lead to trans-endothelial migration. VCAM-1 works with other adhesion molecules to regulate immune surveillance and inflammation. However, when the

stimulation is not properly eliminated, this beneficial reaction can lead to chronic and detrimental inflammatory processes, such as rheumatoid arthritis, asthma, and psoriasis. It has been reported that VCAM-1 expression on endothelial cells is activated during inflammatory diseases[27]. Structurally, VCAM-1 contains an extracellular domain with six or seven immunoglobulin (Ig)-like domains, a transmembrane domain and a cytoplasmic domain[27]. The soluble ectodomain of VCAM-1 can be released from the cell surface into the circulation via proteolytic cleavage[4, 28]. The soluble forms of cellular adhesion molecules (sCAMs) were observed to correlate with the endothelial surface expression of CAMs and can be used as potential biomarkers for endothelial activation[29]. In our study, serum levels of sVCAM-1 were significantly increased in DM, especially in DM-ILD and sVCAM-1 levels were significantly negatively correlated with lung function, including FVC, DLCO and PaO<sub>2</sub>. This result revealed that the pathogenesis of DM-ILD may be associated with endothelial cell damage. In addition, serum sVCAM-1 level positively correlated with D-dimer and fibrinogen confirmed this view.

Soluble VCAM-1 plays a key role in the onset of synovitis in RA, which is accompanied by the infiltration of T cells and monocytes[30, 31]. Multiple factors, including increased production of proinflammatory cytokines, the presence of autoantibodies, and increased oxidative stress activate endothelial cells, leading to increased expression of VCAM-1[32]. sVCAM-1 recruits pathological levels of neutrophils to injury sites and amplifies lung inflammation during acute lung injury[33]. Bronchoalveolar lavage fluid from a patient with DM-ILD revealed neutrophil infiltration[16]. It has been reported that excessive formation of neutrophil extracellular traps (NETs) by neutrophils in DM caused damage to pulmonary vascular endothelial cells and infiltration of inflammatory cells, leading to the occurrence of ILD[34]. Therefore, we hypothesized that the pathogenesis of DM-ILD may be related to VCAM-1 and neutrophil infiltration. Ibbotson et al. first discovered that neutrophil recruitment was depended on the VLA-4/VCAM-1 pathway in human disease[35]. We thus explored VLA-4 expression on surface of neutrophils in patients with DM. Our findings manifested that VLA-4 expression on neutrophils were elevated in DM and significantly negatively correlated with PaO<sub>2</sub>. Patients with DAD patterns had higher VCAM-1 levels in sera and VLA-4 expression than those without DAD. The results may confirm the hypothesis that neutrophils infiltrated via VCAM-1/VLA-4 pathway in DM-ILD. Besides, in our cohort, 10 patients were diagnosed with CADM among 30 patients. We compared sVCAM-1 level in sera and VLA-4 expression on neutrophils surface in classic DM and CADM and found no significant differences. Due to the small sample size, we did not divide them into subgroups for further comparison.

## Conclusions

The present study showed that serum VCAM-1 level was increased in DM individuals, especially in DM-ILD. On the other hand, VLA-4 expression on neutrophil surface rised significantly. It is possible that serum sVCAM-1 level may be a useful biological marker to reflect disease activity. Our results highlight the involvement of neutrophils in the pathogenesis of DM-ILD.

## Abbreviations

VCAM-1:vascular cell adhesion molecule-1; VLA-4:very late antigen-4; DM:dermatomyositis; ILD:interstitial lung disease; ELISA:enzyme-linked immunosorbent assay; DAD:diffuse alveolar damage; HRCT:high-resolution computed tomography; RA:rheumatoid arthritis; MS:multiple sclerosis; JDM:juvenile dermatomyositis; RP-ILD:rapidly progressive interstitial lung disease; CADM:clinical amyopathic dermatomyositis; PM:polymyositis; EDTA:ethylenediamine tetra acetic acid; NSIP:non-specific interstitial pneumonia; OP:organizing pneumonia; UIP:usual interstitial pneumonia; Fg:fibrinogen; D-D:D-dimer; CK:creatin kinase; IgG:immunoglobulin G; IgA:immunoglobulin A; IgM:immunoglobulin M; FITC:fluorescein isothiocyanate; APC:allophycocyanin; ANOVA:one-way analysis of variance; LSD:least significant difference test; HC:healthy controls; LY:lymphocyte; NE:neutrophilic granulocyte; CRP:C-reactive protein; LDH:lactate dehydrogenase; VC:vital capacity; FVC:forced vital capacity; DLCO:carbon monoxide diffusing capacity; PaO<sub>2</sub>:partial pressure of arterial oxygen; LFA-1:lymphocyte function-associated antigen-1.

## **Declarations**

### **Ethics approval and consent to participate**

The study was supported by the Ethics Committee of the First Affiliated Hospital of China Medical University (No. 2018-214-3), and was conducted according to the principles expressed in the Declaration of Helsinki. All participants signed an informed consent prior to the start of the study.

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

The datasets analysed during the current study are available from the corresponding author on reasonable request.

### **Competing interests**

The authors declare that they have no competing interests.

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### **Authors' contributions**

ML, CY and PY conceived the design of the study. ML and CY carried out the experiments and statistical analysis. XH and JX obtained clinical data. XL and BT evaluated CT scores. ML drafted the manuscript.

All authors read and approved the final manuscript.

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Not applicable.

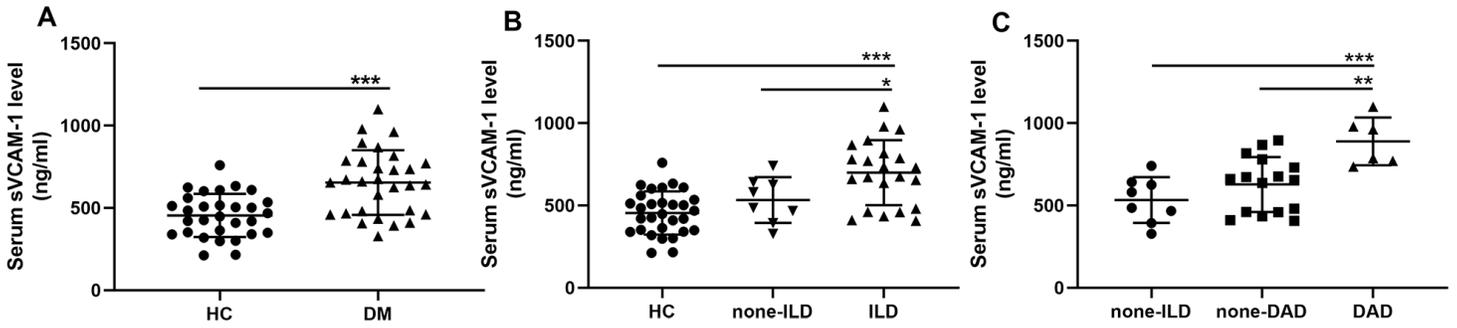
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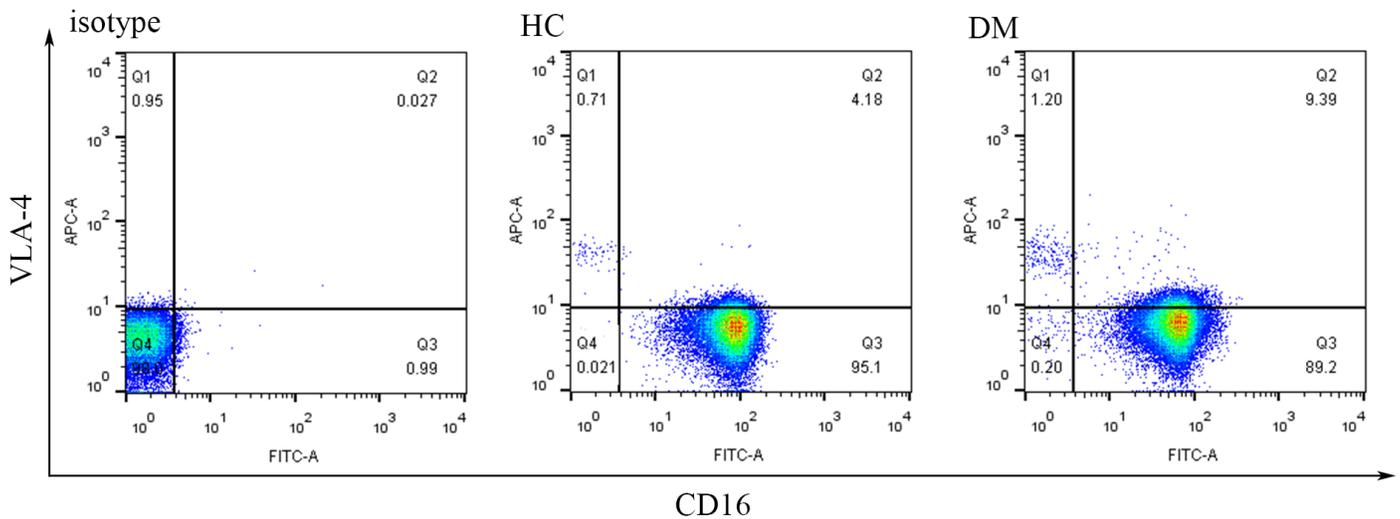
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## Figures



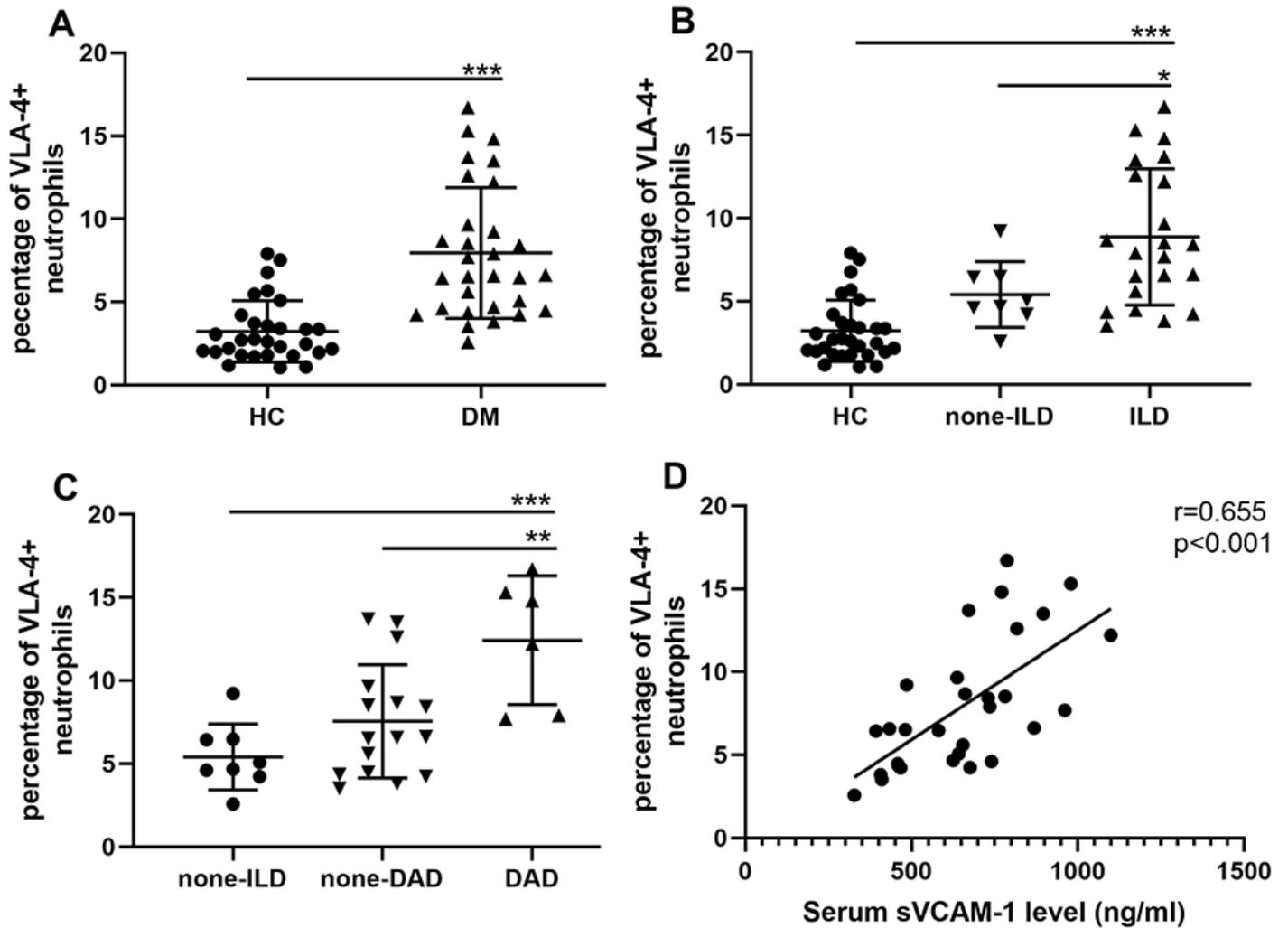
**Figure 1**

Comparison of serum VCAM-1 levels between healthy controls and DM patients. (A) Serum VCAM-1 levels in DM patients and healthy controls. (B) Serum VCAM-1 levels among DM patients with ILD and none-ILD. (C) Serum VCAM-1 levels among DM patients with DAD and none-DAD. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ . VCAM-1: vascular cell adhesion molecule-1, HC: healthy control, DM: dermatomyositis, ILD: interstitial lung disease, DAD: diffuse alveolar damage.



**Figure 2**

FACS dot plots of CD16+VLA-4+ for isotype, control and DM patients. VLA-4: very late antigen-4, HC: healthy control, DM: dermatomyositis.



**Figure 3**

VLA-4 expression on the surface of neutrophils. (A) The percentage of CD16+VLA-4+ neutrophils in controls and DM patients. (B) The percentage of CD16+VLA-4+ neutrophils in DM patients with ILD and none-ILD. (C) The percentage of CD16+VLA-4+ neutrophils in DM patients with DAD and none-DAD. (D) Correlation between the percentage of CD16+VLA-4+ neutrophils and serum VCAM-1 level. \*:  $p<0.05$ ; \*\*:  $p<0.01$ ; \*\*\*:  $p<0.001$ . VCAM-1: vascular cell adhesion molecule-1, VLA-4: very late antigen-4, HC: healthy control, DM: dermatomyositis, ILD: interstitial lung disease, DAD: diffuse alveolar damage.