Expansion of exhausted CD8+ T cells associates with increased pulmonary fungal infection risk in anti-melanoma differentiation associated gene 5 dermatomyositis

Y.-T. Xu[1-3], Z.-Y. Cao[4], S. Lin[1-3], X.-M. Shu[1], X. Lu[1], G.-C. Wang[1-3], Q.-L. Peng[1-3]

[1]Department of Rheumatology, Key Myositis Laboratories, China-Japan Friendship Hospital, Beijing; [2]Graduate School of Peking Union Medical College, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing; [3]Beijing Key Lab for Immune-Mediated Inflammatory Diseases, China-Japan Friendship Hospital, Beijing; [4]Department of Haematology, China-Japan Friendship Hospital, Beijing, China.

Abstract

Objective

Anti-MDA5+ dermatomyositis was associated with poor prognosis due to the high incidence of rapid progressive interstitial lung disease, pulmonary infection. The aim of this study is to investigate the abundance and clinical relevance of exhaustion markers on peripheral CD8 T cells from patients with idiopathic inflammatory myopathy (IIM).

Methods

Twenty-nine healthy controls (HCs) and 71 patients with IIM were enrolled, including 42 with anti-MDA5+ and 18 with anti-MDA5- dermatomyositis (DM) and 11 with anti-synthetase syndrome (ASS). Flow cytometry was applied to detect PD-1, TIM-3 and LAG-3 in CD8 T cells. The clinical associations of the CD8 T cell exhaustion phenotype in patients with anti-MDA5+ DM were analysed.

Results

CD8 T cells from patients with anti-MDA5+ DM showed significantly increased LAG-3, TIM-3 and PD-1 compared to those from patients with anti-MDA5- IIM (18 with anti-MDA5- DM and 11 with ASS) or HCs (adjusted p all <0.05). CD8 T cells with distinct exhaustion levels were all significantly increased in anti-MDA5+ DM patients compared with HCs (p all <0.05). Patients with high level of PD-1+ TIM-3+LAG-3+ CD8+ T cells had a significant higher incidence of pulmonary fungal infections but lower counts of CD4+ and CD8+ T cells. ROC analysis revealed that the frequency of PD-1+TIM-3+LAG-3+CD8+ T cell significantly predicted pulmonary fungal infections (area under the curve: 0.828).

Conclusion

CD8 T cells from patients with anti-MDA5+ DM show significant exhausted phenotype, and increased exhausted CD8 T cells were associated with high risk of pulmonary fungal infection.

Key words

T cell exhaustion, dermatomyositis, fungal infection, anti-melanoma differentiation associated gene 5 antibody

Yue-Tong Xu, MD
Zheng-Yi Cao, PhD
Sang Lin, MD
Xiao-Ming Shu, MD
Xin Lu, MD
Guo-Chun Wang, PhD
Qing-Lin Peng, PhD

Please address correspondence to:
Qing-Lin Peng
Department of Rheumatology,
Key Myositis Laboratories,
China-Japan Friendship Hospital,
Graduate School of Peking
Union Medical College,
Yinghua East Road, Chaoyang District,
Beijing, 100730, China.
E-mail: pqinglin@163.com

ORCID iD: 0000-0002-1271-1729

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Introduction

Dermatomyositis (DM) is a subgroup of idiopathic immune-mediated inflammatory diseases that mainly affect the skin, muscle, and lungs (1). Anti-melanoma differentiation associated gene 5 (MDA5) + DM, as a subtype of DM, is typically characterised by lymphopenia and a high risk of rapid progressive interstitial lung disease (RP-ILD) (2, 3). Patients with Anti-MDA5+ DM tend to be complicated with pulmonary infections during the disease duration, which is a significant factor affecting prognosis (4, 5). Previous studies have suggested that the incidence of pulmonary infection in patients with anti-MDA5+ DM is related to lymphopenia (4, 6), highlighting the need to identify the potential cause of the decrease in T lymphocytes in these patients.

T cell exhaustion is characterised by progressive loss of effector function, decreased proliferation after T cell receptor stimulation, and persistently high levels of multiple inhibitory receptors, including programmed cell death (PD-1), lymphocyte-activation gene 3 (LAG3), T cell immunoglobulin domain, and mucin domain-containing protein 3 (TIM-3) (7, 8). In addition, the intensity and number of immune inhibitory receptors expressed by exhausted T cells positively correlates with the severity of exhaustion (9, 10). Previous studies have shown that a possible relevance between T lymphocyte decrease and T cell exhaustion in coronavirus disease of 2019 (COVID-19) and sepsis (11, 12). Moreover, T cell exhaustion was associated with susceptibility to infection and reduced survival in sepsis (13-15). However, exhaustion of peripheral T cells in idiopathic inflammatory myopathy (IIM) has not yet been assessed.

In this study, we analysed the levels of exhaustion markers expressed on CD8 T cell of patients with IIM using flow cytometric analysis, and further investigate the clinical relevance of the exhausted CD8 T cell phenotype.

Materials and methods

Study design and patients

This cross-sectional study was conducted from February to September 2022, and 71 adult patients with IIM who were admitted to the China-Japan Friendship Hospital were included. All patients with DM fulfilled the 2017 American College of Rheumatology/European League Against Rheumatism classification criteria (16). Connors criteria was used for anti-synthetase syndrome (ASS) (17). Patients with malignancy, viral hepatitis and other autoimmune diseases were excluded from the study. Interstitial lung disease (ILD) was identified based on a combination of respiratory symptoms with pulmonary high-resolution computed tomography (HRCT), and pulmonary function tests (PFTs) were utilised to confirm the ILD diagnosis in patients who underwent PFTs (18). Rapid progressive ILD (RP-ILD) is defined as radiographic deterioration of interstitial lesions within three months of the initial respiratory symptoms, accompanied by severe dyspnoea symptoms (19). All patients with IIM were tested to identify whether myositis specific antibodies including (anti-SRP, anti-Jo-1, anti-PL-12, anti-PL-7, anti-EJ, anti-Mi-2, anti-MDA5, anti-TIF1-γ, anti-NXP2, and anti-SAE) and myositis associated antibodies (including anti-Ku, anti-PM-Scl 100, anti-PM-Scl 75, and anti-Ro-52) were positive by immunoblotting (DL1530-1601-4G, EUROIMMUN, Lubeck, Germany). Peripheral blood samples were collected from all patients using heparin sodium anticoagulant. Clinical manifestations and laboratory data were obtained from electronic medical records. All patient data were anonymised and written informed consent was obtained from all participants. This study was approved by Ethics Review Committee of the China-Japan Friendship Hospital (2016-117).

Definition of infection

Infection complications of the patients with anti-MDA5+ DM were identified by supportive clinical characteristics, radiological imaging examination, anti-infection treatment reaction and positive microorganism detection in the blood, sputum, bronchoalveolar lavage fluid (BALF) culture or histological materials (20).

Flow cytometric analysis

A multicolour immunophenotyping approach was used to identify and analyse the percentage of cells positive for a particular immunophenotypic marker on CD8+ cells. The following anti-human antibodies were used to stain the cells: Peridin-chlorophyll protein (PerCP)-CY5.5-labelled anti-human CD3, phycoerythrin (PE)-CY7-labeled anti-human CD8, APC anti-human CD279 (PD-1), Brilliant Violet 421 anti-human CD223 (LAG-3), and FITC anti-human CD366 (TIM-3), purchased from BioLegend (San Diego, CA, USA). V500-C anti-human CD45, antibody was purchased from BD Biosciences (Franklin Lakes, NJ, USA).

Studies were performed on cells remaining after red blood cell lysis of 200 μL of whole blood. The cells were stained for membrane markers (at room temperature in the dark for 30 min) using antibody cocktails according to the manufacturer's instructions. The samples were processed using a Canto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The results were analysed using the Kaluza software (Beckman Coulter, Calif, USA).

Table I. Clinical and laboratory features of anti-MDA5+ DM patients.

Variables	HC (n=29)	anti-MDA5+ DM (n=42)	anti-MDA5- IIM (n=29)	t/z/X2	*p* value
Age, y, mean (SD)	47.9 (6.2)	50.5 (10.8)	49.93 (12.15)	0.566	0.57
Female, n (%)	17 (58.6)	24 (57.1)	18 (72.4)	1.896	0.388
Disease duration, months, median (IQR)	NA	2 (1-3)	27 (5.5-43)	6.047	<0.001
Myalgia, n (%)	NA	27 (64.3)	14 (48.3)	1.802	0.179
Muscle weakness, n (%)	NA	27 (61.9)	23 (79.3)	2.43	0.119
heliotrope rash, n (%)	NA	27 (64.3)	11 (37.9)	4.79	0.029
Mechanic's hands, n (%)	NA	16 (38.1)	10 (34.5)	0.096	0.756
Gottron's sign, n (%)	NA	35 (83.3)	13 (44.8)	11.614	0.001
V-sign, n (%)	NA	15 (35.7)	7 (24.1)	1.075	0.3
Shawl sign, n (%)	NA	16 (38.1)	11 (37.9)	0	0.989
Dyspnoea n (%)	NA	31 (73.8)	7 (24.1)	17.015	<0.001
RP-ILD n (%)	NA	21 (50)	0 (0)	20.59	<0.001
LDH, IU/L mean (SD)	NA	284.4 (125.4)	249.8 (67.7)	1.354	0.18
CK, IU/L, median (IQR)	NA	39.5 (25.25-73.75)	117 (51.5,249.5)	3.639	<0.001
CRP, mg/dl, median (IQR)	NA	0.38 (0.19-1.09)	0.3 (0.13-1.00)	0.965	0.334
ESR, mm/h, median (IQR)	NA	14 (8.75-20)	11 (7-22.25)	1.131	0.258
Ferritin, ng/ml, median (IQR)	NA	211.7 (45.9-776)	121.4 (54.35,215.38)	1.363	0.173
CD4+T cell, cells/uL, median (IQR)	NA	510 (367-879)	860.5 (583.25-1138)	2.493	0.013
CD8+T cell, cells/uL, median (IQR)	NA	303 (168.5-561)	350(238-513)	0.606	0.505
treatment naïve n (%)	NA	12 (28.6)	8 (27.6)	0.008	0.928
Corticosteroids steroids n (%)	NA	30 (100)	15 (100)	/	/
immunosuppressants n (%)	NA	19 (63.3)	21 (100)	9.817	0.002
biological agents n (%)	NA	7 (23.3)	0 (0)	5.68	0.017
IVIG n (%)	NA	2 (6.7)	3 (14.3)		0.637

IQR: interquartile range; LDH: lactate dehydrogenase; CK: creatine kinase; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; IVIG: intravenous immunoglobulin; DM: dermatomyositis; RP-ILD: rapidly progressive interstitial lung disease; IIM: idiopathic inflammatory myopathy.

Statistical analysis

Categorical variables are expressed as counts and percentages, and continuous variables as mean ± standard deviation or median (interquartile range [IQR]). For comparisons between two groups, the Mann-Whitney U-test was used for analysis of abnormally distributed samples, and unpaired t-test was used for analysis of normally distributed samples. For comparisons between multiple groups, the Kruskal-Wallis H test was used for abnormally distributed samples, and One-Way ANOVA was used for normally distributed samples. The false discovery rate (FDR) adjustment method was used to address the issue of multiple group comparisons. For classified variables, data were compared using the chi-squared or Fisher's exact tests. The Spearman's correlation test was used for association analysis. The sensitivity and specificity represented by receiver operator characteristic curves (ROCs) were analysed, and the discriminability of indicators was identified based on the area under the curve (AUC). SPSS (v. 26.0, IBM, Armonk, N.Y., USA) was used for statistical analysis, and Prism (v. 8.0, GraphPad, San Diego, CA, USA) was used for plotting figure. A *p*-value of <0.05 denoted statistical significance.

Results

Clinical characteristics of patients

The clinical characteristics and laboratory parameters of patients with IIM are summarised in Table I. In this study, 29 healthy controls (HCs), 42 patients with anti-MDA5+ DM, 29 patients with anti-MDA5- IIM were recruited. Among 71 patients with IIM, 20 patients were treatment-naive and 51 were receiving treatment at the time of blood sampling. The patients' age and sex among the groups were not significantly different ($p>0.05$, Table I). Among 42 patients with anti-MDA5+ DM, 12 patients were treatment-naive and 30 were receiving treatment at the time of blood sampling. Among the patients who were receiving treatment, all patients received corticosteroids therapy, 19 patients received immunosuppressant therapy (9 receiving Ciclosporin A, 7 receiving Tacrolimus, 2 receiving Mycophenolate mofetil, 1 receiving methotrexate), and 7 patients received biological agent therapy, 2 patients received intravenous immunoglobulin therapy.

CD8 T cells from patients with anti-MDA5+ DM display elevated level of exhaustion markers

We quantified the frequency of CD8 T cells expressing PD-1, TIM-3, and LAG-3 in different groups of patients and HCs using flow cytometric analysis. The gating strategy is illustrated in Figure 1. Representative fluorescence-activated cell sorting plots are shown in Figure 2A. We found that CD8 T cells from patients with anti-MDA5+ DM showed significantly increased levels of LAG-3, TIM-3 and PD-1 compared to those from patients with anti-

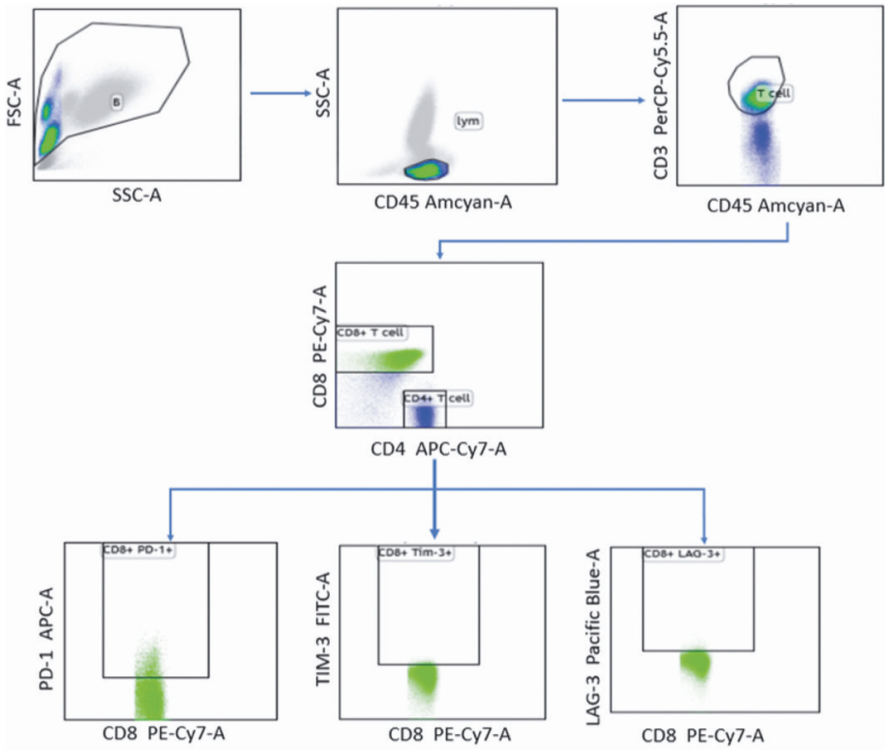

Fig. 1. Gating strategy for assessing the cell surface expression of PD-1, LAG-3 and TIM-3 on CD8+ T cells.

MDA5- IIM or HCs (adjusted $p<0.05$) (Fig. 2B-2D).

In addition, to address the potential impact of immunosuppressive therapy on the expression of these exhaustion markers in patients with anti-MDA5+ DM, we compared the levels of PD-1, LAG-3, and TIM-3 in treatment-naive patients and those who were receiving immunosuppressive therapy. We found that, compared with patients who were receiving treatment, treatment-naive patients showed significantly increased levels of LAG-3 and PD-1 on CD8 T cells (all $p<0.05$) (Fig. 2E and 2F), and the median levels of TIM-3 in CD8 T cells in treatment-naive patients also tended to be higher than in patients who were receiving therapy, although the difference did not reach statistical significance (Fig. 2G).

Patients with anti-MDA5+ DM exhibit increased CD8 T cells consistent with distinct exhaustion levels

To further assess different CD8 T cell phenotypes consistent with distinct exhaustion levels in anti-MDA5+ DM patients, we compared the frequencies of CD8 T cells consistent with a low exhaustion phenotype (1+ marker: PD-1+TIM-3-LAG-3-, PD-1-TIM-3+LAG-3-, PD-1-TIM-3-LAG-3+), an intermediate exhaustion phenotype (2+markers: PD-1+TIM-3+LAG-3-, PD-1+TIM-3-LAG-3+, PD-1-TIM-3+LAG-3+) or a severe exhaustion phenotype (3+markers: PD-1+ TIM-3+LAG-3+). The data showed that CD8 T cells consistent with the low exhaustion, intermediate exhaustion, and severe exhaustion phenotypes were all significantly increased in patients with anti-MDA5+ DM compared with HC group (all $p<0.05$) (Fig. 3A-C).

Patients with anti-MDA5+ DM and high levels of exhausted PD-1+TIM-3+LAG-3+ CD8 T cells show significant lymphopenia and a higher incidence of pulmonary fungal infections

We defined the cut-off value for the level of exhausted PD-1+TIM-3+LAG-3+ CD8 T cells as the 95% confidence interval (non-normal distribution) of healthy control samples. Consequently, patients with anti-MDA5+ DM were subdivided into two groups: one with a high level of exhausted PD-1+TIM-3+LAG-3+ CD8 T cells, and the other with normal levels. We found that, compared with those with normal levels of exhausted PD-1+TIM-3+LAG-3+ CD8 T cells, patients with high levels of exhausted PD-1+TIM-3+LAG-3+ CD8 T cells had a significantly higher incidence of pulmonary fungal infections, a higher level of lactate dehydrogenase, and serum ferritin, and lower counts of CD4 T cells and CD8 T cells (Table II). Interestingly, we can see that the incidence of RP-ILD in high level of PD-1+TIM-3+LAG-3+ CD8+ T cell group was higher than normal level group (56.7% *vs*. 33.3%), although the difference is not significant (Table II). In addition, by comparing the level of PD-1+TIM-3+LAG-3+ CD8+ T cell between the RP-ILD and non-RP-ILD groups, we found that the level of PD-1+TIM-3+LAG-3+ CD8+ T cell was higher in RP-ILD group [0.16 (0.08,0.76) *vs*. 0.1 (0.02,0.14), $p<0.05$]. Furthermore, we found that the increase in CD8 T cells, consistent with distinct exhaustion levels, was inversely correlated with counts of CD8 T cells in patients with anti-MDA5+ DM (Supplementary Fig. S1).

High levels of exhausted PD-1+ TIM-3+LAG-3+ CD8 T cells is an effective marker for indicating pulmonary fungal infections

Pulmonary fungal infections were identified in 13 of the 42 patients with anti-MDA5+ DM. Although three patients who did not obtain pathogenic evidence of fungal infection from BALF or sputum samples, we considered that these three patients also had pulmonary fungal infection based on their positive galactomannan (GM) test results, clinical symptoms, and antifungal treatment response. Examination results of fungal infections are shown in Supplementary Table S1, and more than half of patients suffered from Pneumocystis jirovecii infection (8/13, 61.5%). In anti-MDA5+ DM patients with fungal infection, 7 patients were treatment-naive and 6 were receiving corticosteroids and/or immunosuppressant treatment. Moreover, among the anti-MDA5+ DM patients with fungal

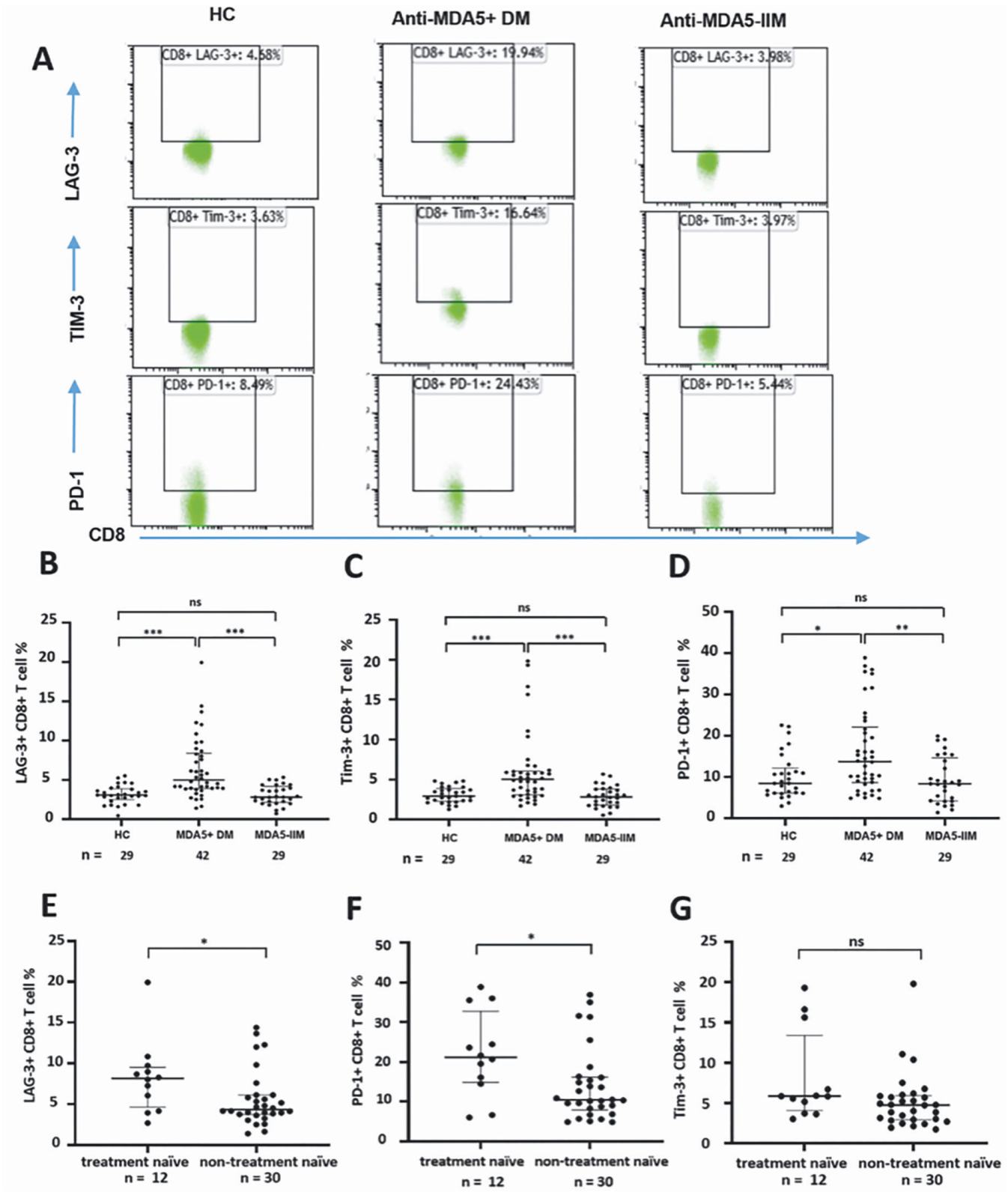

Fig. 2. CD8+ T cells of patients with anti-MDA5+ DM bear high levels of exhaustion markers.
A. Expression of LAG-3, TIM-3, and PD-1 in CD8+ T cells of HC and patients. **B-D**. Frequency of LAG-3 (**B**), TIM-3 (**C**), and PD-1 (**D**) levels in CD8+ T cells of healthy controls (HCs) and patients. **E-G**. Frequency of LAG-3 (**E**), PD-1 (**F**), and TIM-3 (**G**) levels of CD8+ T cells in treatment naive and non-treatment naive patients with anti-MDA5+ DM.
DM: dermatomyositis; IIM: idiopathic inflammatory myopathy.
For comparisons between two groups, the Mann-Whitney U-test was used.
*p<0.05. For comparisons between multiple groups, the Kruskal-Wallis H test was used and adjusted by false discovery rate (FDR). FDR-adjusted p<0.05 (*) p<0.01 (**) and p<0.005 (***).

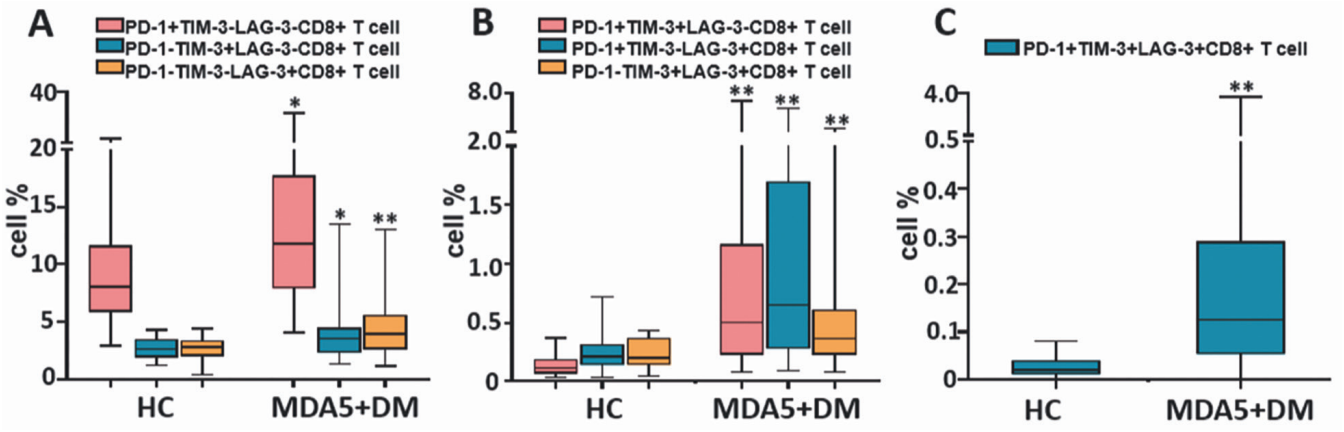

Fig. 3. Patients with anti-MDA5+ DM exhibit increased CD8 T cells consistent with distinct exhaustion levels.
Frequency of CD8+ T cells with a phenotype consistent with low exhaustion (1+ marker) (**A**), intermediate exhaustion (2+ markers) (**B**), or severe exhaustion (3+ markers) (**C**) in patients with anti-MDA5+ DM and HCs.
*p<0.05 compared to the HCs group; **p<0.01 compared to the HCs group. Comparisons were performed using the Mann-Whitney U test.
DM: dermatomyositis.

infection, 6 were receiving TMP/SMZ prophylaxis and 7 were not receiving TMP/SMZ prophylaxis.

We evaluated the discriminative power of the frequency of CD8 T cells consistent with distinct exhaustion levels in uncovering the presence of pulmonary fungal infections in patients with anti-MDA5+ DM. ROC analyses were performed, and the results showed that CD8 T cells with a phenotype consistent with low (AUC for PD-1-TIM-3+LAG-3-CD8 T cells, 0.699), intermediate (AUC for PD-1+TIM-3-LAG-3+ and PD-1-TIM-3+LAG-3+ CD8 T cells, 0.732 and 0.704, respectively), and severe exhaustion (AUC for PD-1+TIM-3+LAG-3+ CD8 T cells, 0.828) could discriminate patients with pulmonary fungal infection from those without (all p<0.05), but CD8 T cells consistent with other phenotype could not (all p>0.05). Not surprisingly, PD-1+TIM-3+LAG-3+ CD8 T cells were most effective in indicating fungal infections in patients with anti-MDA5+ DM, owing to its greatest AUC value (Fig. 4).

Table II. Characteristics of anti-MDA5+ DM subgroups with normal and high level of exhausted PD-1+TIM-3+LAG-3+ CD8+ T cells.

Variables	High level group (n=30)	normal level group (n=12)	t/z/X^2	p value
Age at onset, y, mean (SD)	50.3 (12.0)	46.5 (8.2)	0.993	0.327
Disease duration, months, median (IQR)	3 (1.4-3.3)	2 (1-2.75)	1.557	0.12
Female, n (%)	16 (53.3)	5 (66.7)	0.622	0.43
Myalgia, n (%)	17 (56.7)	10 (83.3)	2.655	0.103
Muscle weakness, n (%)	17 (56.7)	9 (75)	0.568	0.451
heliotrope rash, n (%)	20 (66.7)	7 (58.3)	0.259	0.611
Mechanic's hands, n (%)	10 (33.3)	6 (50)	1.01	0.315
Gottron's sign, n (%)	24 (80)	11 (91.7)	0.21	0.647
V-sign, n (%)	10 (33.3)	5 (41.7)	0.259	0.611
Shawl sign, n (%)	13 (43.3)	3 (25)	0.568	0.451
Dyspnoea n (%)	24 (80)	7 (58.3)	0.082	0.149
Fever	11 (36.7)	5 (41.7)	0.091	0.763
Pulmonary infection	18 (60)	6 (50)	0.35	0.554
Virus infection	12 (40)	6 (50)	0.35	0.554
Bacterial infection	9 (30)	3 (25)	0	1
Fungal infection	13 (43.3)	0 (0)		0.008
RP-ILD onset, n (%)	17 (56.7)	4 (33.3)	1.867	0.17
LDH, IU/L mean (SD)	311 (137)	217 (48)	3.039	0.026
CK, IU/L, median (IQR)	38.5 (26.8-73.8)	51.5 (22-74)	0.125	0.9
CRP, mg/dl, median (IQR)	0.44 (0.2-1.26)	0.28 (0.14-0.8)	1.35	0.177
ESR, mm/h, median (IQR)	14 (8.75-25.25)	15 (7.75-41.25)	0.237	0.813
Ferritin, ng/ml, median (IQR)	458.1 (78.1-1266.5)	47.35 (25.8-193.2)	2.123	0.003
CD4 T cell, cells/uL, median (IQR)	480 (292-611)	1041 (458-1498)	2.603	0.009
CD8 T cell, cells/uL, median (IQR)	277 (114-415)	697 (205-947)	2.422	0.015

The cut-off value calculated as >95% confidence interval (non-normal distribution) of 29 healthy control (HC) samples. A high level indicates the frequency of the CD8+ T cell phenotype consistent with severe exhaustion greater than the cut-off value (0.07%), and a normal level indicates a frequency not higher than the cut-off value.
IQR: interquartile range; LDH: lactate dehydrogenase; CK: creatine kinase; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; RP-ILD: rapidly progressive interstitial lung disease.

Discussion

This study is the first to report significant CD8 T cell exhaustion in patients with anti-MDA5+ DM. We demonstrated a highly increased expression of the exhaustion markers, PD-1, TIM-3, and LAG-3 on peripheral CD8 T cells in patients with anti-MDA5+ DM. Further analyses showed significantly elevated frequencies of CD8 T cells consistent with low exhaustion, intermediate exhaustion, and high exhaustion phenotypes in patients with anti-MDA5+ DM. In addition, high levels of severely exhausted CD8 T cells were associated with an increased incidence of pulmonary fungal infections, and decreased counts of peripheral T cells.

T cell exhaustion is characterised by the increased levels of inhibitory receptors and loss of effector functions. CD8 T cell exhaustion induced by cancer or chronic infection has been well studied, and blocking of inhibitory receptors has

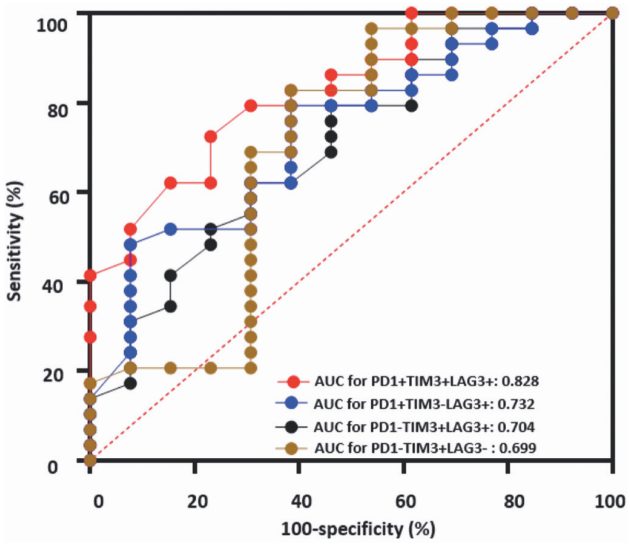

Fig. 4. Receiver operating characteristic (ROC) curve indicating pulmonary fungal infection of patients with anti-MDA5+ DM.
ROC analyses showed that the discriminative power of frequency of PD-1-TIM-3+LAG-3- CD8+ T cells, PD-1+TIM-3-LAG-3+ CD8+ T cells, PD-1-TIM-3+LAG-3+ CD8+ T cells, and PD-1+TIM-3+LAG-3+ CD8+ T cells regarding fungal infection in patients with anti-MDA5+ DM was significant (p<0.05).
DM: dermatomyositis; AUC: area under the curve.

been used as a therapeutic strategy (9). In autoimmune diseases, upregulation of PD-L1 and PD-L2 has been confirmed in type I diabetes, autoimmune encephalomyelitis, and autoimmune liver diseases (21, 22), and T cell exhaustion has been found to be related to disease activity and favourable prognosis of RA, AAV, SLE and pSS (23-25). Interestingly, our study showed that the expression levels of PD-1, TIM-3 and LAG-3 on CD8 T cells were significantly increased in patients with anti-MDA5+ DM compared to those in HCs and other myositis subtypes, providing significant evidence of T cell exhaustion regarding this life-threatening type of myositis. Lymphopenia is a hallmark of patients with anti-MDA5+ DM and associates with RP-ILD, pulmonary infection and poor prognosis of patients with anti-MDA5+ DM (4, 27). However, the mechanism underlying of lymphopenia remains unclear. Our previous study showed that highly expressed RIG-I may contribute to T cell lymphopenia by inducing apoptosis and inhibiting proliferation of T cells (28). Exhausted T cells show decreased proliferative capacity (29), and terminally exhausted T cells undergo subsequent physical deletion (30), suggesting an association between T cell exhaustion and lymphopenia. In this study, we found that the increased levels of exhaustion markers were associated with lower counts of CD8 T cells. Based on the many similarities in pulmonary clinical imaging features and biomarkers between COVID-19 and the anti-MDA5+ DM, the viral infection may be a possible trigger for anti-MDA5+ DM (31). Interestingly, the increase in PD-1 levels on CD4 T cells has been reported to correlate with a decrease in the CD4 T cell count in COVID-19 (11). Moreover, increased abundance of inhibitory receptors (PD-1, and TIM-3) was reported to be related to T cell apoptosis in sepsis (12, 32). Thus, our findings of increased levels of exhaustion markers imply a role for T cell exhaustion in lymphocytopenia observed in patients with anti-MDA5+ DM.

The high incidence of pulmonary infections in patients with anti-MDA5+ DM has attracted increasing clinical attention. Pulmonary fungal infection with Pneumocystis jirovecii has a disturbing high incidence and caused significant mortality in patients with anti-MDA5+ DM (4, 6). Current treatment strategies for Pneumocystis jirovecii pneumonia did not seem to improve the prognosis of patients bearing this particular subtype (6). Glucocorticoids and immunosuppressive treatment was thought to be associated with an increased risk of fungal infections (33-35), in a recent study about Pneumocystis jirovecii pneumonia in anti-MDA5+ DM, the frequency of immunosuppressant used was lower in PJP+ group (36). Interestingly, we found that the levels of exhaustion markers on CD8 T cells from treatment naive anti-MDA5+ DM patients were even higher compared to patients who were receiving immunosuppressive treatment, suggesting that the association between T cell exhaustion and fungal infection in anti-MDA5+ DM patients is independent of immunosuppressive treatment. The lack of understanding of the cause of vulnerability to fungal infections in patients with anti-MDA5+DM results in an unmet medical need for new therapeutic targets to treat fungal infection in these patients. Our study revealed that the CD8 T cells with distinct exhaustion levels in patients with anti-MDA5+ DM bearing a fungal infection were significantly increased compared with patients who did not suffer from fungal infections. Further analysis showed that the frequency of CD8 T cells with severe exhaustion (PD-1+TIM-3+LAG-3+) was an effective marker for indicating fungal infection. Our findings suggest that examination of exhaustion markers on CD8 T cells might be helpful in identifying patients with an increased risk of fungal infection in anti-MDA5+ DM. In a previous study, increased expression levels of the exhausted T cells from patients with COVID-19 were found to be associated with susceptibility to deadly secondary fungal infections (37). It has been reported that patients with cancer receiving checkpoint inhibitor treatment have a higher incidence of dermatologic fungal infections than patients not receiving checkpoint inhibitor treatment (38). In addition, the poor survival rate of fungal sepsis subjects is associated with the levels of PD-1 and cytotoxic T-lymphocyte antigen 4 (CTLA-4) (39, 40). Taken together, our study suggests a role for T cell exhaustion in pulmonary fungal infection in patients with anti-MDA5+ DM and provides therapeutic clues for this life-threatening complication.

Nevertheless, this study had several limitations. First, in this study, only the exhaustion markers of CD8 T cell expression were analysed, and the ability of cytokine secretion and cell proliferation of CD8 T cells needs to be further analysed, which could provide additional evidence of functional exhaustion. Second, the small sample size especially very limited number of treatment-naive patients and patient data from a single-centre may introduce possible biases;

therefore, a multicentre study with larger number of treatment-naive patients is necessary to verify the results. Third, the change in the frequency of exhausted CD8 T cells during follow-up and its association with patient prognosis need to be further analysed. In addition, some patients were identified as having fungal infection using only the GM test, and not all the patients' samples were detected for specific fungi strains, which may overestimate the prevalence of fungal infection.

In conclusion, the present results demonstrate that CD8 T cells from patients with anti-MDA5+ DM exhibit a significant exhaustion phenotype. Our data provide novel pathogenic clues and therapeutic implications for fungal infections in patients with anti-MDA5+ DM.

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