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ORIGINAL RESEARCH

Shared and Distinctive Inflammation-Related Protein Profiling in Idiopathic Inflammatory Myopathy with/without Anti-MDA5 Autoantibodies

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Purpose: Due to the heterogeneous nature of the diseases, treatment efficacy and prognosis vary for idiopathic inflammatory myopathy (IIM) patients with different myositis specific autoantibodies (MSAs). This study aimed to investigate the inflammation-related protein profiling of IIM patients with different MSAs. In addition, the shared and distinctive inflammation-related protein profiling in IIM with/without anti-MDA5 autoantibodies.

Methods: Seventy-seven patients with IIM of different MSAs and 53 gender/age matched healthy controls (HCs) were enrolled in this study. Ninety-two inflammation-related proteins were detected by Olink proteomics. We identified differentially expressed proteins (DEPs), and performed gene set enrichment analysis and KEGG pathway analysis. In addition, correlation between DEPs and serological parameters were performed. The least absolute shrinkage and selection operator (Lasso) regression algorithm of machine learning was used to screen biomarkers related to anti-MDA5+ DM.

Results: Compared with HCs, 36 inflammation-related proteins were identified as DEPs. The top 10 DEPs were CXCL10, CXCL11, CXCL9, CXCL8, S100A12, IL-6, CCL2, CCL8, IL-10 and CCL3. The inflammation-related proteins and cytokine-cytokine receptor interaction pathway were more strikingly expressed in patients with anti-MDA5+ DM patients than in anti-MDA5- IIM patients. In addition, multiple DEPs correlated with serum ferritin, KL-6, muscle enzymes. For the first time, we established that a multi-factor panel comprising CX3CL1, IL-17C, IL-18R1, CCL20, and TNF (AUC = 0.824) serves as a highly efficient diagnostic biomarker for anti-MDA5+ DM.

Conclusion: Plasma profiling revealed that inflammation and inflammatory pathways were extremely elevated in patients with IIM, especially in patients with anti-MDA5 autoantibodies. The shared and distinctive inflammation-related protein signature was demonstrated in patients with/without anti-MDA5 autoantibodies. The expression of CX3CL1 was significantly higher in anti-MDA5+ DM than in patients without anti-MDA5 autoantibodies. In addition, CX3CL1 correlated with ESR, serum ferritin, CK enzymes and disease activity, indicating that CX3CL1 participated in inflammation status of anti-MDA5+ DM.

Keywords: idiopathic inflammatory myopathy, anti-MDA5+ dermatomyositis, myositis specific autoantibodies, inflammation-related biomarkers, CX3CL1

Introduction

Idiopathic inflammatory myopathies (IIM) are rare systemic inflammatory disorders that collectively comprise a heterogeneous group of diseases, including dermatomyositis (DM), anti-synthetase syndrome (ASS), polymyositis (PM), inclusion body myositis (IBM), immune-mediated necrotizing myopathy (IMNM).[1] IIM is characterized by muscle weakness, fatigue, autoimmune muscle inflammation and are frequently accompanied with skin, lung and joints involvement.[2] Myositis-specific autoantibodies (MSAs) are associated with distinct clinical phenotypes which are

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critically useful in the diagnosis of IIM.[3] The distinct organ manifestation, treatment responses and prognosis varied among patients with different MSAs,[4] implicating the different pathophysiological mechanism in each subtype.

The development of the pathogenesis in IIM has achieved great improvement. Adaptive/innate immune system and non-immune mechanism are involved in IIM.[4] Type I and type II interferons participated in IIM, especially in patients with anti-melanoma differentiation-associated gene 5 (MDA5) autoantibodies.[5,6] Signs of inflammation were found in DM/PM,[7] which may induce muscle weakness and tissue damage.[7,8] Inflammatory mediators, such as CXCL10, IL-18, and the IL23/Th17 axis had been established as mediators in IIM.[9] Besides, B cells, CD4+ T cells, dendritic cells and macrophages infiltrated in tissue biopsies of patients with IIM.[2] The role of inflammation-related protein profiling of patients with IIM of different MSAs needs to be elucidated to further understand the nature of this disorder.

Due to the heterogeneous nature of the diseases, treatment efficacy and prognosis vary for IIM patients with different MSAs.[1] For example, the clinical presentation of anti-MDA5+ DM differs substantially from the other forms of DM, according to the predominance of pulmonary, skin-articular or vascular symptoms.[10] Interstitial lung disease (ILD) is a common complication of anti-MDA5+ DM and may develop into rapidly progressive interstitial lung disease (RP-ILD), which can be treatment-resistant and cause progressive respiratory failure, leading to death.[11]

MCP-1 was elevated in PM/DM patients with ILD compared with those without.[12] Galectin-9 and CXCL10 were validated as sensitive and reliable biomarkers for disease activity in juvenile DM.[13] Serum CX3CL1/fractalkine is elevated in patients with polymyositis and dermatomyositis, which is correlated with disease activity.[14] IL-18, sCD206, IFN-α, interferon-inducible protein-10 (IP-10), IL-6, IL-8, IL-10, IL-15 and TNF-α were involved in the pathogenesis of RP-ILD in anti-MDA5+ DM.[15] Proteomic pathways were demonstrated in adult and juvenile DM, a subset of interferon stimulated genes elevated.[16] There were no studies focused on protein profiling of inflammatory related proteins in IIM with different MSAs. In this study, we aim to systemically demonstrate the inflammation-related protein profiling in IIM patients with different MSAs. These investigations may give novel insights into the underlying immunological processes that drive inflammation in patients with IIM. Due to the differed nature of pathogenesis and treatment efficacy of anti-MDA5+ DM and patients with IIM of other MSAs, the inflammation-related protein profiling was also evaluated to investigate the shared and distinctive inflammation profiling between IIM patients with/without anti-MDA5+ autoantibodies.

Materials and Methods

Patient Enrollment

Seventy-seven treatment naïve patients or in active disease condition of patients (discontinuation of glucocorticoids and immunosuppressants for more than two months) with IIM and 53 gender/age matched healthy controls (HCs) from January 2021 to December 2023 were enrolled of the First Affiliated Hospital of Zhengzhou University were enrolled in this study. All patients enrolled were admitted to the ward. IIM was diagnosed according to the criteria of Bohan and Peter or based on the criteria of Sontheimer RD.[17–19] DM was diagnosed according to the 2018 ENMC criteria.[20] Anti-synthetase syndrome (ASS) was defined by the presence of anti-synthetase autoantibodies in DM or PM.[21,22] Patients combined with other autoimmune diseases, complicated with cancer, age of disease onset <18 years, with active HBV or HCV infections, without detection of MSAs or had been treated with glucocorticoids or immunosuppressants previously but with discontinuation of the above drugs, patients with rare MSAs were excluded. All HCs were detected MSAs. No positive MSAs were detected in all HCs. In addition, HCs with titers of ANAs were not enrolled as well. This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (2021-KY-1101). All patients and HCs were written enrolled in informed consent.

Muscle involvement was based on muscle weakness combined with evidences of muscle involvement by magnetic resonance imaging (MRI) of muscles or electromyography or muscle biopsies. Muscles in the upper arms and/or thighs were evaluated by MRI using a 3.0 T unit. MRI displayed subcutaneous adipose tissue oedema, fascial oedema, patchy or diffuse distribution of muscle oedema, muscle atrophy and fatty replacement[23] was recognized as muscle involvement. In our cohort, 44 patients did MRI test. In addition, 17 patients with muscle weakness with abnormal electromyography were also recognized as muscle involvement. Muscle biopsies indicated muscle involvement was seen in nine patients.

The assessment of ILD was based on respiratory symptoms, physical examinations, high-resolution computed tomography (HRCT) and pulmonary function tests. Disease activity assessment was evaluated with the Myositis Intention to Treat Activities Index (MITAX).[24,25] Patients' demographic features, onset symptoms and laboratory parameters and treatment regimens were shown in Table 1.

Table 1 Demographic Features, Clinical Characteristics and Laboratory Parameters of Patients with IIM

Characteristics at Baseline	IIM	MDA5+	TIF-1γ+	Jo-1+	Neg
	(n=77)	(n=34)	(n=15)	(n=10)	(n=10)
Demographic Features					
Age (years)	52±15	54±9	57±19	47±12	48±16
Male/Female ratio	27/50	16/18	5/10	1/9	2/8
Disease duration (month), M (Q_1-Q_3)	3 (2–12)	3(2–6)	3(2–8)	9(2–54)	5(3–36)
Myositis specific antibodies (n%)					
Anti-MDA5	34 (44.2)	161.98±53.70			
Anti-TIF1-γ	15 (19.5)		82.48±43.20		
Anti-Jo-1	10 (13.0)				
Anti-SRP	2 (2.6)				
Negative	10 (13.0)				
Anti-EJ	1(1.3)				
Anti-RO52*	5 (6.5)				
Lung involvement (n%)					
ILD (%)	50 (64.9)	31 (91.2)	3 (20)	8 (80)	4 (40)
Muscle involvement (n%)					
MRI	44 (57.1)	11 (32.4)	12 (80)	7 (70)	10 (100)
Electromyogram	17 (22.1)	3 (8.8)	3 (20)	4 (40)	5 (50)
Muscle biopsy	9 (11.7)	1 (2.9)	1 (6.7)	0 (0)	4 (40)
Symptoms (n%)					
Skin rashes	51 (66.2)	24(70.6)	13(86.7)	3(30)	7(70)
Heliotrope rash	40(51.9)	21(61.8)	12(80)	1(10)	4(40)
Gottron's papules	24 (31.2)	12(35.5)	6(40)	2(20)	3(30)
V-sign rash	26(33.8)	12(35.3)	9(60)	0(0)	3(30)
Shawl-sign rash	23(29.9)	8(23.5)	10(66.7)	0(0)	4(40)
Muscle weakness	38 (49.4)	13(38.2)	10(66.7)	3(30)	7(70)
Fatigue	38 (49.4)	18(52.9)	9(60)	1(10)	6(60)
Chest tightness	35 (45.5)	20(58.8)	2(13.3)	7(70)	2(20)
Cough	24 (31.2)	14(41.2)	3(20)	3(30)	1(10)
Arthralgia	23(29.9)	15(44.1)	1(6.7)	0(0)	2(20)

(*Continued*)

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Table 1 (Continued).

Characteristics at Baseline	IIM	MDA5+	TIF-1γ+	Jo-1+	Neg
	(n=77)	(n=34)	(n=15)	(n=10)	(n=10)
Weight loss	22 (28.6)	10(29.4)	5(33.3)	2(20)	4(40)
Myalgia	21 (27.3)	7(20.6)	6(40)	2(20)	3(30)
Fever	21 (27.3)	11(32.4)	2(13.3)	1(10)	3(30)
Shortness of breath	20 (26.0)	12(35.3)	1(6.7)	4(40)	2(20)
Edema	15(19.5)	4(14.7)	2(13.3)	3(30)	5(50)
Dyspnea	12 (15.6)	7(20.6)	2(13.3)	1(10)	1(10)
Mechanics hand	11(14.3)	9(26.5)	1(6.7)	1(10)	0(0)
Hyperpigmentation	9(11.7)	4(11.8)	3(20)	0(0)	0(0)
Rough skin	6(7.8)	5(14.7)	1(6.7)	0(0)	0(0)
Pharyngalgia	5(6.5)	4(11.8)	1(6.7)	0(0)	0(0)
Dysphagia	4(5.2)	2(5.9)	2(13.3)	0(0)	0(0)
Chocking in drinking water	3(3.9)	1(2.9)	0(0)	0(0)	2(20)
Difficulty in raising head	2(2.6)	0(0.0)	0(0)	0(0)	1(10)
Hoarseness of voice	2(2.6)	1(2.9)	1(6.7)	0(0)	0(0)
Laboratory parameters					
HGB (g/L)	123±16	122±15	125±15	126±15	121±19
WBC (10^9/L)	6.6±3.2	6.2±2.4	5.7±2.1	8.6±4.7	7.0±3.3
PLT (10^9/L)	230±79	230±73	249±98	263±57	226±75
Lymphocytes (10^9/L)	1.19±0.67	1.03±0.56	1.03±0.67	1.63±0.58	1.59±0.88
Neutrophil (10^9/L)	4.75±2.72	4.70±2.24	4.00±1.90	6.04±4.2	4.48±2.37
Neutrophil (%)	70.8±12.1	74.1±10.8	69.3±14.7	65.9±12.1	63.0±8.6
ESR (mm/h), M (Q_1-Q_3)	18 (12–35)	26(13–51)	15(10–17)	34(12–50)	13(8–16)
CRP (mg/L), M (Q_1-Q_3)	2.5(1.4–13.9)	2.2(1.5–19)	3.0(1.5–16.8)	4.1(1.8–13.0)	1.0(1.0–7.9)
ALT (U/L)	57±82	55±63	61±73	81±168	30±37
ALB (g/L)	35.8±4.95	35.0±4.4	37.2±5.4	36.5±4.2	34.7±7.0
KL-6(U/mL), M (Q_1-Q_3)	690(349–1394)	1067(558–1535)	228(195–418)	1176(555–1677)	176(130–349)
Ferritin (ng/mL), M (Q_1-Q_3)	393(162–958)	588(268–2049)	197(106–381)	346(74–778)	261(58–460)
Muscle enzymes (U/L)					
AST, M (Q1-Q3)	31(19–59)	34(24–54)	31(17–149)	22(19–59)	20(16–104)
CK, M (Q1-Q3)	65(30–173)	36(25–86)	197(59–3851)	129(34–506)	115(44–1376)
CK-MB, M (Q1-Q3)	21(15–29)	21(15–26)	19(15–196)	21(16–44)	16(9–47)
LDH, M (Q1-Q3)	294(230–418)	322(231–383)	305(240–574)	273(230–378)	249(171–588)
α-HBDH, M (Q1-Q3)	222 (174–290)	212(174–284)	232(178–478)	202(164–223)	195(134–437)

(*Continued*)

Table 1 (Continued).

Characteristics at Baseline	IIM	MDA5+	TIF-1γ+	Jo-1+	Neg
	(n=77)	(n=34)	(n=15)	(n=10)	(n=10)
Disease activity					
MITAX score	0.343±0.135	0.359±0.147	0.326±0.094	0.293±0.149	0.364±0.170
Treatment (n%)					
Glucocorticoids	73(94.8)	34(100)	14(93.3)	10(100)	8(80)
Tacrolimus	34(44.2)	25(73.5)	1(6.7)	3(30)	2(20)
Tofacitinib	13(16.9)	12(35.3)	0(0)	0(0)	1(10)
IVIG	25(32.5)	21(61.8)	1(6.7)	1(10)	0(0)
SMZ	10(13.0)	9(26.5)	0(0)	0(0)	1(10)
CTX	19(24.7)	11(32.4)	2(13.3)	3(30)	1(10)
Pirfenidone	18(23.4)	8(23.5)	0(0)	5(50)	2(20)
CSA	7(9.1)	4(11.8)	1(6.7)	0(0)	1(10)
AZA	2(2.6)	1(2.9)	0(0)	1(10)	0(0)
Baritinib	2(2.6)	2(5.9)	0(0)	0(0)	0(0)
Recombinant human IL2	1(1.3)	1(2.9)	0(0)	0(0)	0(0)
Thalidomide	7(9.1)	2(5.9)	4(26.7)	0(0)	1(10)
HCQ	14(18.2)	3(8.8)	5(33.3)	1(10)	3(30)
MMF	6(7.8)	0(0)	0(0)	2(20)	2(20)
MTX	8(10.4)	0(0)	7(46.7)	0(0)	1(10)
Tripterygium wilfordii	3(3.9)	0(0)	2(13.3)	0(0)	0(0)

Notes: *Represented positive detection of myositis associated autoantibodies Ro52, and patients without other myositis specific autoantibodies and myositis associated autoantibodies positive.

Abbreviations: IIM, idiopathic inflammatory myopathy; MDA5+, anti-MDA5+ DM; TIF-1γ+, anti- TIF-1γ+ DM; Jo-1, anti-Jo-1+ ASS, Neg patients without MSAs; MSAs, Myositis specific antibodies, Disease duration, the time between disease onset and diagnosed in our hospital; ILD, interstitial lung disease; Q1, quartile 1; Q3, quartile 3, WBC, white blood cell; HgB, hemoglobin; PLT, platelet; EOS, eosinophils; ESR, estimated sedimentation rate; CRP, C-reactive protein; Ig, immunoglobulin; KL-6, Krebs Von den Lungen-6; ALT, alanine transaminase; ALB, alanine transaminase; AST, aspartate transaminase; CK, creatine kinase; CK-MB: creatine kinase MB isoenzyme, LDH, lactate dehydrogenase; α-HBDH, hydroxybutyrate dehydrogenase; MITAX. myositis intention to treat activities index, IVIG, intravenous immunoglobulin; SMZ, Compound Sulfamethoxazole; CTX: cyclophosphamide, CSA, cyclosporin A; AZA, azathioprine; HCQ, hydroxychloroquine; MMF, Mycophenolate mofetil; MTX, methotrexate.

Serological Variables and MSAs Detection

Patients' baseline blood routine test, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), liver/kidney function, Krebs Von den Lungen-6 (KL-6), serum ferritin, muscle enzymes and plasma were detected before systemic treatment given to patients. Anti-MDA5 and anti-TIF1-γ autoantibodies were detected by enzyme-linked immunosorbent assay (MBL, Japan). Anti-Jo-1, anti-EJ, anti-PL-7, anti-PL-12, anti-SRP, anti-Ro52, anti-PM-Scl75, anti-PM-Scl 100, anti-Ku, and anti-Mi-2 antibodies were detected using line immunoassays (EUROIMMUN, Germany).

Olink Proteomics

We used a proximity extension assay (immune-oncology panel, Olink Bioscience, Uppsala, Sweden) to analyze the plasma protein profiling in 77 IIM patients and 53 hCs. In the proximity extension assays, plasma proteins were dually recognized by pairs of antibodies coupled to a cDNA strand that ligates when brought into proximity by its target.

A proximity-dependent DNA polymerization event was caused by the addition of a DNA polymerase, generating a unique PCR target sequence. The generated DNA sequence was detected and quantified using a microfluidic real-time PCR instrument.

Data Analysis and Statistics

All data analysis was performed in R (version 3.4.1). All assay validation data are available on the manufacturer's website (www.olink.com). The R package prcomp was used to perform the principal component analysis (PCA). We applied pairwise PERMANOVA with correction for false discovery rate (FDR) with the R packages corrplot to assess the differences between groups. The volcano plots were calculated using Mann–Whitney *U*-tests with Benjamini-Hochman correction for FDR. For the Gene Ontology (GO) enrichment analysis, FunRich software (https://www.funrich.org) was used with the differential expressed proteins (DEPs). Then we mapped the identified DEPs onto Wikipathways database (https://www.wikipathways.org) and KEGG pathway database (https://www.genome.jp/kegg/pathway.html).

Statistical analyses were conducted through IBM SPSS Statistics version 24.0 software (IBM, Armonk, NY, USA), GraphPad Prism software version 8.0 (GraphPad Software, La Jolla, CA, USA). Results are reported as the mean ± standard deviation (SD) or median (Q1–Q3). For comparison between the IIM and HCs, a two-tailed Student's *t*-test or rank-sum test were used. Correlation analyses were performed using Spearman correlation analysis. A two-tailed *P*-value <0.05 was considered to indicate statistical significance.

Model Building and Validation

Data from anti-MDA5+ DM patients and anti-MDA5- IIM patients were combined for model development. Lasso regression feature selection was used to process the high-dimensional complex collinearity data. We used ROC analyses and AUC to assess the discriminatory capacity of a model for separating patients with and without anti-MDA5 autoantibodies. Decision curve analysis (DCA) was used to report the clinical net benefit of each model compared to biomarker-all and biomarker-none strategies. The selected variables were entered into a stepwise logistic regression algorithm (SLR) to screen clinical predictors and develop the clinical prediction model. All evaluations were conducted on the initial biopsy and subsequent biopsy groups separately and combined. For calculating the confidence intervals and significance tests, the bootstrap resampling procedure was used. All data analyses were conducted in R version 3.4.1 software.

Results

Identification of DEPs in the Plasma of Patients with IIM and HCs

To identify the DEPs in the plasma of patients with IIM, a total of 92 inflammation–related proteins were detected. Figure 1A shows the general workflow of this study. PCA using the first two principal components showed the inflammation-related proteins were perturbed of patients with IIM compared with HCs (Figure 1B), indicating the immense inflammation condition of patients with IIM. The expression estimate of each protein was determined by comparing the IIM and HC groups (Estimate = (protein NPX level of IIM) - (protein NPX level of HC)). *P*-value <0.05 and estimate > 0.5 or <-0.5 were defined as up- or down-regulated, respectively. Thirty-six inflammation-related proteins were identified as DEPs between IIM and HC groups, containing 29 upregulated and 7 downregulated DEPs (Figure 1D). The top 20 significantly changed proteins were CXCL10, CXCL11, CXCL9, CXCL8, S100A12, IL-6, CCL2, CCL8, IL-10, CCL3, CX3CL1, FGF21, CDCP1, CXCL5, OSM, AXIN1, TGFA, SIRT2, STAMBP, IL-18 respectively (Table S1).

The Venn diagram in Figure 1C shows the shared overlapping proteins among the DEPs identified between the HC group and MSA-typed IIM patients, from which there were some DEPs expressed uniquely in MSA-typed groups, and most of the DEPs were shared in common. The top 15 DEPs identified in different MSA-typed IIM patients were shown in Figure 1E.

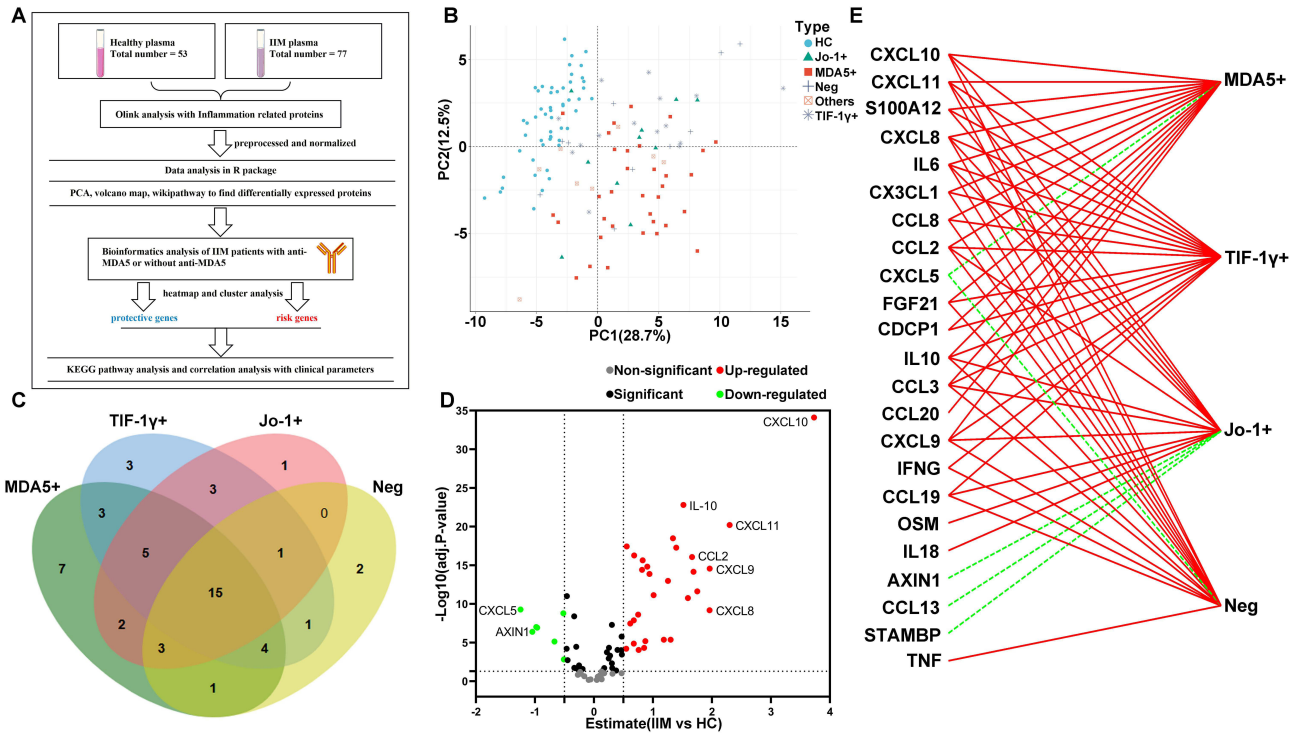

Figure 1 Characterization of the protein profiles in plasma of patients with IIM. (**A**) Experimental workflow used in this study. (**B**) Principal component analysis (PCA) of the first 2 principal components. (**C**) Venn diagram showing the overlap of DEPs identified by comparing the HC group and IIM patient subgroups (anti-MDA5+, anti-TIF1-γ+, anti-Jo-1+, and antibody-negative IIM subgroups) ($p < 0.05$, |Estimate| >0.5). (**D**) Volcano map for DEPs (adj. $p < 0.05$, and |Estimate| >0.5) in plasma of IIM patients and HCs with inflammation-related proteins. (**E**) Top 15 DEPs identified by comparing the IIM patient subgroups with HCs. The solid red and green lines represent the up- and downregulated DEGs separately.

Gene Ontology (GO) and Pathway Enrichment Analysis of DEPs in IIM

The functional annotation of the DEPs was analyzed using the Funrich software. And the top five enriched items were identified according to the *P*-values. The obtained 36 DEPs between IIM and HC groups were annotated to cellular component (CC), molecular function (MF) and biological process (BP) of GO terms (Figure 2A). Immune response, signal transduction, cell communication, and cell proliferation were most enriched in BP. For CC, extracellular, extracellular space, extracellular region, and cell surface were enriched. Chemokine activity, cytokine activity, growth factor activity, and receptor binding were enriched in MF.

Furthermore, among the terms enriched for the DEPs, hypersensitivity, pneumonia, lung injury, inflammation, pulmonary fibrosis ranked in the top five reliability through correlation analysis with human diseases (Figure 2B). And pneumonia, lung injury, and pulmonary fibrosis are closely related to lung injury in the IIM.

To provide insights into the biological pathways involved in IIM, the web-based Gene Set Analysis Toolkit was employed to map the gene symbol of DEPs to the Wiki pathway database. Wiki Pathway functional analysis revealed the cytokine–cytokine receptor interaction pathway and chemokine signaling pathway were the most crucial pathways (Figure 2C).

Among the affected proteins involved in the cytokine–cytokine receptor interaction pathway (Figure 2D), CX3CL1, CXCL9, CXCL10, CXCL11, CCL19 and CCL20 were significantly upregulated in the plasma of patients with IIM, while CXCL5 was downregulated in IIM. And chemokine signaling pathway also revealed the upregulation of CX3CL1, CXCL9, CXCL10, CXCL11, CCL19 and CCL20 (Figure S1).

The above results revealed that inflammation is related to the pathogenesis of IIM.

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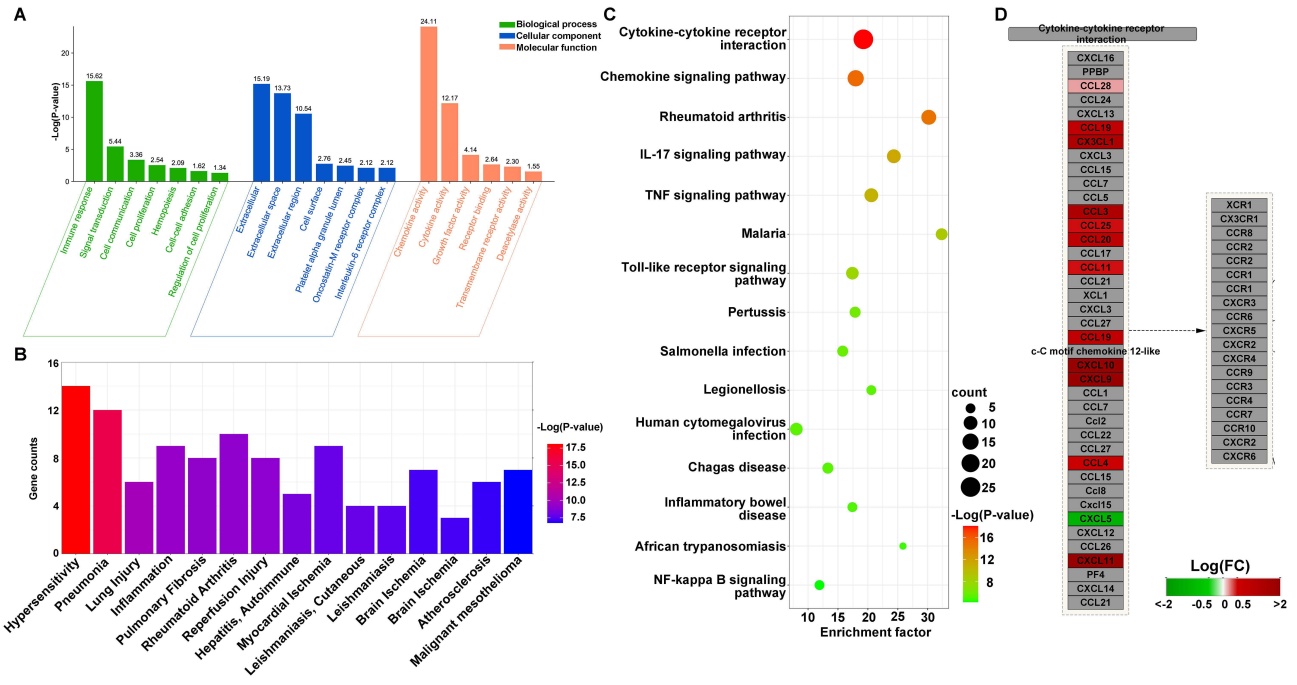

Figure 2 Gene ontology and pathway analysis of DEPs in plasma of patients with IIM. (**A**) GO (gene ontology) analysis of DEPs in plasma of patients with IIM. The seventh most enriched categories in biological process, seventh most enriched categories in cellular components, and sixth most enriched categories in molecular functions are shown. And the enrichment significance (log (p-values), $p < 0.05$) are also shown. (**B**) The DEP correlation analysis with human diseases. (**C**) Bubble chart for pathway analysis of DEPs. The number of proteins identified, enrichment significance (-log (p-values), $p < 0.05$) and enrichment factor in each category are indicated. (**D**) Wiki pathway analysis of the chemokine signaling pathway in plasma of patients with IIM. Red indicates upregulated and green indicates downregulated protein expression values.

Classification of DEPs in IIM Patients with/without Anti-MDA5 Autoantibody

Comparing the anti-MDA5+ and HC groups, 41 inflammation-related proteins were identified as significantly changed proteins, containing 29 upregulated (*P*-value <0.05 and estimate > 0.5) and 12 downregulated (*P*-value < 0.05 and estimate <-0.5) proteins (Figure 3A). And comparing the anti-MDA5- and HC groups, 38 inflammation-related proteins were identified as significantly changed proteins, containing 32 upregulated (*P*-value <0.05 and estimate > 0.5) and 6 downregulated (*P*-value < 0.05 and estimate <-0.5) proteins (Figure 3B). There were 36 common significantly changed proteins among the plasma of IIM group, anti-MDA5- and anti-MDA5+ group (Figure 3C).

To further delineate the changes of protein profiles in IIM patients with/without anti-MDA5 antibodies, we conducted heatmap and cluster analysis on the collection of differentially expressed genes in anti-MDA5- and anti-MDA5+ group mentioned above. Heatmap of the hierarchical clustering analysis was shown in Figure 3D. There were remarkable changes in inflammation-related proteins between HCs and IIM patients with/without anti-MDA5 antibodies. The clustering results divided the protein expression profile in the plasma of HC group, anti-MDA5- group and anti-MDA5+ group into several subgroups. Notably, there were two clusters of inflammation-related proteins inversely expressed in IIM patients with/without anti-MDA5 autoantibodies. IL-18R1, IL17C, LIFR, IL22RA1, CCL25, TNFSF10, CCL20, CXCL11, CX3CL1, CCL23, CDCP1, S100A12, TNFRSF1 and FCF21 were significantly increased in anti-MDA5+ DM compared with HCs and patients without anti-MDA5 autoantibodies. While CXCL1, SIRT2, STAMPBP, NTF3, AXIN1, TNFSF12, KITLG, EIF4EBP1, CXCL5, CD244, FGF19, MMP10, LTA, CD6, and TNFSF11 were significantly lower in anti-MDA5+ DM compared with HCs and anti-MDA5- IIM patients. The expression levels of DEPs in cluster 1 show a downregulated trend, while the expression levels of DEPs in cluster 2 show the opposite trend. Therefore, we propose a hypothesis that DEPs in cluster 1 and cluster 2 may be potentially involved in the pathogenesis of patients with anti-MDA5+ antibodies.

All the DEPs in clusters 1 and 2 were analyzed using the FunRich software. And the top six enriched items were identified for the GO terms of CC, MF and BP according to the *P*-values (Figure 3E). For BP, cell communication, response to stimulus, and biological regulation were both most enriched in clusters 1 and 2. For CC, extracellular space,

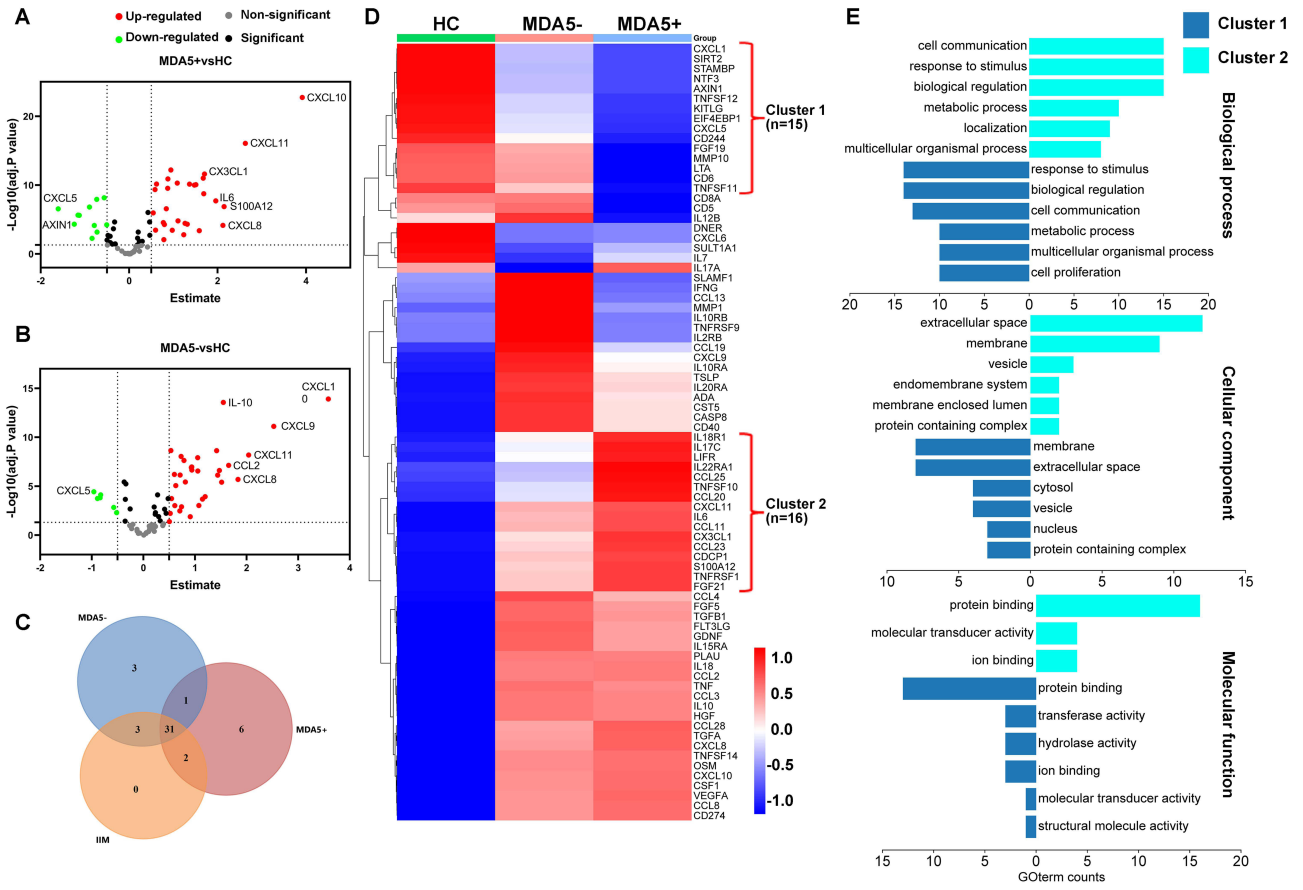

Figure 3 Volcano map, heatmap and go analysis of proteome expression profiles in plasma of anti-MDA5-, anti-MDA5+ IIM patients and HCs with inflammation-related proteins.(**A**) Volcano map for DEPs in plasma of anti-MDA5+ DM patients compared with HCs. (**B**) Volcano map for DEPs in plasma of anti-MDA5- IIM patients compared with HCs. (**C**) Venn diagram showing the overlap of identified proteins in IIM patients, anti-MDA5-, anti-MDA5+ IIM patients. (**D**) Heatmap of the hierarchical clustering analysis of proteome expression profiles in HCs, anti-MDA5-, anti-MDA5+ IIM patients with inflammation-related proteins. (**E**) GO analysis of DEPs in cluster1 and cluster2. The sixth most enriched categories in biological processes, cellular components, and molecular functions are shown.

membrane, protein containing complex were enriched. Protein binding and molecular transducer activity were enriched in MF.

KEGG Pathway Analysis of DEPs in Cluster 1 and Cluster 2 of IIM Patients with/without Anti-MDA5 Autoantibodies

To gain a better understanding of the biological pathways of DEPs in IIM plasma with/without anti-MDA5 autoantibody, we used the web-based Gene Set Analysis Toolkit (http://www.webgestalt.org) to search the KEGG Pathway databases. KEGG Pathway analysis revealed the activation of several immunity pathways in IIM patients with/without anti-MDA5 autoantibodies. The enriched pathways are classified into environmental information processing, organizational systems, and human diseases according to the KEGG pathway classification.

The analysis results revealed that cytokine–cytokine receptor interaction, rheumatoid arthritis, hematopoietic cell lineage, TNF signaling pathway, breast cancer, type I diabetes mellitus and PI3K-AKT signaling pathway were the most enriched pathways of DEPs in cluster 1 (Figure 4A). CXCL5, AXIN1, CXCL1, TNFSF12, EIF4EBP1, NTF3, TNFSF11, and KITLG played an important role in the above KEGG pathways (Figure 4C). The above proteins are downregulated in both anti-MDA5- and MDA5+ IIM patients.

And in the DEPs of cluster 2, cytokine–cytokine receptor interaction, chemokine signaling pathway, IL-17 signaling pathway, TNF signaling pathway, JAK-STAT signaling pathway were enriched in anti-MDA5+ DM patients (Figure 4B).

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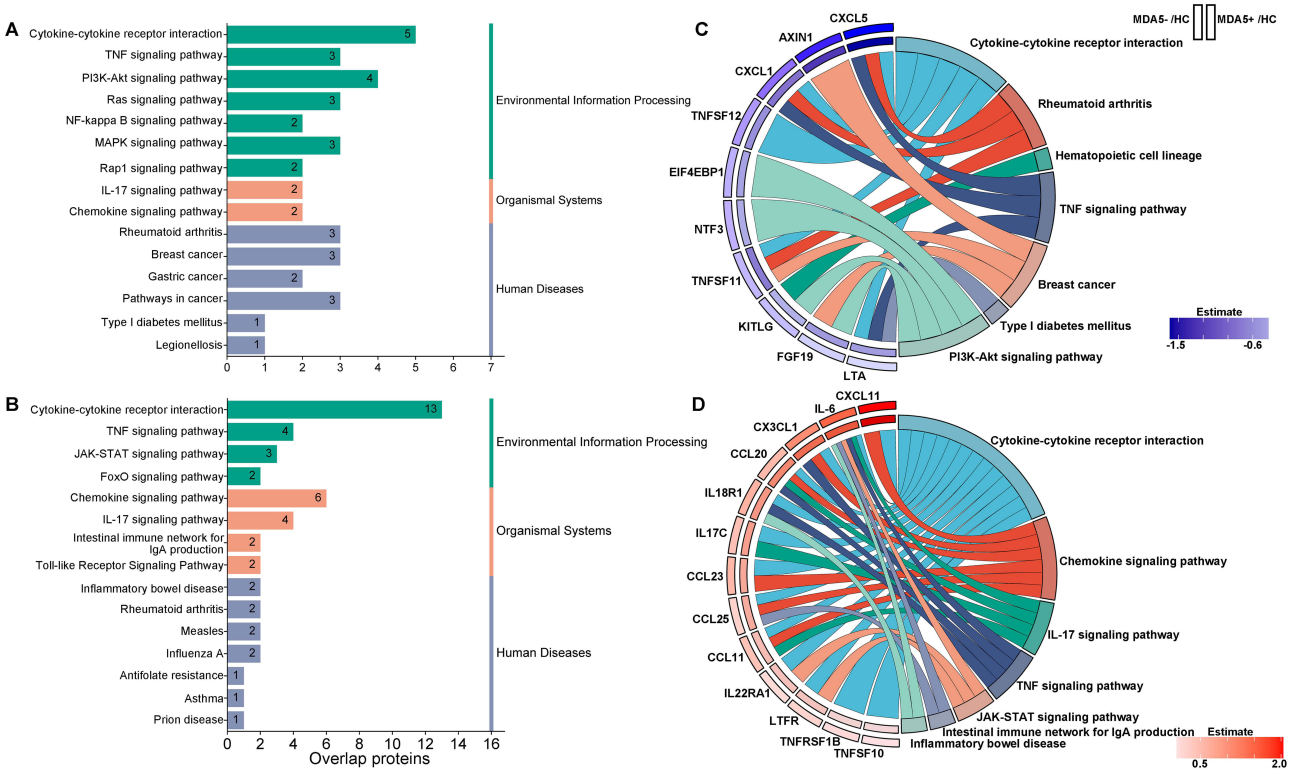

Figure 4 KEGG pathway analysis of DEPs in cluster1 and cluster2. (**A**) KEGG pathway enrichment analysis of the DEPs in cluster1 in plasma of IIM patients with anti-MDA5- or anti-MDA5+. (**B**) KEGG pathway enrichment analysis of the DEPs in cluster 2 in plasma of IIM patients with anti-MDA5- or anti-MDA5+. (**C**) Chord plot of DEPs in cluster 1 of enriched KEGG pathway. The color of the outer ring was marked as MDA5-/HC ratios, and the color of the inner ring was marked as MDA5+/HC ratios. (**D**) Chord plot of DEPs in cluster 2 of enriched KEGG pathway.

CXCL11, IL-6, CX3CL1, CCL20, IL-18R1, IL-17C, CCL23, CCL25, CCL11 were significantly increased in anti-MDA5 + DM patients compared with anti-MDA5- IIM patients and HCs (Figure 4D).

The Cytokine System Is Activated in the Plasma of Patients with Anti-MDA5+ DM

To gain a better understanding of the underlying mechanism in anti-MDA5+ DM, we performed a KEGG Pathway analysis of all significant differentially expressed proteins in the plasma of IIM patients with/without anti-MDA5 autoantibodies. The pathview package was then used to visualize the activation of signaling pathways. Compared with HCs and anti-MDA5- IIM patients, CX3CL1, IL-18R1, CCL20, IL-17C, CCL23, CXCL11, and IL-6 were significantly upregulated in the plasma of DM patients with anti-MDA5 autoantibodies, while the CXCL5 and CXCL6 were downregulated (Figure 5). These findings suggested that the cytokine–cytokine receptor interaction pathway is activated and that these cytokines participate in the pathogenesis of anti-MDA5+ DM.

Among the proteins involved in the IL-17 signaling pathway (Figure S2A), IL-17C, CXCL8, CXCL10, CCL20, and IL-6 were markedly upregulated in the plasma of anti- MDA5+ DM patients compared with that detected in HCs and anti-MDA5- IIM patients, while there was a significant downregulation in the expression of CXCL1 and CXCL5 in patients with anti-MDA5 autoantibodies. And in the TNF signaling pathway (Figure S2B), the expression level of CCL20, CXCL10, CX3CL1 and IL-6 were upregulated remarkedly in anti-MDA5+ DM patients compared with HCs and IIM patients without anti-MDA5 autoantibodies. By contrast, CXCL1, CXCL5 were downregulated in anti-MDA5+ DM patients. These results demonstrated that as a downstream pathway, the TNF signaling pathway was affected by the IL-17 signaling pathway, which both participated in the pathogenesis of MDA5+ DM.

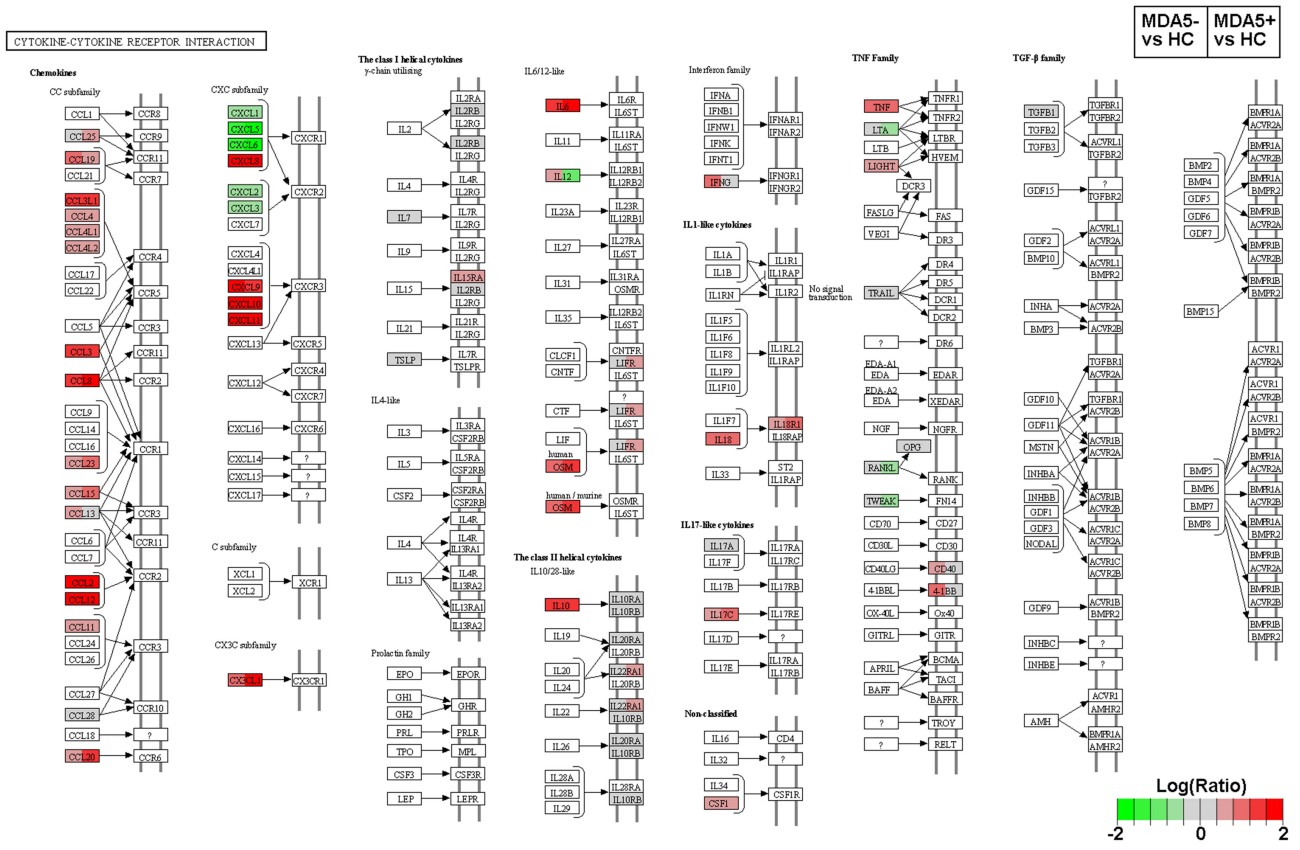

Figure 5 Pathway analysis of the cytokine-cytokine receptor interaction pathway in IIM patients with/without anti-MDA5+ autoantibodies. The DEPs of the anti-MDA5- subgroup and anti-MDA5+ subgroup were marked in the cytokine-cytokine receptor interaction pathway. Proteins marked in red were upregulated against HCs, while those marked in green were downregulated.

Correlation Analysis of Top Increased DEPs with Clinical Parameters

To determine the clinical significance of the protein alterations, the correlation between plasma CX3CL1, IL-17C, IL-18R1, CCL20 and TNF with laboratory parameters were conducted (Figure 6). The analysis results revealed CX3CL1 correlated positively with ESR, ferritin, LDH, α-HBDH and MITAX score, $p = 0.00052$, $R = 0.40$; $p = 0.000014$, $R = 0.52$; $p = 0.000076$, $R = 0.45$; $p = 0.0014$, $R = 0.39$; $p = 0.00041$, $R = 0.39$ respectively (Figure 6B–F). In addition, IL-17C correlated positively with ferritin (Figure 6G) $p = 0.0023$, $R = 0.37$. And IL-18R1 was associated positively with ESR, ferritin, LDH, and α-HBDH, $p = 0.000029$, $R = 0.48$; $p = 6\times10^{-8}$, $R = 0.62$; $p = 0.000015$, $R = 0.49$; $p = 0.0048$, $R = 0.35$ respectively (Figure 6H–K). Besides, CCL20 correlated positively with ferritin level, $p = 0.0073$, $R = 0.33$ (Figure 6L). And TNF was associated positively with LDH, $p = 0.00013$, $R = 0.44$ (Figure 6M).

To establish a diagnostic biomarker panel, Lasso binary logistic regression model was used to select the features (Figure 7A). The selection of tuning parameter (λ) in the Lasso model used tenfold cross-validation based via minimum criteria. The area binomial deviance curve was plotted versus log (λ). The dotted vertical lines were drawn at the optimal values by 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 (Figure 7B). Finally, the 5 DEPs related to anti-MDA5+ DM were selected (Figure 7C). They were IL-18R1, CCL20, CX3CL1, TNF and CXCL5.

A multiple logistic regression model was established using the 5 DEPs selected. The results predicted by the model after incorporating the final 5 biomarkers into the model were shown in Figure 7D. The AUC of the model was 0.824 (95% CI:0.729–0.918). And Figure 7E shows results of the multivariate logistic regression analysis. The DCA curve was performed to assess the clinical net benefits, and the threshold probabilities of the new model had excellent net benefits and enhanced performance for predicting the patients with anti-MDA5 autoantibodies (Figure 7F).

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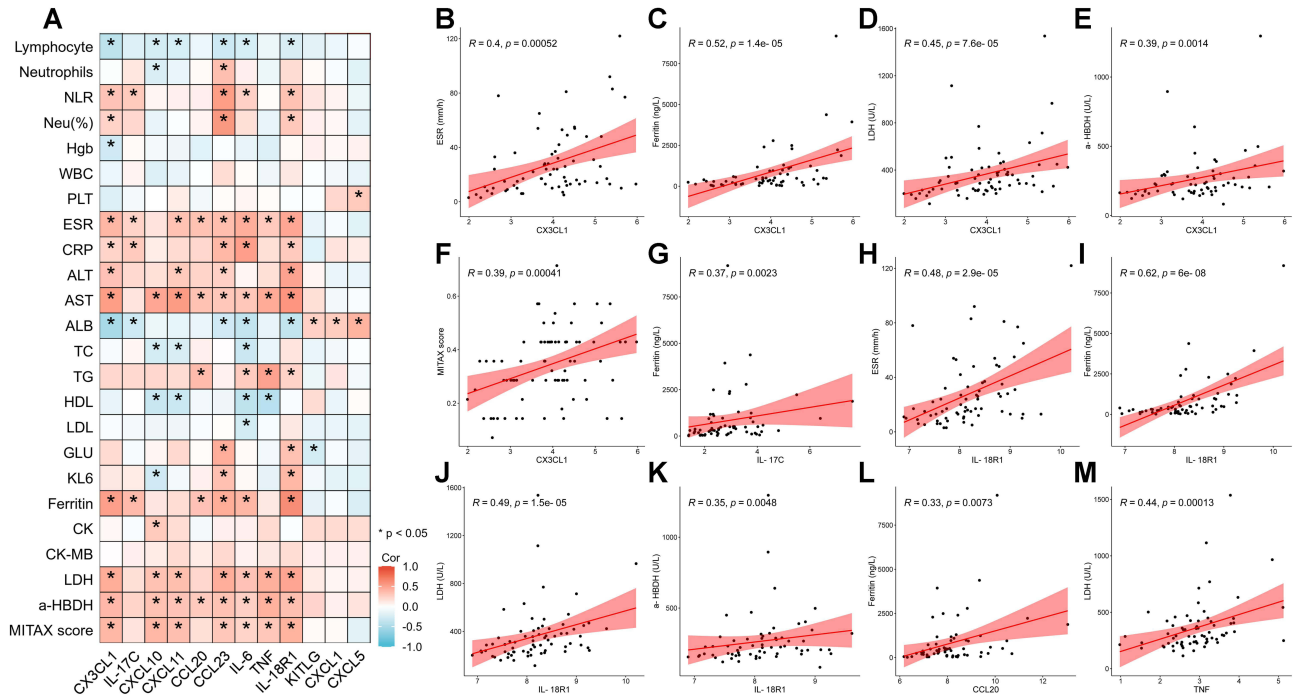

Figure 6 Correlation analysis of DEPs in the plasma of IIM patients and HCs. (**A**) Pairwise Spearman correlations between DEPs and laboratory parameters. The color bar indicates the correlation coefficients. (**B–M**) Correlation analysis of significantly increased protein levels with laboratory parameters in patients with IIM. (**B–F**) Correlation of CX3CL1 with ESR, serum ferritin, LDH, α-HBDH and MITAX score. (**G**) Correlation of IL-17C with serum ferritin. (**H–K**) Correlation of IL-18R1 with ESR, serum ferritin, LDH, α-HBDH. (**L**) Correlation of CCL20 with ferritin. (**M**) Correlation of TNF with LDH.

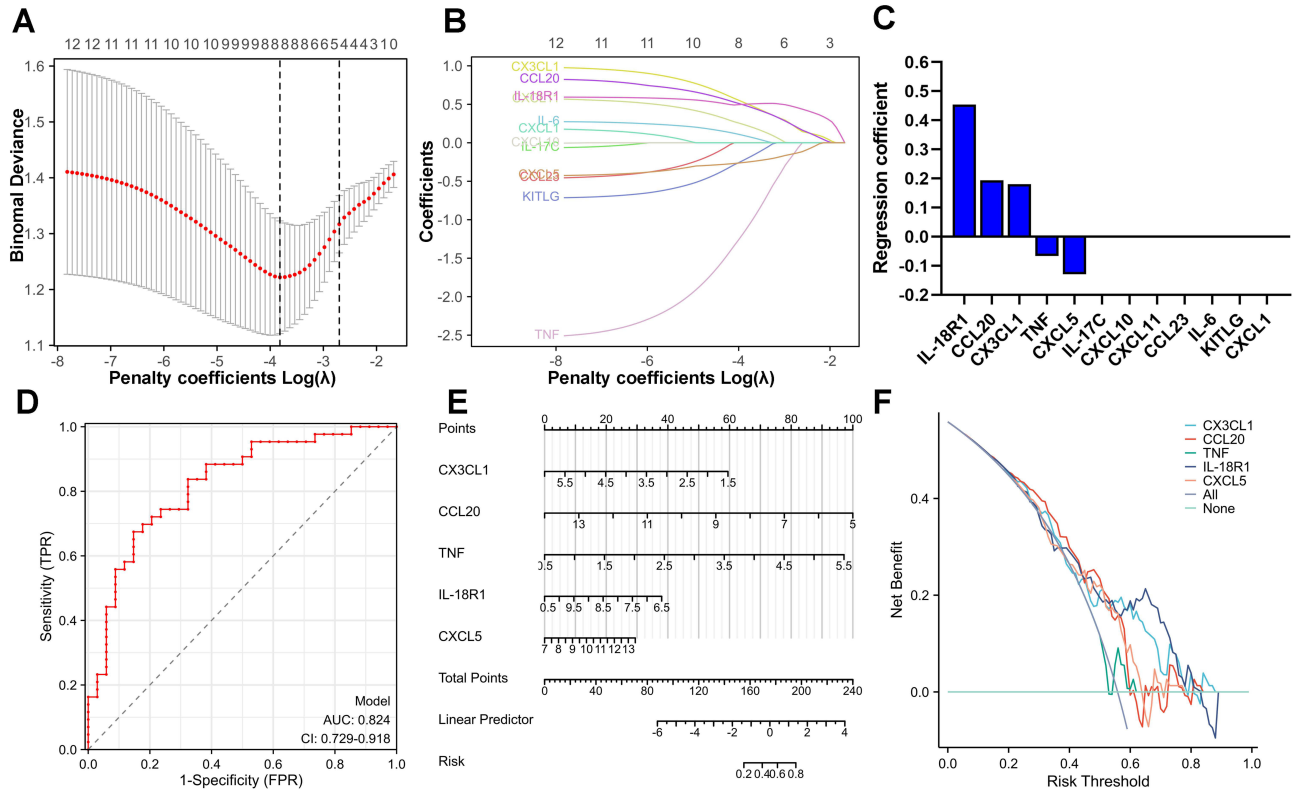

Figure 7 The Lasso binary logistic regression model and a multiple logistic regression model using the examination biomarkers selected. (**A**) The selection of tuning parameter (λ) in the LASSO model used tenfold cross-validation via minimum criteria. (**B**) The graph of changes in 12 marker coefficients with the penalty parameter (λ). (**C**) Based on the selected best penalty parameter (λ), 12 marker coefficients obtained. (**D**) Receiver operating characteristic (ROC) curve with area under the curve values for Lasso regression. (**E**) Study population for multivariate logistic regression analyses. (**F**) Decision curve analysis (DCA) of the novel nomogram for predicting diagnostic markers of anti-MDA5+ DM patients.

Discussion

Our study revealed the shared and distinctive inflammation-related protein signature in patients with IIM of different MSAs. Excessive inflammation was prominently elevated in anti-MDA5+ DM patients compared with HCs and patients without anti-MDA5+ autoantibodies. Cytokine–cytokine receptor interaction pathway and IL-17 signaling pathway were activated in anti-MDA5+ DM. Altered DEPs correlated with multiple laboratory parameters. IL-18R1, CCL20, CX3CL1, TNF and CXCL5 were demonstrated as diagnostic markers for anti-MDA5+ DM by Lasso regression.

Cytokines and pro-inflammatory factors have been investigated in patients with IIM.[26] IL-15, IL-8, IFN-α, IFN-γ, MCP-1, MCP-2, MCP-3, IL-6, IL-33, S100A12 were elevated in patients with IIM.[6,11,27–29] IL-15 and CCL22 could be a predictive marker for RP-ILD development in anti-MDA5+ DM patients.[11] Consistent with previous research, our study also revealed the altered profiling of inflammation-related proteins in patients with IIM of different MSAs. Instead, we also found novel inflammation-related proteins, such as IL18R1, CCL25, CCL23 and CDCP1 were elevated in anti-MDA5+ DM. IL-18R1, upregulated in serum and bronchoalveolar lavage fluid of patients with severe asthma,[30] could be a prognostic marker for idiopathic pulmonary fibrosis. CCL25 contributed to the development of acute lung injury and inflammation.[31] CDCP1 participated in the development of Kawasaki disease in mice models.[32] The significantly elevated inflammatory proteins in anti-MDA5+ DM could be associated with lung damage. The mechanism of the altered inflammatory proteins in lung involvement of anti-MDA5+ DM needs further investigation.

There were similarities and differences in patients with IIM of different MSAs, including the clinical symptoms, serological parameters and the underlying pathogenesis. Type 1 interferon signature was a distinctive feature in IIM, especially in anti-MDA5+ DM. The aberrant stimulation of interferons drives systemic inflammation and autoinflammatory disorders.[33,34] Compared with anti-MDA5- IIM patients, we found shared and distinctive inflammation-related protein profiling in anti-MDA5+ DM patients in our study. The excessive and distinctive inflammation condition in anti-MDA5+ DM may be associated with the poor prognosis of this disorder. As a diagnostic marker, anti-MDA5 autoantibodies correlated positively with disease activity.[26] We previously revealed that plasma KRT19 was a potential biomarker in anti-MDA5+ DM.[35] In this study, we first demonstrated that IL-18R1, CCL20, CX3CL1, TNF and CXCL5 could be new diagnostic biomarkers for anti-MDA5+ DM.

CX3CL1, a transmembrane chemokine expressed on macrophages and monocytes, represents as a marker of TGF-β expression. A growing interest has been focused on the role of CX3CL1 in regulating cell adhesion, chemotaxis and immune response.[36,37] Functions as an adhesion molecule, CX3CL1 is the only chemokine facilitating easier penetration of immune cells to the area of inflammation.[38] CX3CL1 increases the expression of M1-type macrophage markers and inflammation factors through the NF-κB signaling pathway in osteoclast differentiation.[39] In lung tissues from patients with idiopathic pulmonary fibrosis (IPF), the expression of CX3CL1 and its receptor CX3CR1 may participate in the pathophysiology of IPF.[40] Serum level of soluble CX3CL1 was elevated in 14 patients with PM and DM, which is correlated with disease activity.[14] In our study, we revealed the expression of CX3CL1 was significantly higher in anti-MDA5+ DM than in patients without anti-MDA5 autoantibodies. In addition, CX3CL1 correlated with ESR, serum ferritin, CK enzymes and disease activity, indicating that CX3CL1 participated in inflammation status of anti-MDA5 + DM.

The discovery of elevated CX3CL1 in anti-MDA5+ patients suggests divergent molecular pathways underlying distinct clinical phenotypes. This aligns with the observed disparity of Type I interferon disorder in MDA5+DM. Longitudinal analysis revealed that CX3CL1 correlated positively with ESR, ferritin, LDH, α-HBDH and MITAX score. This provides a potential tool for early intervention in high-risk patients. Our findings redefine IIM heterogeneity by linking autoantibody-defined subgroups to unique proteomic signatures. These insights not only fill mechanistic gaps but also pave the way for stratified therapies targeting subtype-specific pathways.

There were some limitations in our study. Firstly, the sample size was relatively small. Second, the altered inflammation-related proteins were not detected in IIM patients with different MSAs during follow-up. Third, disease duration may also affect the results. Fourth, the mechanism of altered inflammation-related proteins in the pathogenesis of IIM with different MSAs was not conducted.

Conclusions

Plasma profiling revealed that inflammation and inflammatory pathways were extremely elevated in patients with IIM, especially in patients with anti-MDA5 autoantibodies. The shared and distinctive inflammation-related protein signature was demonstrated in patients with/without anti-MDA5 autoantibodies. Cytokine–cytokine receptor interaction, chemokine signaling pathway, IL-17 signaling pathway, TNF signaling pathway, JAK-STAT signaling pathway were enriched in anti-MDA5+ DM patients compared with anti-MDA5- IIM patients. CX3CL1, IL-17C, IL-18R1, CCL20 and TNF were screened as biomarkers of anti-MDA5+ DM.

Abbreviations

IIM, idiopathic inflammatory myopathies; DM, dermatomyositis; ASS, anti-synthetase syndrome; IBM, inclusion body myositis; IMNM, immune-mediated necrotizing myopathy; MSAs, myositis-specific autoantibodies; anti-MDA5, anti-melanoma differentiation-associated gene 5; ILD, interstitial lung disease; RP-ILD, rapidly progressive interstitial lung disease; HCs, healthy controls; MRI, magnetic resonance imaging; HRCT, high-resolution computed tomography; MITAX, Myositis Intention to Treat Activities index; ESR, erythrocyte sedimentation rate; CRP, C-relative protein, KL-6, Krebs Von den Lungen-6; PCA, principal component analysis; FDR, false discovery rate; GO, gene ontology; DEPs, differential expressed proteins; DCA, decision curve analysis; SLR, stepwise logistic regression; Cis, confidence intervals; CC, cellular component; MF, molecular function; BP, biological process; IPF, idiopathic pulmonary fibrosis.

Data Sharing Statement

Data are available on reasonable request from the corresponding author.

Ethics Approval and Consent to Participate

The studies involving human participants were reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Consent for Publication

All authors approved the publication of this study.

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Disclosure

The authors declare that they have no relevant conflicts of interest for this work.

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