Dipeptidyl peptidase 4/CD26 expression in human idiopathic inflammatory myopathies reveals skeletal muscle injury and vascular inflammation

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Abstract

Objective

We performed a retrospective and prospective observational study to investigate whether the T lymphocyte activation antigen dipeptidyl peptidase 4 (DPP4)/CD26 is expressed in the skeletal muscle of patients with idiopathic inflammatory myopathies (IIM) and whether its expression offers clues to understand the events taking place in the tissue.

Methods

CD26 expression in the muscle, evaluated by immunofluorescence, was assessed in 32 patients with IIM and 5 healthy controls and compared among patients with dermatomyositis (DM), immune-mediated necrotising myopathy (IMNM), inclusion body myositis (IBM), and polymyositis (PM). The relationship of CD26 expression and localisation with clinical, serological and histological features was determined.

Results

CD26 is selectively expressed in the skeletal muscle of patients with IIM. The highest levels of CD26 are found in the skeletal muscle from patients with DM and in particular in those characterised by tissue necrosis and vascular inflammation. CD26 expression is associated with decreased muscle performance and independently predicts the number of treatments before reaching disease stabilisation or improvement (odds ratio, OR=1.2, $p<0.05$).

Conclusions

CD26 is expressed in the IIM skeletal muscle and may represent a target of molecular intervention for patients with treatment-refractory myositis.

Key words

CD26, DPP4, myositis, idiopathic inflammatory myopathies, skeletal muscle, inflammation, immune response

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Introduction

The idiopathic inflammatory myopathies (IIM) comprise immune-mediated diseases of the skeletal muscle, polymyositis (PM), dermatomyositis (DM), inclusion body myositis (IBM) and immune-mediated necrotising myopathy (IMNM) representing major subtypes (1). Gene expression profiles in muscle biopsies are characteristic for each type of IIM, indicating that muscle damage in each of these diseases is supported by independent mechanisms (2). However, the rarity and heterogeneity of IIM make it difficult to identify these mechanisms and develop targeted therapies able to contain disease activity and muscle damage (3).

Both humoral and cellular immune mechanisms participate to muscle inflammation, and repeated cycles of tissue necrosis and regenerations are hallmarks of IIM (4). Muscle-infiltrating T lymphocytes play a pathogenic role (5). However, their phenotype and function are poorly understood and may be regulated differently reflecting adaptation to diverse environmental conditions. CD26 (also called dipeptidyl peptidase 4, DPP4) is a membrane glycoprotein that cleaves dipeptides from the N-terminus of polypeptides bearing proline or alanine at penultimate position (6). CD26 is found on the surface of many cell types, such as lymphocytes, endothelial cells and epithelial cells (7). CD26 might also be shed as an enzymatically active soluble form from cellular membranes (8). CD26 exerts its roles either via enzymatic activity or via interactions with a variety of binding partners (8). It has also a role in T cell activation and antigen presentation (9-11). CD26 is considered to be a contributing factor in autoimmune diseases (12), but little is known on whether it is expressed in IIM muscle and contributes to IIM pathogenesis (13). Here we verified whether CD26 is expressed in the inflamed skeletal muscle of patients with IIM and whether the extent and location of CD26 reflect clinical, serological and histological characteristics of the disease.

Patients and methods

Patients and controls.

Consecutive individuals (n=37) who underwent muscle biopsy at a single centre, the San Raffaele University Hospital in Milan, Italy, between 2010 and 2017 were included in the study. Thirty-two patients were classified as having DM, PM, IBM or IMNM according to established criteria (14, 15). Five participants had no evidence of muscle inflammation nor of other diseases and served as healthy controls. Written informed consent was obtained from all participants. The study protocol conforms to the Declaration of Helsinki and has been approved by the Institutional Ethics Committee (MYODPP).

Clinical features (presence and degree of muscle weakness, myalgias, interstitial lung disease, dysphonia, dysphagia, arthralgias, arthritis and DM rash, defined as the presence of heliotrope rash or Gottron's sign) and serum muscle enzyme levels (creatine kinase, CK, and aldolase) of patients were documented at the time of biopsy. Twenty-two patients (8 with DM, 9 with PM, 1 with IBM and 4 with IMNM) were longitudinally followed at the Myositis Clinic of our Institution with clinical and laboratory assessments for a total of 202 visits, a median [interquartile range, IQR] of 8 [7-11.25] visits per patient and a median follow-up time of 2.63 [1.58-3.54] years. The Medical Research Council (MRC) scale was used by the examining physician to serially quantify muscle strength and calculate the manual muscle test 8 (MMT-8) score at each visit. For analysis purposes, MRC scale was converted to Kendall's 0-10 scale as described (16). Right- and left-side strength assessments of deltoids, *biceps brachii* muscles, wrist extensors, hip flexors and extensors, quadriceps and ankle dorsiflexors were merged, and the average was used for computations. Myositis Disease Activity Assessment (MYOACT) tool, Myositis Damage Index (MDI) and Health Assessment Questionnaire (HAQ) were used to evaluate disease activity, disease-related damage and quality of life, respectively (16). Muscle enzyme levels obtained >6 weeks before or after strength evaluation were excluded for statistical analyses. Autoantibodies were also determined in longitudinally followed patients.

Prior to analysis, data were cross-checked with medical charts in the

Table I. Demographic, clinical, serologic and histological features of patients with IIM.

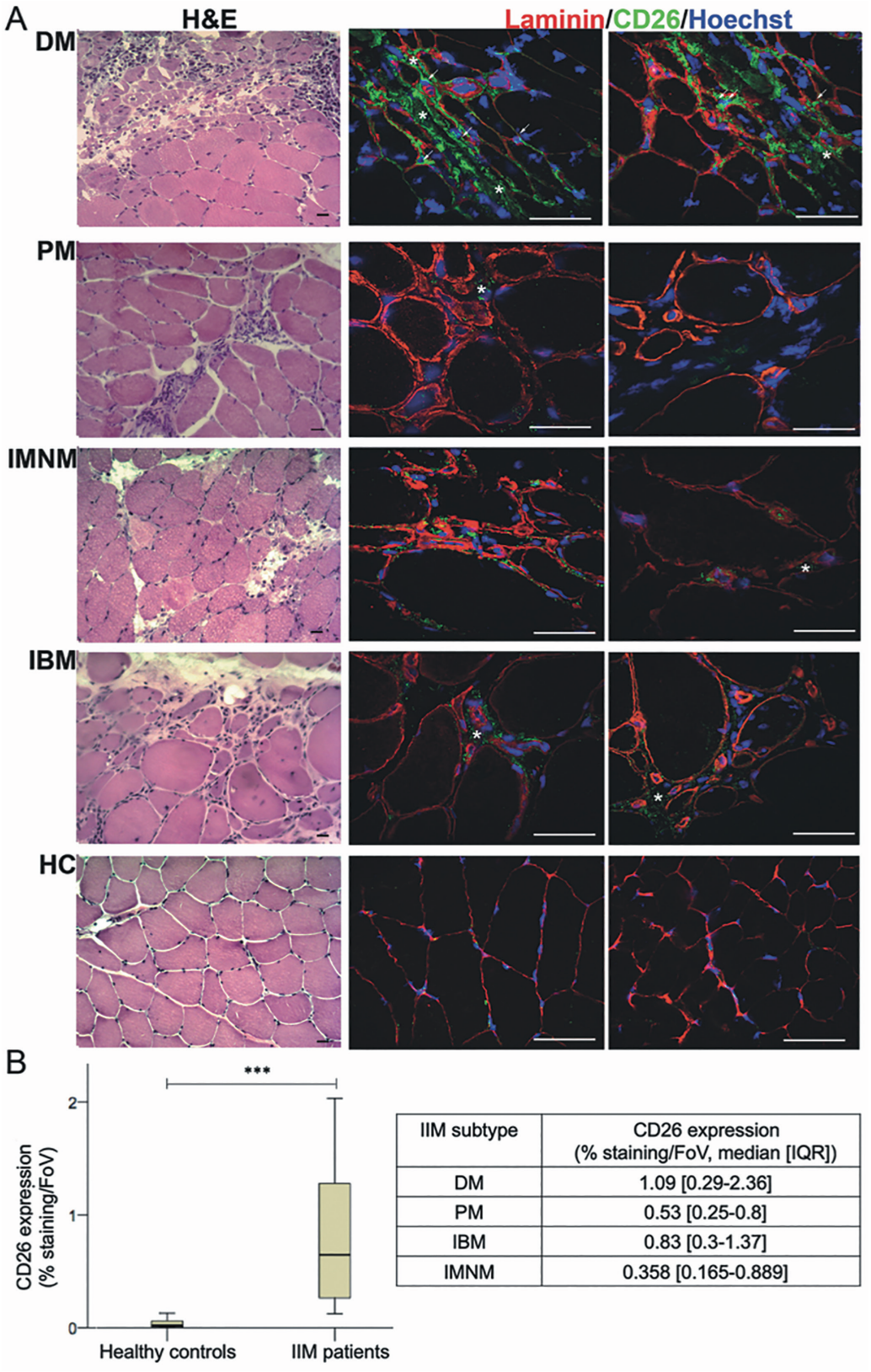
	DM (n=10)	PM (n=12)	IBM (n=4)	IMNM (n=6)
Caucasians	70% (7)	100% (12)	100% (4)	100% (6)
Female sex	70% (7)	58.3% (7)	75% (3)	50% (3)
Time of follow-up (years)	1.9 [1.1-3]	3.4 [1.9-4.2]	5	1.9 [1.6-4.5]
Age at onset	56.7 [42.3-69]	51.2 [49.1-62.6]	68.8 [63.5-68.8]	65 [42.6-72.7]
Clinical features				
Proximal weakness	90% (9)	100% (12)	100% (4)	100% (6)
Distal weakness	10% (1)	8.3% (1)	75% (3)	-
Myalgias	70% (7)	58.3% (7)	50% (2)	50% (3)
DM rash	100% (10)	-	-	-
ILD	40% (4)	-	-	16.6% (1)
Dysphonia	10% (1)	8.3% (1)	-	33.3% (2)
Dysphagia	30% (3)	16.6% (2)	50% (2)	33.3% (2)
Arthralgias	30% (3)	8.3% (1)	-	33.3% (2)
Arthritis	20% (2)	-	-	-
Mean MYOACT score (0-1)†	0.05 [0.04-0.15]	0.05 [0.02-0.07]	0.04	0.07 [0.05-0.08]
Mean total MDI score >0†	87.5% (7)	66.7% (6)	100% (1)	100% (4)
Mean HAQ score (0-3)†	0.6 [0.09-1.5]	0.6 [0.2-0.7]	1.5	0.3 [0.2-0.6]
Treatments				
MMF	50% (5)	16.6% (2)	25% (1)	16.6% (1)
AZA	20% (2)	25% (3)	25% (1)	33.3% (2)
MTX	20% (2)	66.6% (8)	-	33.3% (2)
IVG	20% (2)	16.6% (2)	-	16.6% (1)
TAC	20% (2)	-	-	-
RTX	10% (1)	8.3% (1)	-	16.6% (1)
Autoantibody positivity†				
Anti-Mi2	30% (3)	-	-	16.6% (1)
Anti-HMGCR	-	-	-	16.6% (1)
Anti-Jo1	10% (1)	-	-	-
Anti-PL12	-	-	-	16.6% (1)
Anti-PM/Scl	10% (1)	-	-	-
Histological features				
Perifascicular atrophy	40% (4)	-	-	-
Myofibre necrosis	70% (7)	83.3% (10)	75% (3)	100% (6)
Endomysial infiltrates	40% (4)	41.7% (5)	25% (1)	50% (3)
Macrophages	40% (4)	41.7% (5)	25% (1)	50% (3)
T cells	30% (3)	25% (3)	25% (1)	33.3% (2)
Perimysial infiltrates	30% (3)	33.3% (4)	-	-
Macrophages	30% (3)	33.3% (4)	-	-
T cells	30% (3)	25% (3)	-	-
Perivascular infiltrates	50% (5)	41.7% (5)	25% (1)	-
Macrophages	50% (5)	41.7% (5)	25% (1)	-
T cells	50% (5)	25% (3)	-	-
MHC-I	90% (9)	75% (9)	50% (2)	66.7% (4)
MAC	90% (9)	25% (3)	25% (1)	83.3% (5)

Dichotomous variables were expressed as percentage (count) and continuous variables as median [IQR].DM, dermatomyositis.
PM: polymyositis; IMNM: immune-mediated necrotising myopathy; IBM: inclusion body myositis; ILD: interstitial lung disease; MYOACT: Myositis Disease Activity Assessment; MDI: Myositis Damage Index; HAQ: Health Assessment Questionnaire; MMF: mycophenolate mofetil; AZA: azathioprine; MTX: methotrexate; IVG: intravenous immunoglobulins; TAC: tacrolimus; RTX: rituximab; MHC-I: major histocompatibility complex I; MAC: complement membrane attack complex.
†Data available for the twenty-two longitudinally followed patients (see Patients and methods).

presence of both data managers and clinicians for accuracy.

Immunohistochemistry and immunofluorescence

Perifascicular atrophy, presence of necrosis and regeneration, and characteristics of inflammatory infiltrates were identified by immunohistochemistry and immunofluorescence (17) in all biopsies. CD26 expression was evaluated on 7μm-thick slices using the monoclonal antibody (mAb) clone D6D8K (Abcam, dilution 1:50) and Begelomab (BEGEDINA®, 40 μg/ml) (kindly provided for this study by ADIENNE Pharma & Biotech, Switzerland) after acetone fixation. Antigen expression was then revealed by immunofluorescence using anti-mouse AlexaFluor488 IgG2b secondary antibody as second-step reagent. Placental tissue, which expresses large amounts of the antigen (18), was used as positive control. Isotype-matched control antibody was employed in parallel to verify the specificity of the staining. Nuclei were revealed by 4´, 6-diamidino-2-phenylindole (DAPI), and anti-laminin (LSBio, dilution 1:200) followed by anti-chicken Al-



IIM subtype	CD26 expression (% staining/FoV, median [IQR])
DM	1.09 [0.29-2.36]
PM	0.53 [0.25-0.8]
IBM	0.83 [0.3-1.37]
IMNM	0.358 [0.165-0.889]

Fig. 1. CD26 expression in skeletal muscle of IIM and controls. **A**: Histological features (H&E) and CD26 expression (immunofluorescence, green) in skeletal muscle biopsies of representative patients with DM, PM, IBM and IMNM and of a healthy control (HC). In immunofluorescence images, nuclei are counterstained with Hoechst (blue) while fibres are identified by laminin (red). Asterisks indicate CD26 expression in the extracellular matrix. Arrows point to DPP4/CD26 expression in infiltrating cells. Scale bars= 50 μm. **B**: CD26 expression is indicated as median, interquartile range and 95% confidence interval. Outliers are not shown. ***$p<0.001$.

exaFluor633 secondary antibody was used to identify muscle fibres. Images were captured using Ultraview Perkin Elmer laser scanning confocal microscope and analysed. CD26 expression was quantified using the Java-based image processing programme ImageJ, which calculates CD26 positive areas as % of field of view (FoV). At least four representative images were collected for each of the 37 participants. The mean of the percentages obtained for each muscle biopsy was then used for statistical analyses. Biopsies with a CD26 expression (% staining/ FoV)

Table II. Impact of demographic and clinical features on CD26 expression.

	CD26 expression Median [IQR]	*p*
Demographic or disease features n. of patients		
DM		
10 Yes	1.09 [0.29-2.36]	0.17
22 No	0.53 [0.23-0.83]	
PM		
12 Yes	0.53 [0.25-0.80]	0.77
20 No	0.71 [0.25-1.47]	
IMNM		
6 Yes	0.36 [0.16-0.89]	0.17
26 No	0.75 [0.28-1.37]	
IBM		
4 Yes	0.83 [0.30-1.37]	0.89
28 No	0.56 [0.25-1.30]	
Sex		
20 Females	0.72 [0.30-1.16]	0.83
12 Males	0.43 [0.22-1.85]	
Ethnicity		
29 Caucasian	0.61 [0.23-1.42]	0.67
3 Non-Caucasian	0.96 [0.32-0.96]	
Distal weakness		
5 Present	0.91 [0.58-3.62]	0.12
21 Absent	0.45 [0.22-1.09]	
Myalgias		
19 Present	0.45 [0.22-1.23]	0.43
7 Absent	0.76 [0.35-1.33]	
DM rash		
10 Present	1.09 [0.29-2.36]	0.17
22 Absent	0.53 [0.23-0.83]	
ILD		
5 Present	0.32 [0.19-1.14]	0.37
22 Absent	0.71 [0.32-1.30]	
Dysphonia		
4 Present	0.38 [0.22-1.62]	0.56
22 Absent	0.75 [0.23-1.25]	
Dysphagia		
9 Present	0.91 [0.38-1.78]	0.20
17 Absent	0.45 [0.22-0.87]	
Arthralgias		
6 Present	0.34 [0.18-0.83]	0.15
20 Absent	0.75 [0.27-1.47]	
Treatments N. of patients		
MMF		
9 Yes	1.33 [0.48-3.34]	0.06
16 No	0.38 [0.23-0.76]	
AZA		
8 Yes	0.95 [0.49-2.89]	0.07
17 No	0.36 [0.21-0.88]	
MTX		
12 Yes	0.61 [0.36-0.92]	0.61
13 No	0.61 [0.21-1.42]	
IVG		
5 Yes	1.33 [0.29-3.34]	0.37
20 No	0.51 [0.23-0.92]	
RTX		
3 Yes	0.96 [0.18-0.96]†	0.72
22 No	0.53 [0.23-1.25]	

CD26 expression was compared between patients with IIM with (Yes) or without (No) the depicted demographic and clinical features. DPP4/CD26 expression was indicated as median [IQR] and bivariate comparisons were made using Mann-Whitney's U-test.
DM: dermatomyositis; PM: polymyositis; IMNM: immune-mediated necrotising myopathy; IBM: inclusion body myositis; ILD: interstitial lung disease; MMF: mycophenolate mofetil; AZA: azathioprine; MTX: methotrexate; IVG: intravenous immunoglobulins; TAC: tacrolimus; RTX: rituximab.

Table III. Impact of histological features on CD26 expression.

	CD26 expression Median [IQR]	*p*
Histological features n. of patients		
Perifascicular atrophy		
4 Yes	1.27 [0.27-4.80]	0.44
27 No	0.61 [0.24-1.23]	
Myofibre necrosis		
26 Yes	0.75 [0.36-1.52]	0.005
5 No	0.22 [0.18-0.28]	
Endomysial infiltrates		
13 Yes	0.41 [0.26-1.14]	0.54
19 No	0.76 [0.24-1.52]	
Endomysial macrophages		
13 Yes	0.41 [0.26-1.14]	0.54
19 No	0.76 [0.24-1.52]	
Endomysial T cells		
9 Yes	0.75 [0.24-1.59]	0.96
23 No	0.61 [0.24-1.23]	
Perimysial infiltrates		
7 Yes	1.33 [0.41-4.66]	0.08
25 No	0.5 [0.23-0.93]	
Perimysial macrophages		
7 Yes	1.33 [0.41-4.66]	0.08
25 No	0.5 [0.23-0.93]	
Perimysial T cells		
6 Yes	1.59 [0.66-4.93]	0.04
26 No	0.48 [0.23-0.92]	
Perivascular infiltrates		
11 Yes	0.96 [0.50-3.35]	0.01
21 No	0.36 [0.21-0.86]	
Perivascular macrophages		
11 Yes	0.96 [0.50-3.35]	0.01
21 No	0.36 [0.21-0.86]	
Perivascular T cells		
8 Yes	1.14 [0.57-4.33]	0.02
24 No	0.43 [0.22-0.88]	
MHC-I		
24 Yes	0.71 [0.33-1.30]	0.23
8 No	0.30 [0.17-1.33]	
MAC		
18 Yes	0.64 [0.27-1.37]	0.89
14 No	0.60 [0.23-1.14]	

CD26 expression was compared between patients with IIM with (Yes) or without (No) the depicted histological features. DPP4/CD26 expression was indicated as median [IQR] and bivariate comparisons were made using Mann-Whitney's U-test.
MHC-I: major histocompatibility complex I; MAC: complement membrane attack complex.

higher than 0.5 were selected for further immunofluorescence analyses. The threshold of 0.5% staining/FOV was arbitrarily chosen based on signal visibility. Specifically, eight biopsies out of 37 (four DM, two PM, one IBM and one IMNM) were analysed by immunofluorescence for the simultaneous expression of CD3 and CD31 antigens to identify T lymphocytes and endothelial cells, respectively. The rabbit anti-human CD31 mAb (LSBio, dilution 1:100) and the rabbit anti-human CD3 antibody (Abcam, dilution 1:50) were used as primary antibodies, while the goat anti-rabbit AlexaFluor546-conjugated mAb and the goat PE-conjugated anti-rabbit mAb were respectively employed as second-step reagents.

Statistical analyses

Categorical variables were expressed as percentages and absolute frequencies, while continuous variables as medians [IQR]. Two-tailed Mann-Whitney's U-test was used to compare the level of muscular CD26 expression among patients with or without each clinical and histologic feature. Kruskal-Wallis non-parametric ANOVA was employed for the same comparison analysis among patients with different myositis subtypes. Correlations between CD26 expression and continuous variables including MMT-8 score, the level of muscle weakness in each muscle group, and CK and aldolase serum levels were performed using two-tailed Spearman's Rank test. For comparisons of categorical and continuous variables between patient groups, we used Chi-square or Fisher's exact test, as appropriate, and Mann-Whitney's U-test, respectively. The contribution of categorical or continuous variables in predicting the extent of CD26 expression in skeletal muscle was determined using generalised linear models with gamma distribution of the dependent variables and log function as a link function for multivariate analysis. Univariable and multivariable Poisson regression analyses were used to define whether CD26 expression predicts the number of steroid-sparing immunosuppressive agents attempted before achieving disease stabilisation or improvement. Data were analysed using IBM SPSS® v. 21. A 2-sided p value <0.05 was considered statistically significant.

Data availability statement

All the data included are available upon request.

Results

Selective CD26 expression in IIM

The skeletal muscle of 37 consecutive individuals followed at a single academic reference centre between 2010 and 2017 were analysed for CD26 ex-

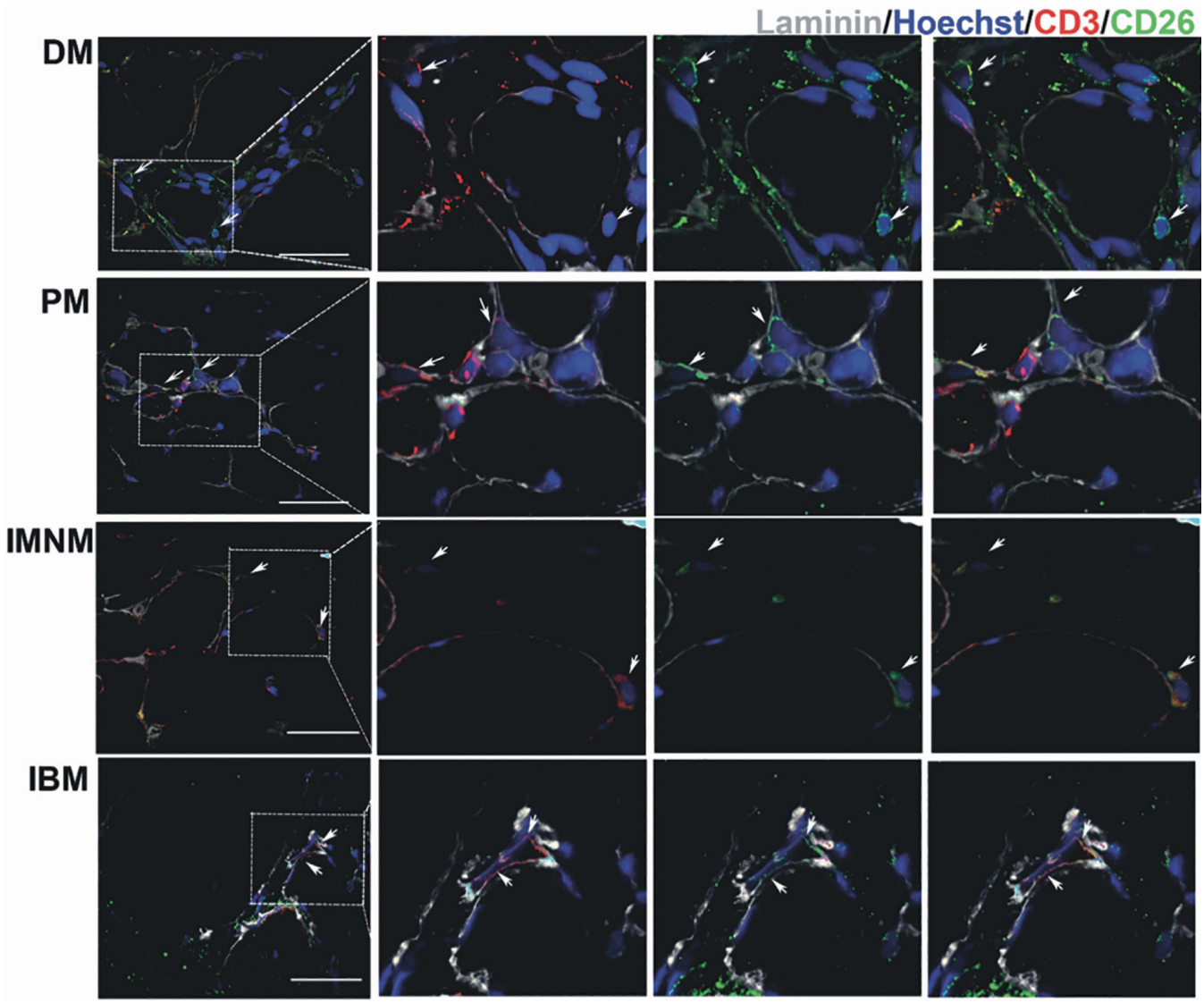


Fig. 2. CD26 expression by infiltrating T cells in IIM skeletal muscle.
Expression of DPP4/CD26 (green) and CD3 (red) antigens in the skeletal muscle of representative patients with DM, PM, IMNM and IBM. Nuclei are counterstained with Hoechst (blue). Fibres are identified by laminin (grey).
Scale bars = 50 µm. Arrows indicate $CD26^+$ $CD3^+$ cells.

pression. Out of these, thirty-two patients were identified as having IIM, while five participants had no detectable histological feature of muscle inflammation and were otherwise healthy. Ten (31%) patients with IIM had DM, twelve (37.5%) PM, four (12.5%) had IBM and six (19%) IMNM. Twenty-three patients were not treated pharmacologically at the time of the biopsy. Nine had been receiving steroids for a median [IQR] number of years of 0.19 [0.1-0.35], one patient had been taking methotrexate (MTX) for five months and two patients azathioprine (AZA) for 2 and 23 months respectively. Table I summarises the main demographic, clinical and histopathological features of patients.

CD26 was expressed in all biopsies from patients with IIM, albeit with varying degrees of expression and different localisations. In contrast, CD26 was never detectable in the skeletal muscle of healthy controls (Fig. 1). CD26 was consistently expressed in the muscle of all patients with IIM (n=32) by cells. CD26 was also present within the extracellular matrix (ECM) of IIM patients (Fig. 1, panel A). Median [IQR] CD26 expression (% staining/ FoV) was 0.645 [0.251–1.302] for patients with IIM *vs.* 0.02 [0.0045–0.095] for healthy controls ($p<0.001$, Fig. 1, panel B). Among IIM subtypes, patients with DM expressed the highest levels of CD26 (Fig. 1).

No difference in the expression of CD26 was found in patients with different sex or ethnicity. CD26 expression did not significantly change with varying age at onset or disease duration (not shown), and was similar in patients with or without distal weakness, myalgia or extra-muscular manifestations (Table II).

CD26 expression reflects necrosis and vascular inflammation

The extent of CD26 expression was significantly higher in biopsies with areas of myofibre necrosis (Table III).

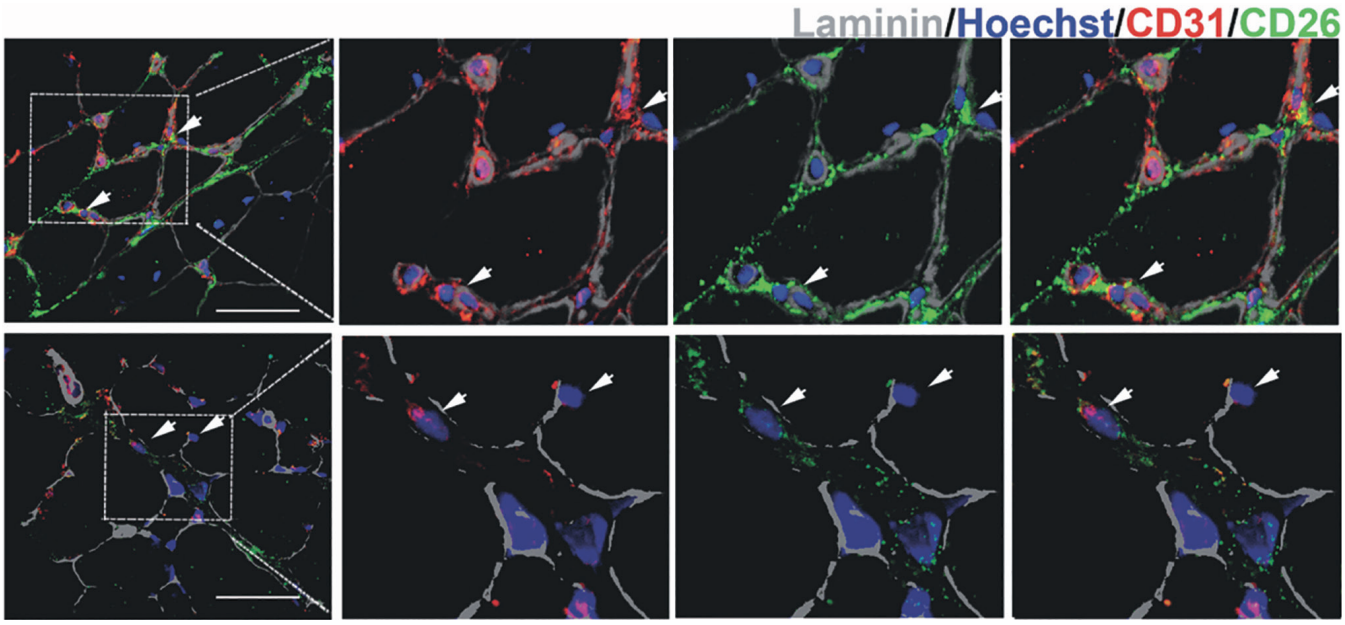


Fig. 3. CD26 expression by endothelial cells in IIM skeletal muscle.
Expression of DPP4/CD26 (green) and CD31 (red) in muscle biopsy sections of representative patients. Nuclei are counterstained with Hoechst (blue). Fibres are identified by laminin (grey).
Scale bars = 50 μm. Arrows indicate CD26$^+$ CD31$^+$ cells.

Table IV. Muscle strength in patients with different CD26 expression levels.

	High CD26	Reduced CD26
Mean MMT-8	138.2 [129-144.9]	145.7 [136-149.2]
MMT-8 at last visit	140.5 [129-145.2]*	149 [139-150]
Mean neck flexors strength	9.1 [8.1-10]	10 [9.3-10]
Neck flexors strength at last visit	10 [9-10]	10 [10-10]
Mean arm abductors strength	8.9 [6.9-9.7]	9.5 [8.4-10]
Arm abductors strength at last visit	9.5 [7.9-10]	10 [8.5-10]
Mean biceps strength	8.8 [8-9.6]**	10 [9.4-10]
Biceps strength at last visit	9.5 [8.3-10]	10 [10-10]
Mean wrist extensors strength	9.5 [7.5-9.8]*	10 [9.5-10]
Wrist extensors strength at last visit	9.2 [7-10]	10 [10-10]
Mean hip flexors strength	6 [5-8.2]**	8.7 [8-9.2]
Hip flexors strength at last visit	6 [4.5-8.5]**	9 [8-10]
Mean quadriceps strength	9.4 [7.7-9.7]**	10 [9.9-10]
Quadriceps strength at last visit	8.7 [7.8-10]*	10 [10-10]

*p<0.05, **p<0.01.
Variables were expressed as medians [IQR]. Bivariate comparisons were made using Mann-Whitney's U-test. MMT-8: manual muscle test 8.

Of interest, mean and maximum CK or aldolase levels did not correlate with the degree of CD26 expression (not shown). Infiltrating cells expressing CD26 were frequent in areas of vascular inflammation. The antigen was, in fact, significantly more expressed in tissues of patients showing perimysial CD3$^+$ T cells than in those without, and in tissues of patients with perivascular infiltrates comprising CD68$^+$ macrophages and CD3$^+$ T cells compared with those without (Table III). The presence of perimysial T lymphocytes (OR=4.66, p=0.01) and perivascular infiltrates (OR=3.42, p=0.03) survived as independent predictors of the degree of CD26 expression in skeletal muscle at multivariate analysis. No difference was found in the expression of CD26 in patients with or without perifascicular atrophy, major histocompatibility I, complement membrane attack complexes or endomysial infiltrates, either macrophages or T cells.

In the investigated biopsies, CD26 was expressed by CD3$^+$ T lymphocytes, CD31$^+$ endothelial cells and within the ECM. Interestingly, not all infiltrating T cells expressed CD26 (Fig. 2), in agreement with its differential regulation in various T cell populations. CD31$^+$ endothelial cells represent a second source of CD26 in muscle, in particular in DM. CD26 was constantly expressed throughout the microvasculature of the skeletal muscle, regardless of the presence of perivascular infiltrating inflammatory cells (Fig. 3).

CD26 expression as a biomarker of muscle performance and response to therapy

Patients were subdivided into two groups based on whether the percentage of CD26-positive FoV at muscle biopsy was higher or lower than 0.645, the median value among all patients in study. For the purpose of the analyses, patients with a value ≥0.645 were defined as having a *higher* or *more prominent* CD26 expression, while those with a value <0.645 were considered as having *reduced* levels of CD26 in skeletal muscle. Muscle strength at the time of biopsy was not influenced by the degree of CD26 expression. In contrast, MMT-8 at last visit was lower in patients with higher CD26 expression at diagnosis (Table IV). Accordingly, the strength in several muscle groups, including biceps,

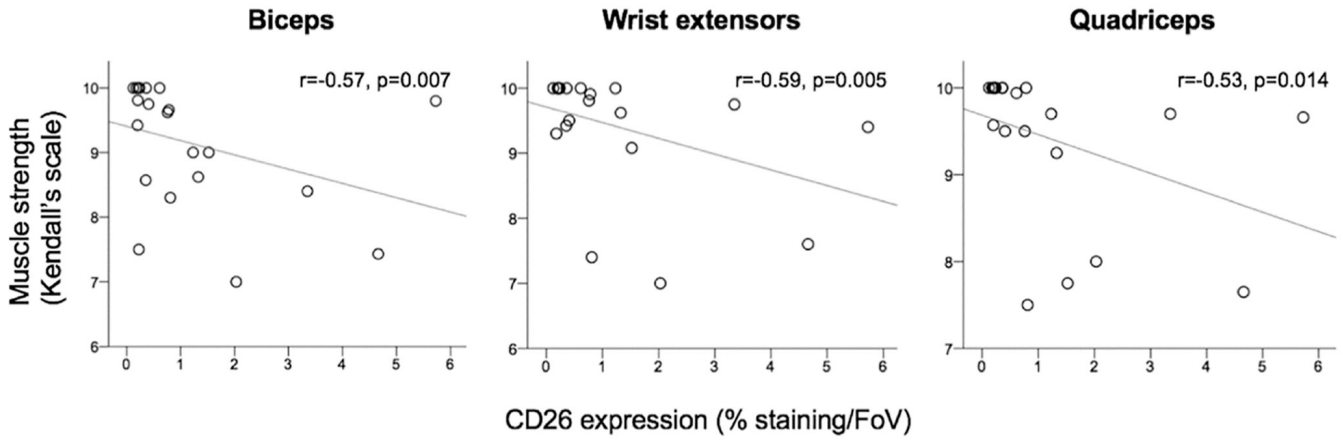


Fig. 4. Correlation of CD26 expression with muscle strength. Spearman Rho correlation (r = correlation coefficient) between muscular CD26 expression and strength in biceps, wrist extensors and quadriceps.

wrist extensors, hip flexors and quadriceps was reduced in patients with higher CD26 expression at diagnosis (Table IV). Mean strength in these muscles (Fig. 4) as well as strength in neck flexors (not shown) at last visit decreased with increasing percentages of CD26-positive FoV at diagnosis (all $p<0.02$).

All patients were treated after biopsy and diagnosis with steroids and a variety of disease-modifying anti-rheumatic agents (DMARDs) such as mycophenolate mofetil (MMF), azathioprine (AZA) and methotrexate (MTX). Intravenous immunoglobulins (IVG), tacrolimus (TAC) and the anti-CD20 monoclonal antibody rituximab (RTX) were also used in selected cases (Table I). Patients with a more prominent CD26 expression at muscle biopsy more frequently received MMF therapy in the following years (7 of 12, 58% *vs*. 2 of 13, 15%, respectively; $\chi^2=5$, $p<0.05$). CD26 expression was similar in patients treated or not with AZA, MTX, IVG or RTX (Table V).

Patients requiring either combined immunosuppressive regimens or treatment switch during the disease course had a higher expression of CD26 at muscle biopsy compared with those who responded to first-line monotherapy ($p<0.05$). Poisson regression analysis confirmed that the number of therapeutic attempts before reaching disease stabilisation or improvement increases as CD26 muscular expression increases (OR=1.2, $p<0.05$), independently of the time from disease onset, IIM type, age at onset or time of follow-up.

Discussion

This is the first study providing evidence that CD26 is selectively expressed in the skeletal muscle of patients with IIM and not of healthy controls. Although expressed in muscle biopsies of all patients with IIM, the highest expression of CD26 was found in DM muscle. Higher CD26 levels were observed when perimysial and perivascular inflammatory infiltrates were detected in muscle tissue, independently of the IIM subtype, indicating that the molecule may contribute to inflammation. We found that CD26 expression is increased in muscles with histological signs of myofibre necrosis, but it does not seem to be influenced by CK or aldolase serum levels, in agreement with the notion that events taking place in the tissue may not be faithfully reflected by bloodstream abnormalities (14).

Patients with higher CD26 expression had decreased muscle performance, with strength in neck flexors, biceps, wrist extensors, hip flexors and quadriceps decreasing with increasing muscle CD26 expression. Interestingly, patients who received MMF therapy showed a more prominent CD26 expression at muscle biopsy while having decreased muscle strength compared with patients not receiving this immunosuppressive agent (not shown). These findings, together with the observation of an increased risk of therapeutic failure with higher CD26 expression, suggests that a prominent expression of CD26 may identify a group of patients experiencing a more severe disease. Further longitudinal studies following larger patient cohorts are needed to confirm this hypothesis.

CD26 plays a role in T cell activation and signal transduction (19), and T lymphocytes are major effectors in muscle inflammation and injury in myositis (20, 21). The first suggestion of CD26 involvement in myositis came in 1990 with a report of CD26 expression on circulating mononuclear cells in patients with active IIM independently of prior immunosuppressive treatment (13). Previous reports demonstrated that CD26 prompts the trans-endothelial migratory ability of T cells (22, 23), which is confirmed by the abundance of $CD26^+$ T cells in inflamed tissues in various autoimmune diseases, such as rheumatoid arthritis and inflammatory bowel disease (24).

CD26 expression has been implicated in T_H17 and T_H1 polarisation (12, 25-29). $CD26^{high}$ $CD4^+$ T cells respond maximally to recall antigens, competently migrating to inflammatory tissues and activating B cells to generate antibodies (10, 30). Therefore, they may contribute to the memory response, perpetuating and accelerating immune-mediated tissue damage.

IFN-γ, whose production increases upon CD26-mediated co-stimulation of $CD8^+$ T cells (31), is known to enhance the expression of high mobility group protein B1 (HMGB1) and major histocompatibility complex type I (MHC-I) on regenerating muscle cells (32). It could ultimately lead to the activation

of effector T cells, the production of autoantibodies, and the migration of immune cells into skeletal muscle (33). MHC-I up-regulation in turn causes muscle degeneration and death through protein accumulation and misfolding in the endoplasmic reticulum (ER) (34, 35). The resulting necrotic material further sustains the immune response translating into persistent muscle inflammation and injury (36). Accordingly, clusterin, a protein involved in the clearance of cellular debris and apoptosis (37), is upregulated in serum and muscle of IIM patients and correlate with disease severity (38). Indeed, the preferential expression of CD26 in muscle characterised by necrosis and vascular inflammation is in line with these observations.

We observed that CD26 is expressed also within the ECM. Cytotoxic T lymphocyte degranulation has been proposed as a major source of proteolytically active soluble CD26 (39). $CD8^+$ T cells play a major role in the inflamed tissues of patients with IIM. Whether they represent a source of the antigen expressed in the ECM in muscle tissue deserves specific investigation.

Various pathways including activation of the complement cascade cause endothelial activation and damage in IIM (40). HMGB1 is a prototypical DAMP signal expressed in myofibres of patients with IIM independently of muscle inflammation (32, 41) and endowed with angiogenic properties (42). Importantly, HMGB1 is a substrate of CD26 enzymatic activity and upon cleavage loses its ability to restore capillary density (43). Accordingly, CD26 inhibitors improve wound healing in type II diabetes through enhanced HBGB1 function (43). In DM muscle biopsies, CD26 was also expressed at the endothelial level and may contribute to vascular damage.

This study is not without limitations. Muscle biopsies were all performed at time of diagnosis, one biopsy being available for each patient. Consequently, potential modifications in CD26 expression following changes in therapy or disease activity were not assessable. Moreover, the small sample size prevented us from investigating potential differences in CD26 expression according to the autoantibody specificity and may imperil the generalisability of the evidences provided, implying the need of further studies to validate our findings. On the other hand, the inclusion of a well characterised population with the same healthcare access minimises the risk of ascertainment bias. Despite these limitations, it is tempting to hypothesise that CD26 jeopardises the regeneration of inflamed skeletal muscle in IIM and may represent a target of molecular intervention for these disabling conditions, which are often refractory to currently available pharmacological treatments.

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