Peripheral blood immunophenotypic diversity in patients with anti-MDA5+ dermatomyositis and its impact on prognosis

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Abstract

Objective

To explore the heterogeneity and the corresponding clinical significance of lymphocyte subsets in dermatomyositis patients with anti-melanoma differentiation-associated gene 5 positive autoantibody (anti-MDA5+ DM).

Methods

268 anti-MDA5+ DM patients and 536 gender-age matched healthy controls (HCs) were retrospectively enrolled. Patients' clinical data, serological parameters, peripheral blood lymphocyte subsets, imagological examinations, treatment regimens and follow-up were collected. Cluster analysis based on peripheral blood lymphocyte subsets was conducted in anti-MDA5+ DM patients.

Results

The absolute number of $CD3^+$ T lymphocytes, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD3^-CD19^+$ B cells and $CD16^+CD56^+$ NK cells were significantly reduced in anti-MDA5+ DM patients compared with HCs. The absolute counts of the above cell subsets were remarkably reduced in non-survivors compared to the survivors of anti-MDA5+ DM. Cluster analysis based on lymphocyte subsets divided anti-MDA5+ DM patients into cluster 1(n=125) and cluster 2 (n=143). Patients in cluster 1 presented with lower counts of $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD3^-CD19^+$ B cells and NK cells compared with cluster 2. Notably, RP-ILD rate, three-month and six-month death rate in cluster 1 were dramatically higher than in cluster 2, $p<0.001$, respectively.

Conclusion

Lymphocytes and their subsets were significantly altered in anti-MDA5+ DM patients. There was remarkable heterogeneity of lymphocyte subsets in anti-MDA5+ DM patients between survivors and non-survivors. Anti-MDA5+ DM patients were divided into two groups with distinct symptoms and survival rate by cluster analysis based on lymphocyte subsets.

Key words

anti-MDA5+ dermatomyositis, lymphocyte subsets, cluster analysis, $CD3^+CD4^+$ cells, NK cells, prognosis

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Introduction

Anti-melanoma differentiation-associated gene 5 (anti-MDA5) positive dermatomyositis (anti-MDA5+ DM) is a unique subtype of DM, which is frequently associated with rapidly progressive interstitial lung disease (RP-ILD) (1). RP-ILD occurs in 39%-100% of anti-MDA5+ DM patients in East Asia, higher than that of the Caucasian populations (2). The presence of RP-ILD in anti-MDA5+ DM is associated with high mortality (3), the six-month mortality rate for anti-MDA5+ DM with RP-ILD is as high as 30%-60% (1, 4, 5). Therefore, it is pivotal to investigate the heterogeneity of clinical characteristics and pathological mechanism of anti-MDA5+ DM patients to develop personalised therapy (6).

Although relapse occurs, the long-term survival of anti-MDA5+ DM who survived the remission induction phase is generally favourable (4). Therefore, the identification of useful markers predictive of poor outcomes is required for anti-MDA5+ DM patients (7). Muscle involvement, arthritis, were recognised as a favourable prognostic marker (8, 9). However, old age, male gender, RP-ILD, high C-reactive protein (CRP), hypoxemia, low forced vital capacity, high levels of serum ferritin, creatine kinase, lactate dehydrogenase (LDH) and lymphocytopenia are risk factors for mortality in patients with anti-MDA5+ DM (1, 3, 10-12). Alterations in lymphocytes are common manifestations in autoimmune diseases (13). The immune signatures in peripheral T and B cells have been investigated. High frequency of circulating $ISG15^+CD8^+$ T cells at baseline predicts poor one-year survival in MDA5+ DM patients (14). Therefore, the investigation of lymphocyte subsets in anti-MDA5+ DM is crucial in clinical practice.

Alterations in lymphocytes associated with clinical symptoms of anti-MDA5+ DM patients. Patients with severe lymphocytopenia had skin ulcers and higher proportions of RP-ILD (5). In patients of anti-MDA5+ DM with ILD, deceased $CD3^+CD8^+$ cell count and $CD3^-CD19^+$ count were independent predictors of mortality (15). In our study, we focused on investigating the alterations and heterogeneity of lymphocyte subsets in a large cohort of anti-MDA5+ DM patients in China. Cluster analysis based on lymphocyte subsets was also performed to further demonstrate lymphocyte subsets in the clinical practice of anti-MDA5+ DM.

Methods and materials

Enrolment of patients

345 anti-MDA5+ DM patients and 536 age-gender matched healthy controls (HCs) aged ≥18 years old were retrospectively enrolled in the first affiliated hospital of Zhengzhou University between February 2019 and March 2023. 268 anti-MDA5+ DM patients fulfilled the inclusion criteria were enrolled in our research (Fig. 1). This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Zhengzhou University (KY-2021-1101). All patients and HCs approved to participate in this study in accordance with the Declaration of Helsinki.

Definition of anti-MDA5+ DM patients

The definition of DM was according to the 2020 European Neuromuscular Centre DM criteria. Besides, patients were retrospectively reconfirmed according to the 2017 European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR) idiopathic inflammatory myopathy classification criteria. Further, anti-MDA5+ autoantibody was positive for all anti-MDA5+ DM patients. The exclusion criteria were as follows: (1) Age of disease onset <18 years; (2) Patients combined with other autoimmune diseases, malignant cancers, active HBV or HCV infections; (3) Lack of peripheral lymphocyte subsets data.

Definition of ILD

ILD was identified on the basis of respiratory symptoms with HRCT findings, pulmonary function test results were also utilised to confirm the ILD diagnosis. RP-ILD was defined as displaying the worsening of radiological interstitial changes with progressive hypoxemia and dyspnoea within 1 month of respiratory symptoms onset.

Clinical characteristics and laboratory parameters collection

The demographic features, baseline and final follow-up clinical characteristics, serological parameters, radiographic examination, pulmonary function, treatment strategy, three-month and six-month survival were collected. Serological parameters included blood routine test, liver/renal function, erythrocyte sedimentation rate (ESR), C-reactive protein, muscle enzymes, immunoglobulin G/A/M, complement 3 (C3), C4, Krebs von den Lungen-6 (KL-6), serum ferritin, IL-6 and peripheral blood lymphocyte subsets were collected. NLR represented the neutrophil/lymphocyte ratio. The antinuclear antibody test was performed for all patients. Blood routine test was detected by a fully automatic haematology analyser and microscopic analysis. Total protein, ALB, GGT, BNU and UA were detected by the colorimetric method. ALT, AST, ALP and muscle enzymes were detected by enzyme kinetics assay. Bilirubin was detected by diazo reaction. ESR was detected by the Laser-based method. Serum ferritin, IL-6 were detected by electrochemiluminescence. CRP, IgG, IgA, IgM, C3, C4 were tested by turbidimetric inhibition immunoassay. KL-6 was detected by chemiluminescence. The anti-nuclear antibody was detected by indirect immunofluorescence. Myositis specific autoantibodies (MSAs) and myositis associated autoantibodies (MAAs) were conducted using line immunoassays for all patients (EUROIMMUN, Germany). The serum level of anti-MDA5 autoantibody was detected using an enzyme-linked immunosorbent assay (MBL, Japan). Data on peripheral blood lymphocyte subsets were collected.

The treatment regimens were also collected. Dural therapy represented glucocorticoids (GCs) therapy combined with one type of immunosuppressants, including cyclophosphamide (CTX), cyclosporin (CsA), tacrolimus, mycophenolate mofetil (MMF), Janus kinase inhibitors and biologics. Triple therapy represented GCs therapy combined with two kinds of immunosuppressants.

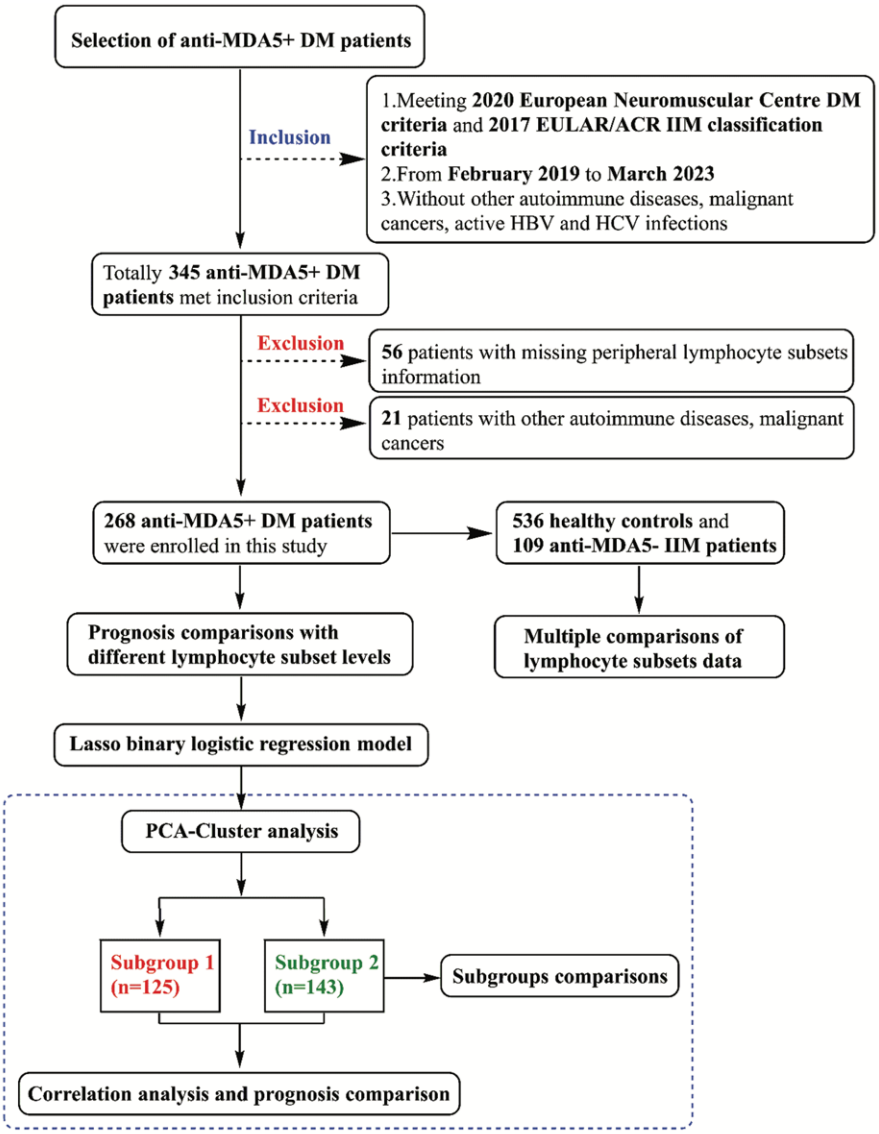

Fig. 1. Outline of the analysis workflow used in this study.

Cluster analysis and PCA

Data analysis was performed in R (version 4.2.1; https://www.R-project.org/). Principal component analysis (PCA) was performed using the package prcomp. Hierarchical cluster analysis was performed by the package cluster with the Ward method. The flow chart of this study is shown in Figure 1.

Statistical analysis

Statistical analysis was performed by SPSS (Version 25.0), R (Version 4.3.2) and GraphPad Prism (Version 9.0). Quantitative data were described as mean ± SD or median [first quartile (Q1), third quartile (Q3)] according to data distribution. Qualitative data were presented as frequencies. Student's t-test or Mann-Whitney U test was used for continuous data. c^2 or Fisher's exact tests were used for binary data. Multiple comparisons of quantitative data were calculated using one-way ANOVA test or Kruskal-Wallis test. Spearman's correlation test was conducted to analyse the correlation between lymphocyte subsets and laboratory parameters. Two-sided p values <0.05 was considered as statistically significant. Cox-regression was performed to identify the independent mortality associated risk factors for anti-MDA5+ DM patients. HRs and 95% CIs for death were calculated using Cox proportional hazards models. Survival curve was evaluated by Kaplan-Meier analysis using the log-rank test.

Table I. Clinical characteristics of patients with anti-MDA5+ DM, anti-MDA5- IIM and HCs.

Clinical characteristics	Anti-MDA5+ DM patients (n=268)	Anti-MDA5- IIM patients (n=109)	Healthy controls (n=536)
Demographic features			
Age at disease onset(years)	52.5 (46-59)	51 (43-59)	52 (41-60)
Female, n (%)	174 (64.9)	86 (78.9)	359 (67.0)
Disease duration (months)	2.1 (1.1-3.2)	7 (2-24)	
Manifestations of Skin			
Rash, n (%)	245 (91.4)	45 (41.3)	
Skin ulcers, n (%)	47 (17.5)	3 (2.8)	
Heliotrope rash, n (%)	175 (65.3)	29 (26.6)	
Gottron sign, n (%)	154 (57.5)	18 (16.5)	
Mechanic's hands, n (%)	60 (22.3)	20 (18.3)	
Skin roughness, n (%)	74 (27.6)	14 (12.8)	
Hair loss, n (%)	18 (6.7)	0 (0)	
Musculoskeletal involvement			
Myalgia, n (%)	86 (32.1)	44 (40.4)	
Muscle weakness, n (%)	105 (39.2)	54 (49.5)	
Respiratory involvement			
Cough, n (%)	219 (81.7)	33 (30.3)	
Short of breath, n (%)	196 (73.1)	29 (26.6)	
Thoracic tightness, n (%)	180 (67.2)	41 (37.6)	
Dyspnea, n (%)	120 (44.8)	9 (8.3)	
RP-ILD	144 (53.7)	65 (59.6)	
Other clinical manifestations			
Fever, n (%)	143 (53.4)	32 (29.4)	
Fatigue, n (%)	231 (86.2)	39 (35.8)	
Arthralgia/arthritis, n (%)	132 (49.3)	34 (31.2)	
Morning stiffness, n (%)	31 (11.6)	14 (12.8)	
Raynaud phenomenon, n (%)	16 (6.0)	6 (5.5)	
Dysphagia, n (%)	16 (6.0)	17 (15.6)	
Choking on drinking water, n (%)	9 (3.4)	6 (5.5)	
Hoarseness of voice, n (%)	16 (6.0)	7 (6.4)	
Pharyngalgia, n (%)	25 (9.3)	10 (9.2)	
Poor appetite, n (%)	111 (41.4)	36 (33.0)	
Weight loss, n (%)	107 (39.9)	10 (9.2)	
Complications			
Mediastinal emphysema, n (%)	29 (10.8)	1 (0.9)	
Macrophage activation syndrome, n (%)	14 (5.2)	0 (0.0)	
Initial treatment strategy			
Glucocorticoids monotherapy, n (%)	25 (9.3)	14 (12.8)	
Dual therapy, n (%)	109 (40.7)	43 (39.4)	
Triple therapy, n (%)	126 (47.0)	47 (43.1)	
Initial treatment regimens			
Conventional immunosuppressants			
CTX, n (%)	127 (47.4)	30 (27.5)	
CsA, n (%)	57 (21.3)	11 (10.1)	
Tacrolimus, n (%)	124 (46.3)	38 (34.9)	
MMF, n (%)	2 (0.7)	9 (8.3)	
Biological agents			
RTX, n (%)	2 (0.7)	1 (0.9)	
Tocilizumab, n (%)	5 (1.9)	38 (34.9)	
JAK inhibitors	62 (23.1)	16 (14.7)	
Tofacitinib, n (%)	58 (21.6)	14 (12.8)	
Baricitinib, n (%)	4 (1.5)	2 (1.8)	
Intravenous immunoglobulin, n (%)	188 (70.1)	20 (18.3)	

Dual therapy represented glucocorticoids (GCs) therapy combined with one type of immunosuppressants, including cyclophosphamide (CTX), cyclosporin (CsA), tacrolimus, mycophenolate mofetil (MMF), Janus kinase inhibitors (JAKs) and biologics. Triple therapy represented GCs therapy combined with two kinds of immunosuppressants. RTX: rituximab.

We used receiver operating characteristic curve (ROC) analyses and AUC to assess the discriminatory capacity of a model for separating patients with and without anti-MDA5 autoantibody. Decision curve analysis (DCA) was used to report the clinical net benefit of each model compared to biomarker-all and biomarker-none strategies.

Results

Lymphocyte subsets were significantly decreased in anti-MDA5+ DM

A total of 268 anti-MDA5+ DM patients were enrolled, comprising 174 females (64.9%) and 94 males (35.1%), with a median age of 52.5 (46-59) years. Additionally, 109 MDA5– patients were included: 86 females (78.9%) and 23 males (21.1%), with a median age of 51 (43-59) years. The clinical characteristics of the participants are presented in Table I. Lymphocyte subset profiles for anti-MDA5+ DM patients, anti-MDA5- IIM patients, and HCs are displayed in Table II. Compared with anti-MDA5- IIM patients and HCs, there were multiple lymphocyte subset alterations in anti-MDA5+ DM patients. The total lymphocyte counts were significantly decreased in anti-MDA5+ DM, as well as the percentages of $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD16^+CD56^+$ NK cells. Strikingly, the counts of $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD3^-CD19^+$ B cells and $CD16^+CD56^+$ NK cells were dramatically decreased in anti-MDA5+ DM patients compared to anti-MDA5- IIM patients and HCs (Fig. 2A-F). To further demonstrate the clinical relevance between peripheral lymphocyte subsets and laboratory parameters, Spearman's correlation was conducted. Lymphocyte counts, $CD3^+$ T cell counts, $CD3^+CD4^+$ T cell counts, $CD3^+CD8^+$ T cell counts, $CD19^+$ B cell counts and NK cells correlated with multiple serological parameters, including AST, ALB, CK, LDH, CRP and serum ferritin (Fig. 2G).

Lymphocyte subsets distribution of anti-MDA5+ DM patients with/without RP-ILD

And we performed comparative immunophenotyping between anti-MDA5+ DM patients with and without RP-ILD. Patients with RP-ILD showed significantly lower absolute counts of $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and $CD16^+CD56^+$ NK cells compared to those without RP-ILD (Supple-

Table II. Peripheral blood lymphocyte subsets distribution in anti-MDA5+ DM patients, anti-MDA5-IIM and HCs.

Lymphocytes subsets	Anti-MDA5+ DM patients (n=268)	Anti-MDA5- IIM patients (n=109)	HCs (n=536)	*p*-value
Lymphocytes counts/μL	743 (479-1033)	1074 (684-1679)	2004 (1684-2444)	**<0.001**
Proportion of $CD3^+$ T (%)	69.0 (58.9-78.1)	70 (62.0-77.4)	73 (68.2-77.1)	**<0.001**
$CD3^+$ T cell counts/μL	480 (291-699)	716 (435-1141)	1454 (1195-1758)	**<0.001**
Proportion of $CD3^+CD4^+$ T cells (%)	42.6 (32.6-50.0)	38.4 (32.1-48.0)	43.1 (38.0-48.2)	**0.003**
CD3+$CD4^+$ T cell counts/μL	287 (167-446)	405 (254-675)	848 (703-1053)	**<0.001**
Proportion of $CD3^+CD8^+$ T cells (%)	22.9 (16.5-29.5)	24.3 (16.9-32.1)	25 (21.4-29.2)	**0.003**
$CD3^+CD8^+$ T cell counts/μL	148 (93-238)	274 (149-382)	501 (387-639)	**<0.001**
Ratio of $CD4^+T/CD8^+T$ cells	1.86 (1.32-2.67)	1.63 (1.11-2.82)	1.75 (1.39-2.16)	0.110
Proportion of CD3-$CD19^+$ B cells (%)	16.0 (11.0-25.0)	13.8 (8-22)	13.6 (11.0-16.9)	**<0.001**
CD3-$CD19^+$ B cell counts/μL	113 (62-205)	157 (71-300)	279 (208-366)	**<0.001**
Proportion of NK cells (%)	10.0 (5.2-15.0)	11 (8-18)	11 (7.8-16.0)	**0.003**
$CD16^+CD56^+$ NK cell counts/μL	68 (32-122)	126 (77-203)	230 (158-333)	**<0.001**

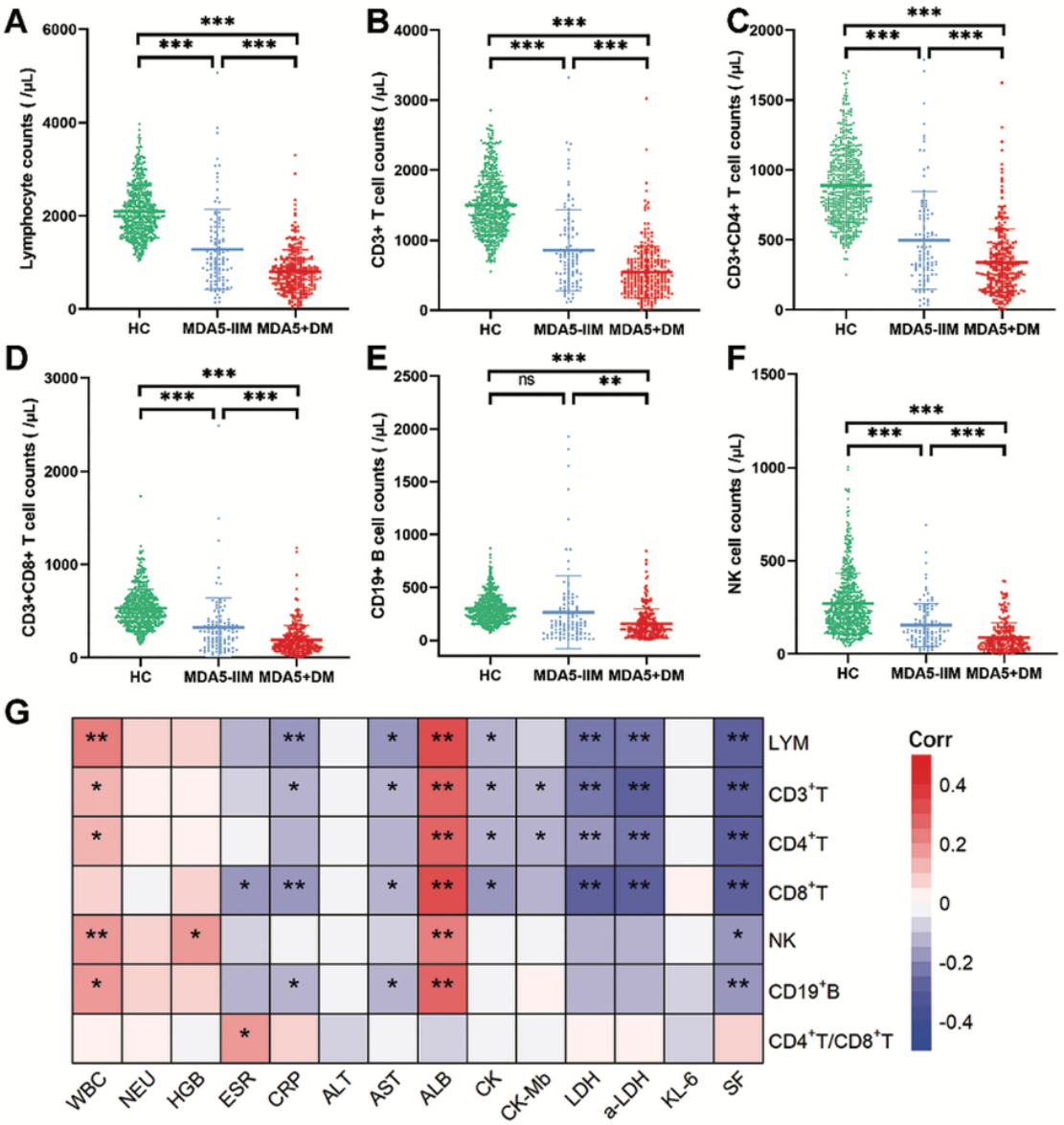

Fig. 2. Comparison of lymphocyte subsets between HCs, anti-MDA5– IIM and anti-MDA5+ DM patients and correlations of lymphocyte subsets with clinical parameters in anti-MDA5+ DM patients.
(A-F) Histogram showing comparisons among the absolute counts of lymphocyte subsets in HCs, anti-MDA5- IIM patients and anti-MDA5+ DM patients. **(G)** Correlation matrix of lymphocyte subsets and laboratory results in patients with anti-MDA5+ autoantibodies. Correlation coefficients for each pair are represented by colour.
Red: positive correlation coefficient; Blue: negative correlation coefficient; White: no significant correlation.

Table III. Lymphocyte subsets comparison between survivors and non-survivors of patients with anti-MDA5+ DM.

Lymphocytes subsets	Non-survivors (n=69)	Survivors (n=195)	*p*-value
Lymphocytes counts/μL	525 (321-796)	812 (561-1673)	**<0.001**
Proportion of CD3+ T (%)	68.8 (52.6-76.1)	69.0 (60.0-79.0)	0.149
CD3+ T cell counts/μL	525 (321-796)	526 (340-731)	**<0.001**
Proportion of CD3+CD4+ T cells (%)	41.0 (28.0-51.0)	42.8 (33.0-50.0)	0.427
CD3+CD4+ T cell counts/μL	217 (112-328)	322 (204-465)	**<0.001**
Proportion of CD3+CD8+ T cells (%)	21.3 (15.1-28.5)	23.6 (17.0-30.0)	0.184
CD3+CD8+ T cell counts/μL	101 (59-178)	165 (106-265)	**<0.001**
Ratio of CD4+T/CD8+T cells	1.75 (1.27-2.67)	1.87 (1.31-2.69)	0.907
Proportion of CD3-CD19+ B cells (%)	16.3 (12.2-25.5)	16.0 (9.9-25.0)	0.393
CD3-CD19+ B cell counts/μL	85 (32-131)	123 (75-216)	**0.001**
Proportion of NK cells (%)	9.9 (5.7-15.1)	10.0 (5.1-15.1)	0.689
CD16+CD56+ NK cell counts/μL	47 (21-83)	73 (41-138)	**<0.001**

mentary Table S1). However, the proportions of these lymphocyte subsets did not differ significantly between groups. This underscores the association between specific immune cell abnormalities and clinical disease severity.

Lymphocyte subsets distribution between six-month survivors and non-survivors in anti-MDA5+ DM patients

The follow-up time of 268 anti-MDA5+ DM patients was 14.5 (4.4-30.5) months. Four patients were lost to follow-up. 69 (26.1%) anti-MDA5+ DM patients died within six months, 195 (73.9%) anti-MDA5+ DM patients survived in six-month follow up. 29 (42%) patients died for the progression of ILD, 26 (37.7%) patients died for infection and the progression of ILD, eight (11.6%) patients died for lung infection, two (2.8%) patients died for mediastinal emphysema, two (2.8%) patients died for ILD progression combined with macrophage activation syndrome (MAS), one (1.4%) patient died

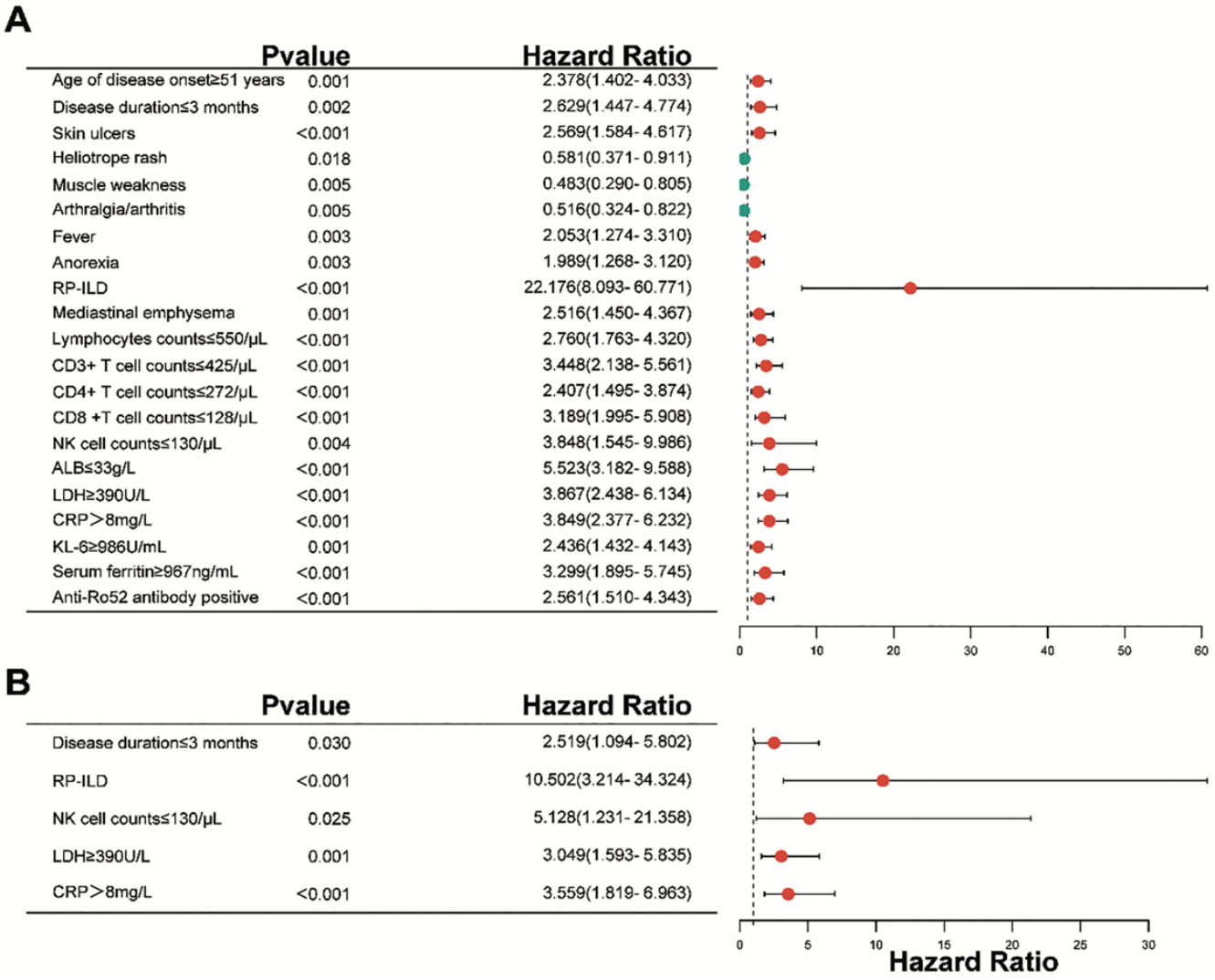

Fig. 3. Univariate and multivariate analysis for mortality associated factors of anti-MDA5+ DM patients.
(A) Univariate analysis for mortality associated factors of anti-MDA5+ DM patients. **(B)** Multivariate analysis for mortality associated factors of anti-MDA5+ DM patients.

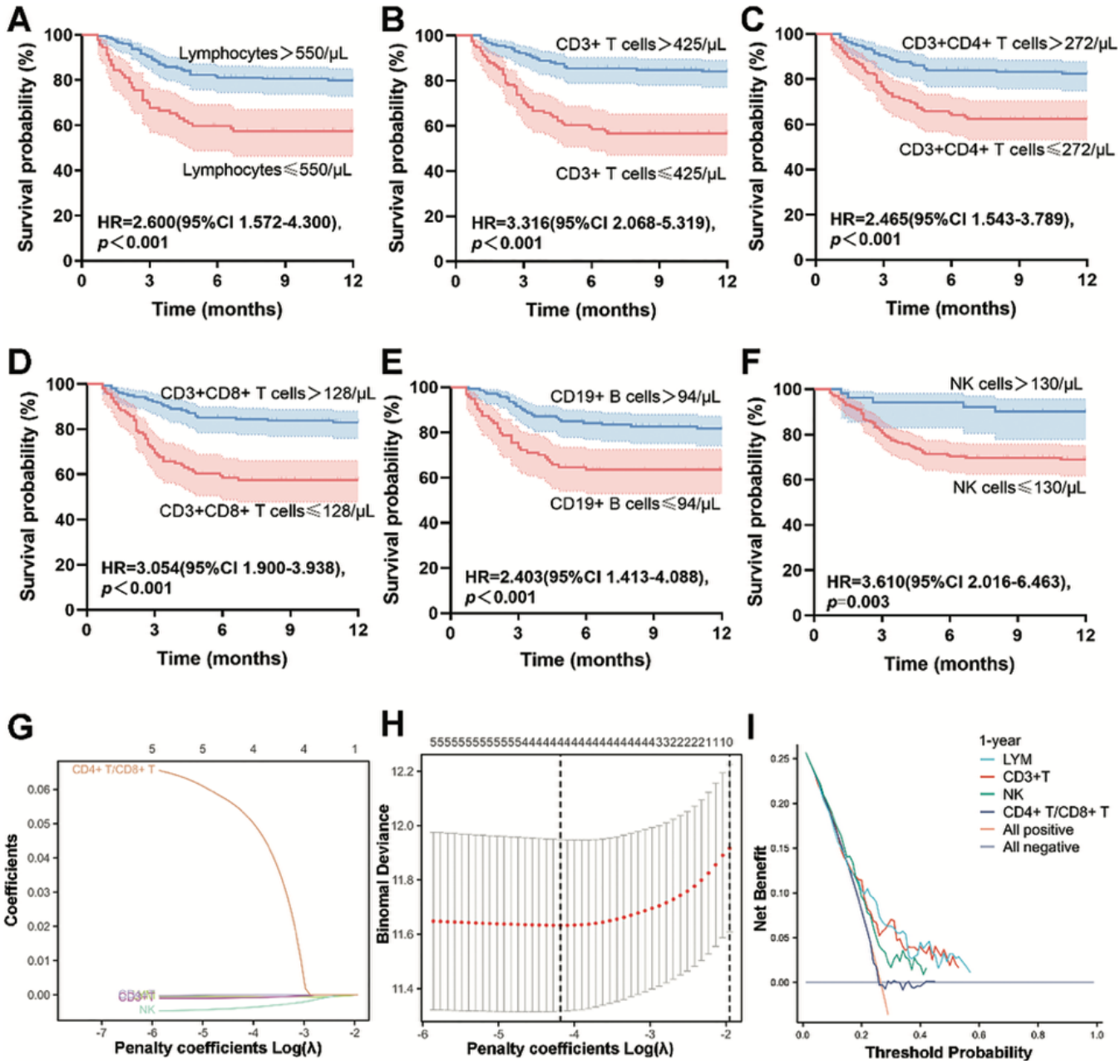

Fig. 4. Survival analysis of anti-MDA5+ DM patients with higher or lower lymphocyte subsets and the Lasso binary logistic regression model. **(A-F)** Kaplan-Meier one-year survival curves in patients with anti-MDA5+ autoantibodies with higher and lower counts of lymphocyte subsets. **(A)** Lymphocytes. **(B)** $CD3^+$ T cells. **(C)** $CD3^+CD4^+$ T cells. **(D)** $CD3^+CD8^+$ T cells. **(E)** $CD19^+$ B cells. **(F)** NK cells. **(G)** Changes in 7 marker coefficients with the penalty parameter (λ). **(H)** Tuning parameter (λ) selection in the Lasso model used tenfold cross-validation based on the minimum criteria. **(I)** Decision curve analysis (DCA) of the novel nomogram for the prognosis of anti-MDA5+ DM patients.

for MAS, and one (1.4%) died for an unknown cause. The comparison of demographic features, clinical characteristics, complications and initial treatment regimens between survivors and non-survivors of anti-MDA5+ DM patients were shown in Supplementary Table S2. Further, the comparison of laboratory parameters between survivors and non-survivors was shown in Supplementary Table S3.

In order to investigate the disparity of lymphocyte subsets in anti-MDA5+ DM patients, the baseline lymphocyte subsets were compared between survivors and non-survivors (Table III). The percentages of $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, CD3-$CD19^+$ B cells and $CD16^+CD56^+$ NK cells were comparable between survivors and non-survivors of anti-MDA5+ DM patients. Whereas, the absolute counts of total lymphocytes, $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD3^-CD19^+$ B cells and $CD16^+CD56^+$ NK cells were dramatically decreased in non-survivors compared with survivors. The above results indicated the decreased counts of lymphocyte subsets associated with the poor prognosis of anti-MDA5+ DM.

Investigate mortality associated factors in anti-MDA5+ DM by Cox-regression

Univariate analysis (Supplementary Table S4) revealed age ≥51 years, skin

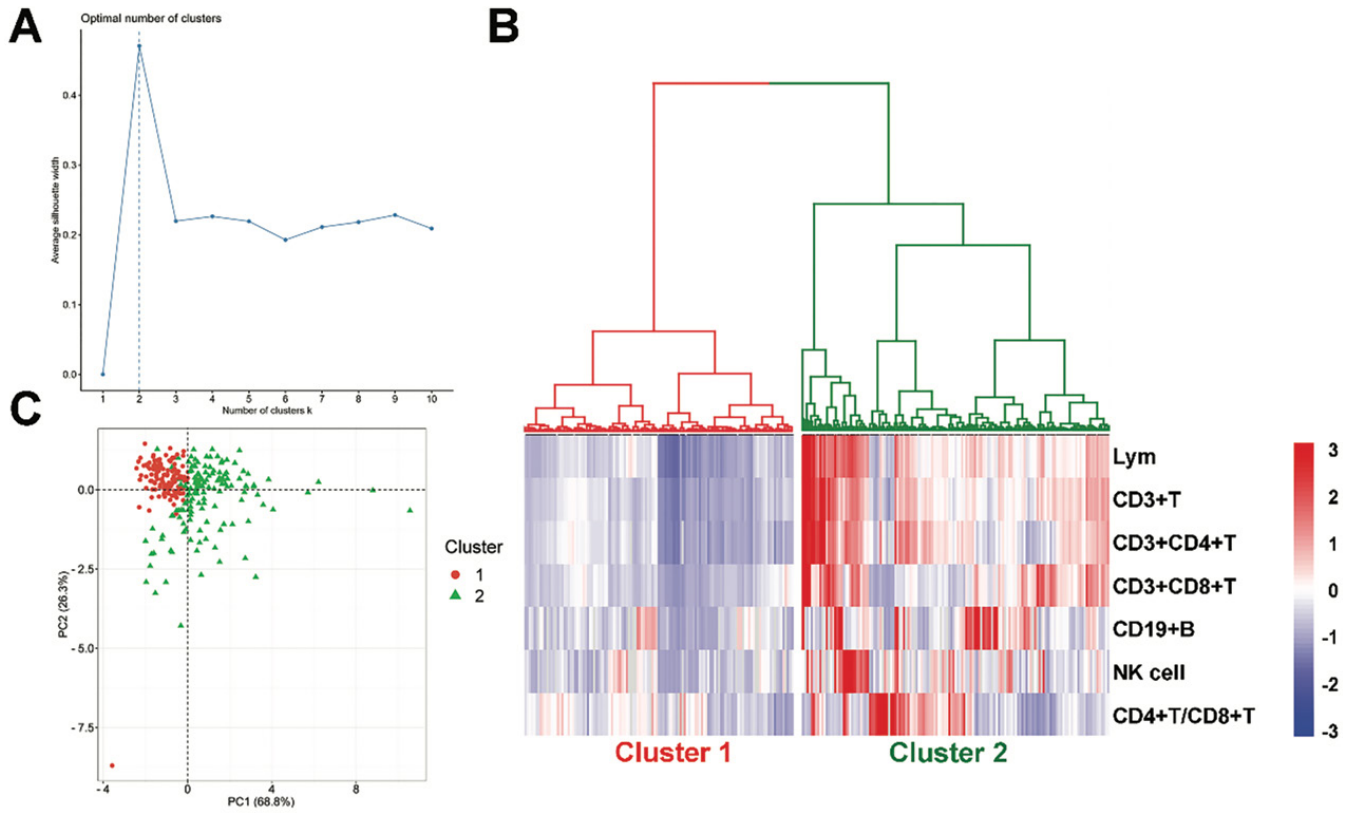

Fig. 5. Cluster analysis based on lymphocyte subsets in patients with anti-MDA5+ autoantibodies.
(A) The number of clusters was chosen by the inflection point in a plot of the sum of squared error as a function of cluster number. **(B)** The tree diagram and heatmap showing hierarchical cluster analysis divided anti-MDA5+ DM patients into two subgroups. **(C)** The PCA plot showing multiple correspondence analysis confirms the presence of two clusters in anti-MDA5+ DM patients.

ulcers, fever, RP-ILD, mediastinal emphysema, lymphocytes≤550/μL, $CD3^+$ T cells≤425/μL, $CD3^+CD4^+$ T cells≤272/μL, $CD3^+CD8^+$ T cells≤128/μL, NK cells≤130/μL, ALB≤33 g/L, LDH ≥390 U/L, CRP>8 mg/L, KL-6≥967 U/mL and anti-Ro52 antibody positive were mortality associated factors for anti-MDA5+ DM patients. However, heliotrope sign, muscle weakness and arthritis were protective factors of death for anti-MDA5+ DM patients. Further, multivariate analysis revealed RP-ILD (HR=10.502, 95%CI 3.214-34.324), NK cells≤130/uL (HR=5.128, 95%CI 1.231-21.358), LDH≥390U/L(HR=3.049, 95%CI 1.593-5.835) and CRP>8 mg/L (HR=3.559, 95%CI 1.819-6.963) were independent mortality associated factors for anti-MDA5+ DM patients (Fig. 3, Supplementary Table S4).

Survival analysis based on lymphocyte subsets of anti-MDA5+ DM patients

We previously demonstrated that the total counts of lymphocytes and multiple lymphocyte subsets were significantly decreased in six-month non-survivors of anti-MDA5+ DM patients. Next, the cut-off value of lymphocytes, $CD3^+$T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD19^+$ B cells, NK cells in six-month death prediction of anti-MDA5+ DM patients were conducted by ROC. Then, the quantitative date of lymphocyte subsets was transformed into bivariate data to perform Kaplan-Meier survival analysis. Results demonstrated that anti-MDA5+ DM patients with either $CD3^+$ T cells ≤425/μL, $CD3^+CD4^+$ T cells ≤272/μL, $CD3^+CD8^+$ T cells ≤128/μL, $CD3^-CD19^+$ B cells ≤94/μL and NK cells ≤130/μL had a poorer survival rate than those without (Fig. 4A-F).

The lymphocyte subsets were selected using the Lasso binary logistic regression model (Fig. 4G-H). The tuning parameter (λ) selection in the Lasso model used tenfold cross-validation based on the minimum criteria. The area under the binomial deviance curve was plotted versus log (λ). Dotted vertical lines were drawn at the optimal values using the minimum criteria and the 1 standard error of the minimum criteria (the 1-SE criteria). A coefficient profile plot was produced against the log (λ) sequence (Fig. 4G). Finally, the ratio of $CD3^+CD4^+$ T cells/$CD3^+CD8^+$ T cells, NK cells, lymphocytes, $CD3^+$ T cells related to anti-MDA5+ DM were selected.

And the DCA curve revealed that the prediction model was useful for threshold probabilities between 25% and 60% and enhanced performance for the prognosis of anti-MDA5+ DM patients (Fig. 4I).

Two subgroups with distinct clinical characteristics were divided by cluster analysis based on peripheral lymphocyte subsets in anti-MDA5+ DM

In order to further investigate the clinical significance of lymphocyte subsets in anti-MDA5+ DM patients, cluster analysis based on lymphocyte subsets was performed. The number of clusters was determined by Sihouette

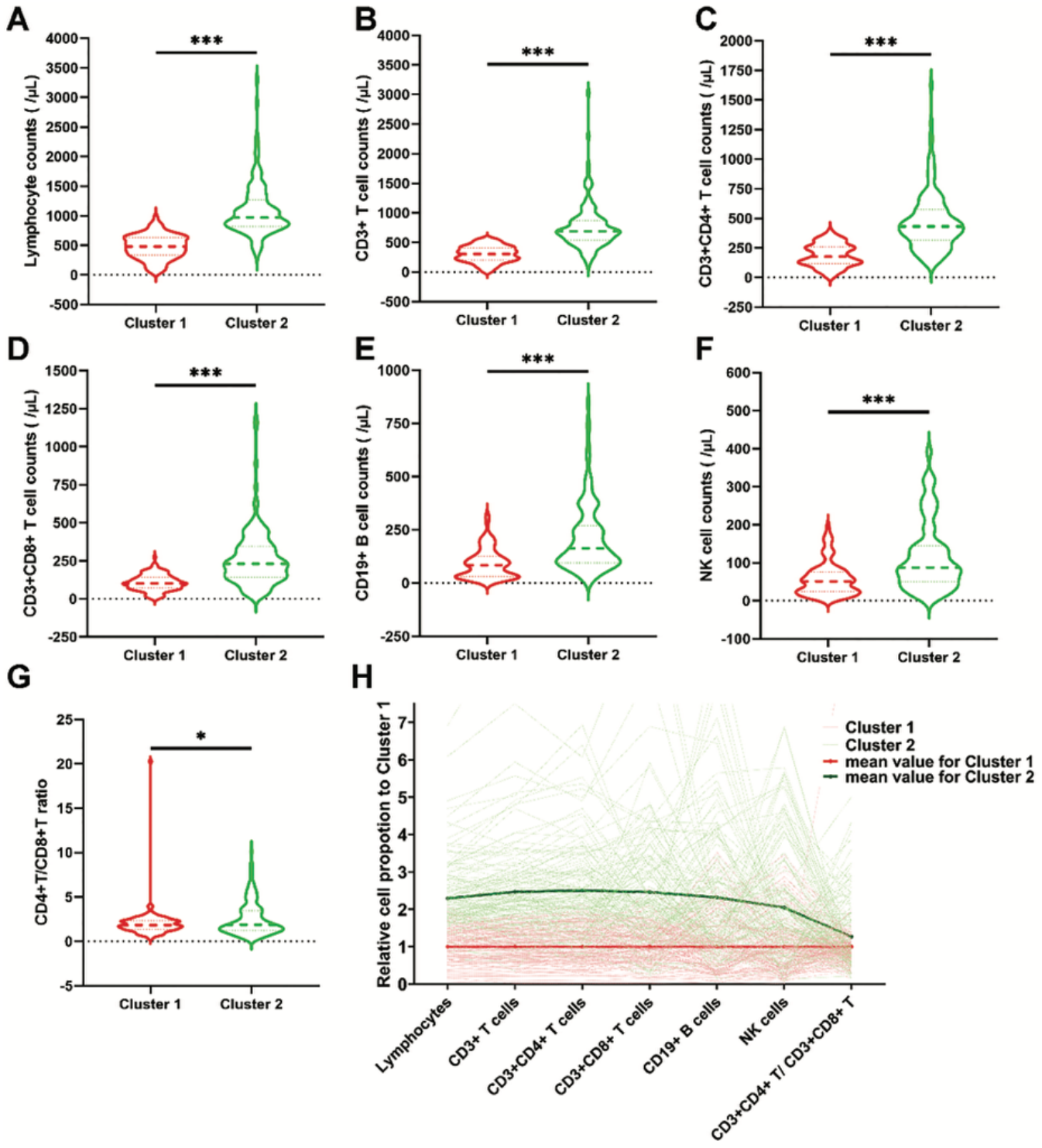

Fig. 6. Comparison of lymphocyte subsets between the subgroups in anti-MDA5+ DM patients.
(A-G) Violin plots showing comparisons among the proportions of lymphocyte subsets between the two clusters of anti-MDA5+ DM patients. **(A)** Lymphocytes. **(B)** $CD3^+$ T cells. **(C)** $CD3^+CD4^+$ T cells. **(D)** $CD3^+CD8^+$ T cells. **(E)** $CD19^+$ B cells. **(F)** NK cells. **(G)** Ratio of $CD3^+CD4^+$ T/ $CD3^+CD8^+$ T cells. **(H)** Line chart for the comparison for the proportions of lymphocyte subsets between two clusters.

method (Fig. 5A). Cluster 1 (n=125) and cluster 2 (n=143) were classified (Fig. 5B). Further, PCA and machine learning was conducted to distinguish and validate the two clusters. 2 PCs were extracted and the distribution of lymphocyte subsets were visualised in patients with IIM. The cumulative percentage of PC1 and PC2 was 95.1%, as shown in Figure 5C. Results revealed a distinct immune signature between cluster 1 and cluster 2.

The distribution of lymphocyte subsets of cluster 1 and cluster 2 was shown in Figure 6A-G. The counts of lymphocytes, $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD19^+$ B cells, NK cells, and the ratio of $CD3^+CD4^+$ T

cells/CD3+CD8+ T cells, were significantly decreased in cluster 1 compared to cluster 2. When the above results were normalised relative to cluster 1, CD3+T cells had the largest increase in lymphocyte subsets between the two clusters (Fig. 6H).

Laboratory parameters comparison between cluster 1 and cluster 2

Baseline serological variables were compared between cluster 1 and cluster 2 (Table IV). The NLR, AST, LDH, HBDH, serum ferritin, serum concentration of anti-MDA5+ autoantibody, ESR, CRP and proportion of anti-Ro52 positive in cluster 1 was significantly higher than in cluster 2, while lymphocytes, hemoglobulin, platelet and ALB was significantly lower in cluster 1 than in cluster 2 otherwise (Table IV, Fig. 7A-F). The above results revealed more severe disease activity in cluster 1 than in cluster 2.

Table IV. Comparisons of serological parameters between cluster 1 and cluster 2 of patients with anti-MDA5+ DM.

Baseline serological parameters	Cluster 1 (n=125)	Cluster 2 (n=143)	*p*-value
WBC, $\times10^9$/L	4.82 (3.43-6.81)	5.3 (4.0-7.4)	0.104
Lymphocytes, $\times10^9$/L	0.53 (0.35-0.70)	0.93 (0.74-1.18)	**<0.001**
Neutrophils, $\times10^9$/L	3.84 (2.53-5.75)	3.78 (2.69-5.52)	0.898
NLR	7.49 (4.43-13.46)	3.76 (2.74-6.00)	**<0.001**
Hemoglobulin, g/L	116.8 ± 17.0	121.6 ± 15.9	**0.017**
Platelet, $\times10^9$/L	192 (144-244)	220 (169-264)	**0.010**
ALT, U/L	41.5 (23.3-78.0)	41.0 (22.0-71.5)	0.673
AST, U/L	56.5 (36.3-85.8)	44.5 (27.0-76.0)	**0.025**
GGT, U/L	67 (27-130)	51 (28-114)	0.145
ALP, U/L	78 (61-101)	76 (61-90)	0.333
ALB, g/L	32.1 ± 4.8	35.1 ± 5.6	**<0.001**
SUA, μmol/L	214 (173-257)	231 (188-293)	**0.014**
CK, U/L	76 (40-143)	58 (34-123)	0.144
LDH, U/L	383 (300-480)	318 (269-388)	**<0.001**
α-HBDH, U/L	264 (218-323)	235 (200-287)	**0.004**
KL-6, U/mL	998 (687-1698)	986 (633-1480)	0.356
Serum ferritin, ng/mL	1171 (513-2110)	659 (264-1211)	**<0.001**
Anti-Ro-52 antibody positive, n (%)	84 (67.2)	74 (51.7)	**0.010**
Levels of anti-MDA5 autoantibodies, U/mL	182 (155-206)	174 (143-197)	**0.047**
IL-6, pg/mL	5.82 (3.01-12.47)	4.18 (2.99-23.32)	0.756
ESR, mm/H	39 (22-54)	24.5 (12-42)	**<0.001**
CRP, mg/L	6.4 (1.9-29.9)	3.1 (1.4-11.3)	**<0.001**
IgG, g/L	13.2 (10.6-15.5)	12.0 (10.0-15.0)	**0.048**
IgA, g/L	2.7 (2.0-3.6)	2.7 (2.0-3.6)	0.939
IgM, g/L	1.3 (0.9-1.8)	1.1 (0.9-1.5)	**0.032**

Distinct clinical characteristics were demonstrated between cluster 1 and cluster 2

To further investigate the differences between cluster 1 and cluster 2, demographic features, clinical characteristics, complications, comorbidity, initial treatment regimens and prognosis were compared (Table V). The age of patients in cluster 1 was older than that in cluster 2, while there was no statistical significance in gender and disease duration between the two clusters. The proportion of thoracic tightness, shortness of breath, dyspnoea was higher in cluster 1 compared with cluster 2, 75.2% *vs*. 60.1%, 79.2% *vs*. 67.8%, 52.8% *vs*. 37.8%, p=0.009, p=0.036 and p=0.014 respectively. The incidence of RP-ILD in cluster 1 (n=81, 64.8%) was significantly higher than that in cluster 2 (n=64, 44.1%), p=0.001. The percentage of fever in cluster 1(n=77, 61.6%) was higher than in cluster 2 (n=66, 46.2%), while Raynaud's syndrome in cluster 1 (n=3, 2.4%) was lower than in cluster 2 (n=13, 9.1%), p=0.011 and p=0.021. The proportions of arthritis/arthralgia, fatigue, sore throat, hoarseness, dysphagia, water aspiration, poor appetite and weight loss were comparable between cluster 1 and cluster 2.

Survival rate comparison in cluster 1 and cluster 2

In Table V, the proportion of glucocorticoids monotherapy was higher in cluster 1 than in cluster 2; other treatment regimens were comparable. The survival rate in cluster 1 and cluster 2 was shown in Figure 7G, the death rate in cluster 1 was higher than in cluster 2, HR=2.588, 95%CI 1.604-4.221, p<0.001, revealing the poor prognosis. Here, we used a supervised random forests algorithm for each cluster to identify key variables that could differentiate individuals and to identify if these key variables remained the same at each cluster.

Comparing classifier strength (mean decrease gini) over the different clusters, we observed that, surprisingly, for lymphocyte subsets, cluster 1 and cluster 2 had different individual-specific signatures (Fig. 7H-I). The proportion of CD3+T cells was the factor with the highest resolving power between individuals in cluster 1, while the proportion of NK cells was the factor with the highest inter individual resolution in cluster 2.

Discussion

Lymphocytopenia and its subsets decline is a distinguishing feature of anti-MDA5+ DM patients. Non-survivors of anti-MDA5+ DM patients suffered remarkably decreased absolute counts of CD3+ T cells, CD3+CD4+ T cells, CD3+CD8+ T cells, CD3−CD19+ B cells and CD16+CD56+ NK cells than those survivors. Cox regression revealed decreased NK cell counts was an independent risk factor for mortality of anti-MDA5+ DM. Cluster analysis demonstrated heterogeneity of lymphocyte distribution and distinctive clinical features, serological parameters and prognosis of anti-MDA5+ DM patients.

Lymphocytopenia was an independent risk factor for RP-ILD and mortality of anti-MDA5+ DM (10, 16, 17). Jin *et al*. revealed anti-MDA5+ DM patients with the severe lymphopenia presented with skin ulceration, higher proportion of RP-ILD and poorer prognosis (5). Ye *et al*. revealed two distinct immune cell signatures with different clinical outcomes in patients with amyopathic DM, patients with enriched activated CD45RA+HLA−DR+CD8+ T cells combined with decreased CD56dim NK cell proportions showed a higher prevalence of RP-ILD and higher mortality (18). Anti-MDA5+ DM patients of the dead group had a higher frequency of CD4+GZMK+GZMB− cells

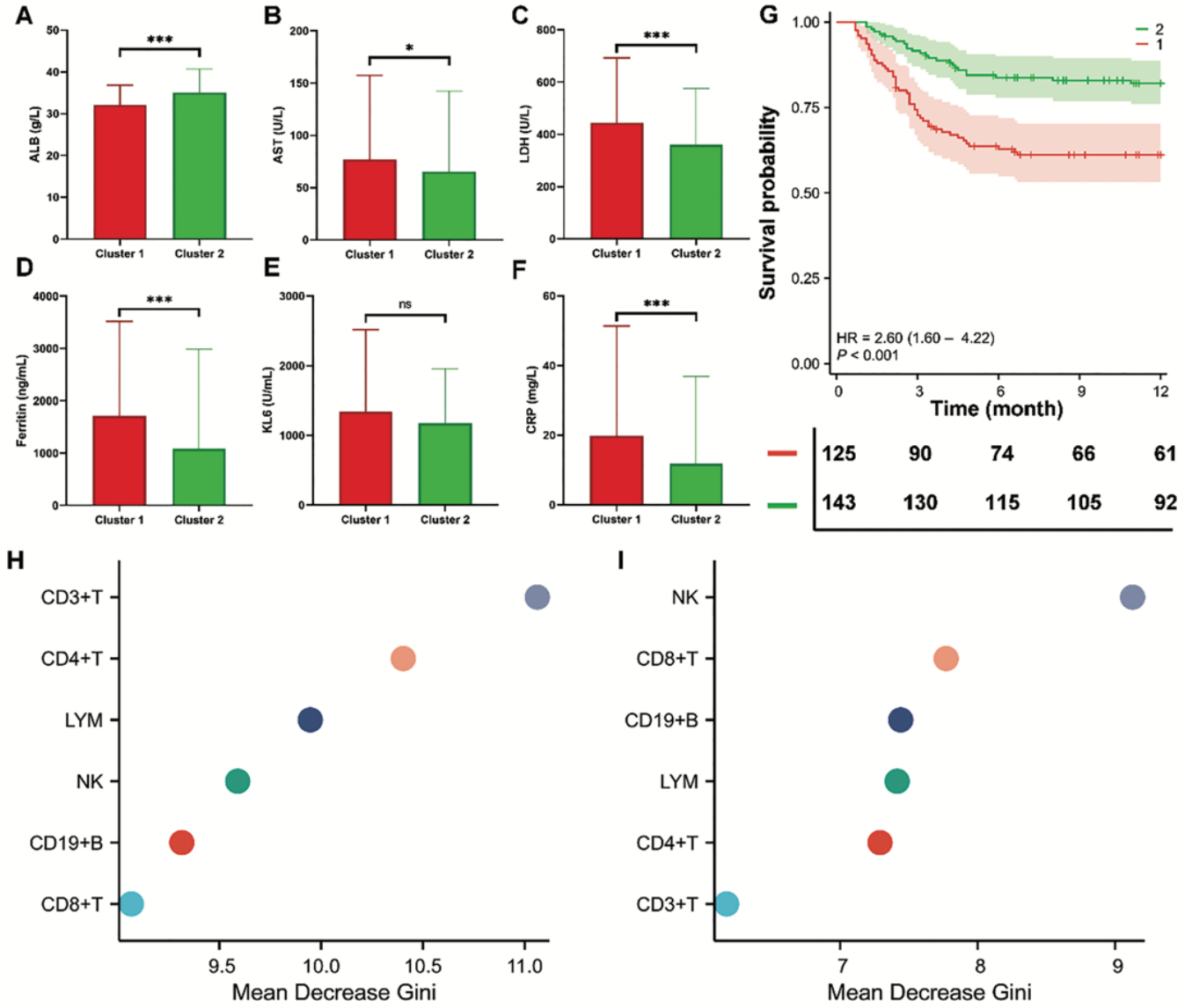

Fig. 7. The random forest importance comparisons of mortality associated factors between the subgroups in anti-MDA5+ DM patients.
(A-F) Histograms showing comparisons of ALB, AST, LDH, ferritin, KL-6, CRP between the two clusters in patients with anti-MDA5+ autoantibodies. **(G)** Kaplan-Meier one-year survival curves by two subgroups of patients with anti-MDA5+ autoantibodies. (**H**-**I**) Variable importance plot of supervised random forest algorithm output in cluster 1 or cluster 2 MDA5+ DM patients. Six lymphocyte subsets in cluster 1 or cluster 2 patients contributing to the model are shown on the y-axis ranked by importance, quantified by the mean decrease gini.

and $CD8^+GZMK^+GZMB^-$ cells (19). Ren *et al*. revealed that peripheral blood lymphocyte count, $CD3^+$ count, $CD3^+CD4^+$ count, $CD3^+CD8^+$ count and CD3-$CD19^+$ B cell count were lower in 59 anti-MDA5+ DM patients than in 194 anti-MDA5- DM patients (15). The frequencies of $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells were comparable in HCs and anti-MDA5+ DM patients in our research. Consistent with previous studies, non-survivors of anti-MDA5+ DM patients suffered decreased total lymphocytes, $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, CD3-$CD19^+$ B cells and NK cells than survivors in our study. Further, our study demonstrated that anti-MDA5+DM patients with either lower count of $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, CD3-$CD19^+$ B cells and $CD16^+CD56^+$ NK cells had a lower survival rate compared with those with higher lymphocyte subsets in a large cohort of Chinese patients. The RIG-I pathway was involved in decreased peripheral T cells in patients with DM (20). Researches focusing on the mechanism of lymphocyte subsets decline needs further investigation.

As a linkage of innate and adaptive immune responses, NK cells participated pivotal roles in the onset of autoimmune diseases (21). Studies have provided evidence that NK cells are involved in SLE, Sjögren's syndrome, systemic sclerosis, and rheumatoid pathogenesis (22). NK cell numbers in IIM patients with lung involvement were reduced compared to non-lung involvement patients (23). Decreased NK cell count was a significant characteristic of DM patients with anti-MDA5+ autoantibody in a cohort of 119 patients (24). In our study, NK cells≤130/μL was an independent risk factor of mortality for patients with anti-MDA5+ DM. In addition, anti-MDA5+ DM patients with

Table V. Clinical features and treatment regimens comparison of anti-MDA5+ DM patients in cluster 1 and cluster 2.

Clinical characteristics	Cluster 1 (n=125)	Cluster 2 (n=143)	*p*-value
Demographic features			
Age at disease onset(years)	54 (47.5-61.5)	52 (45-58)	**0.018**
Female, n (%)	82 (65.6)	92 (64.3)	0.829
Disease duration (months)	1.6 (1.0-3.2)	2.1(1.2-3.2)	0.094
Manifestations of Skin			
Rash, n (%)	116 (92.8)	129 (90.2)	0.450
Skin ulcers, n (%)	26 (20.8)	21 (14.7)	0.189
Heliotrope rash, n (%)	79 (63.2)	96 (67.1)	0.500
Gottron sign, n (%)	71 (56.8)	83 (58.0)	0.837
Mechanic's hands, n (%)	28 (22.4)	32 (22.4)	0.997
Skin roughness, n (%)	39 (31.2)	35 (24.5)	0.219
Hair loss, n (%)	6 (4.8)	12 (8.4)	0.241
Musculoskeletal involvement			
Myalgia, n (%)	39 (31.2)	47 (32.9)	0.771
Muscle weakness, n (%)	47 (37.6)	58 (40.6)	0.658
Respiratory involvement			
Cough, n (%)	106 (84.8)	113 (79.0)	0.222
Short of breath, n (%)	99 (79.2)	97 (67.8)	**0.036**
Thoracic tightness, n (%)	94 (75.2)	86 (60.1)	**0.009**
Dyspnoea, n (%)	66 (52.8)	54 (37.8)	**0.014**
ILD, n (%)	122 (97.6)	134 (93.7)	0.124
RP-ILD	81 (64.8)	64 (44.1)	**0.001**
Other clinical manifestations			
Fever, n (%)	77 (61.6)	66 (46.2)	**0.011**
Fatigue, n (%)	111 (88.8)	120 (83.9)	0.248
Arthralgia/arthritis, n (%)	59 (47.2)	73 (51.0)	0.572
Raynaud phenomenon, n (%)	3 (2.4)	13 (9.1)	**0.021**
Oedema, n (%)	27 (21.6)	36 (25.2)	0.491
Dysphagia, n (%)	9 (7.2)	7 (4.9)	0.427
Choking on drinking water, n (%)	7 (5.6)	2 (1.4)	0.087
Hoarseness of voice, n (%)	6 (4.8)	10 (7.0)	0.450
Pharyngalgia, n (%)	14 (11.2)	11 (7.7)	0.334
Poor appetite, n (%)	61 (48.8)	53 (37.1)	0.053
Weight loss, n (%)	48 (38.4)	59 (41.3)	0.634
Complications			
Mediastinal emphysema, n (%)	15 (12.0)	14 (9.8)	0.561
Macrophage activation syndrome, n (%)	10 (8.0)	4 (2.8)	0.056
Initial treatment strategy			
Glucocorticoids monotherapy, n (%)	19 (15.2)	10 (7.0)	**0.031**
Dual therapy, n (%)	47 (37.6)	62 (43.4)	0.339
Triple therapy, n (%)	57 (45.6)	69 (48.3)	0.664
Intravenous immunoglobulin, n (%)	93 (74.4)	95 (66.4)	0.155
Outcome			
Death within three months, n (%)	34 (27.2)	12 (8.4)	**<0.001**
Death within six months, n (%)	46 (36.8)	23 (16.1)	**<0.001**

Dual therapy represented glucocorticoids (GCs) therapy combined with one type of immunosuppressants, including cyclophosphamide (CTX), cyclosporin (CsA), tacrolimus, mycophenolate mofetil (MMF), Janus kinase inhibitors (JAKs) and biologics. Triple therapy represented GCs therapy combined with two kinds of immunosuppressants.

NK cells≤130/μL had a lower survival rate than those with NK cells >130/μL in our study. Serum ferritin is an important inflammatory disease marker (25), NK cell counts correlated negatively with serum ferritin, indicating that NK cell decline as a poor prognosis of patients with anti-MDA5+ DM. The mechanism of NK cells declines in anti-MDA5+ DM deserves further investigation.

Due to the heterogeneity of patients with anti-MDA5+ DM, studies have been conducted to categorise anti-MDA5+ DM patients into subgroups with different clinical phenotypes. Xu *et al.'s* study classified anti-MDA5+ DM into three clusters by unsupervised hierarchical cluster analysis (1). All-cause mortality rates were higher in cluster 3 than in cluster 2 and 1. Jin *et al.* demonstrated that anti-MDA5+ DM patients with arthritis show the highest lymphocyte counts and best prognosis; the RP-ILD cluster presents the lowest peripheral lymphocyte, high incidence of RP-ILD, and poor prognosis; while patients with the typical DM rash had a moderate peripheral lymphocyte count and an intermediate prognosis (5). The heterogeneity of lymphocyte subset distribution was also varied among anti-MDA5+ DM patients in our study. Cluster analysis based on lymphocyte subsets divided anti-MDA5+ DM patients into two clusters with distinctive clinical symptoms and prognosis. The absolute counts of $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, CD3-$CD19^+$ B cells and NK cells were significantly decreased of anti-MDA5+ DM patients in cluster 1 compared to cluster 2. Notably, RP-ILD rate was higher in cluster 1 than in cluster 2; three-month and six-month survival rate in cluster 1 was dramatically lower than in cluster 2 respectively. The above research indicated the pivotal importance of lymphocyte subsets detection in prognosis prediction and treatment regimen administration of anti-MDA5+ DM patients.

The proportion of lymphocytopenia was higher in cluster 1 than in cluster 2 in our study. Lymphocytopenia is a unique manifestation and predictor of poor prognosis in anti-MDA5+ DM patients (26, 27). In our study, the remarkable heterogeneity of lymphocyte subsets demonstrated the different importance in predicting the prognosis of anti-MDA5+ DM patients. Decreased count of $CD3^+$ T cells, especially $CD3^+CD4^+$ T cells was associated with poorer prognosis for anti-MDA5+ DM patients in cluster 1, while decreased count of NK cells associated with poorer prognosis in cluster 2. The percentage of glucocorticoids monotherapy was higher in cluster 1 than in cluster 2, for anti-MDA5+ DM patients with more severe disease activity died before combined treatment regimens added in our study, indicating that severe lymphocytopenia affects the treatment strategy of anti-MDA5+ DM patients.

Limitations

There were some limitations in our

study. First, this is a retrospective study. Second, external validation from another cohort was not performed. Third, the mechanism concerning lymphopenia was not investigated in this study.

Conclusion

Lymphocytes and their subsets were significantly altered in anti-MDA5+ DM patients. Anti-MDA5+ DM patients with RP-ILD had decreased lymphocyte subsets. Non-survivors of anti-MDA5+ DM patients presented with lower $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD3^-CD19^+$ B cells and $CD16^+CD56^+$ NK cells than those survivors. Anti-MDA5+ DM patients were divided into two groups with distinct symptoms, serological variables and survival rate by cluster analysis based on lymphocyte subsets. The survival rate in cluster 1 was significantly lower than in cluster 2.

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