

Upregulation of the CD155-CD226 Axis Is Associated With Muscle Inflammation and Disease Severity in Idiopathic Inflammatory Myopathies

Wenli Li, PhD, Chuiwen Deng, PhD, Hanbo Yang, PhD, Xiaolan Tian, PhD, Lida Chen, PhD, Qingyan Liu, PhD, Chang Gao, BS, Xin Lu, PhD, Guochun Wang, PhD, and Qinglin Peng, PhD

Correspondence
Dr. Peng
pqinglin@163.com

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Abstract

Background and Objectives

The CD155-CD226/T-cell Ig and immunoreceptor tyrosine-based inhibitory motif (ITIM) domain (TIGIT) pathway plays a critical role in regulating T-cell responses and is being targeted clinically. However, research on the role of this pathway in autoimmune diseases is limited. This study aimed to investigate the expression and tissue-specific roles of CD155-CD226/TIGIT pathway molecules in the inflamed muscles of patients with idiopathic inflammatory myopathies (IIMs).

Methods

Immunohistochemistry, Western blot analysis, and polychromatic immunofluorescence staining were performed to examine the expression of CD155, CD226, and TIGIT in skeletal muscle biopsies from 30 patients with dermatomyositis (DM), 10 patients with amyopathic DM (ADM), 20 patients with immune-mediated necrotizing myopathy (IMNM), 5 patients with dysferlinopathy, and 4 healthy controls. Flow cytometry analysis was used to analyze the functions of T cells with different phenotypes.

Results

Strong expression of CD155 was observed in patients with DM and IMNM, while its expression was largely negative in those with ADM and dysferlinopathy and healthy controls. The costimulatory receptor CD226 was highly expressed on muscle-infiltrating cells, while the coinhibitory receptor TIGIT was expressed at low levels. These infiltrating CD226+ cells were mainly activated effector T cells that localized adjacent to CD155-expressing myofibers, but were faintly detectable within the muscle fascicles lacking CD155. A strong positive correlation between CD155 and CD226 expression scores was also observed. Polychromatic immunofluorescence staining revealed that CD155+ muscle cells coexpressed major histocompatibility complex classes I and II, and tumor necrosis factor alpha expression was detected in CD226+ T cells at their close sites with the myofibers. Furthermore, the expression levels of CD155 and CD226 showed a positive correlation with creatine kinase, lactate dehydrogenase, and the muscle histopathology damage scores and an inverse correlation with the Manual Muscle Testing-8 scores. In addition, CD155 and CD226 expressions were significantly decreased in representative patients who achieved remission posttreatment.

Discussion

These findings demonstrate that the CD155-CD226 axis is highly activated in inflamed muscle tissues of patients with IIM and is associated with muscle disease severity. Our data uncover the immunopathogenic role of the axis in the pathology of IIMs.

From the Department of Rheumatology (W.L., H.Y., X.T., Q.L., C.G., X.L., G.W., Q.P.), Key Myositis Laboratories, China-Japan Friendship Hospital; Department of Rheumatology and Clinical Immunology (C.D.), Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College; and Department of Blood Transfusion (L.C.), China-Japan Friendship Hospital, Beijing.

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Glossary

ADM = amyopathic dermatomyositis; **CK** = creatine kinase; **DM** = dermatomyositis; **IBM** = inclusion body myositis; **IFN- γ** = intracellular interferon- γ ; **IIM** = idiopathic inflammatory myopathy; **IMNM** = immune-mediated necrotizing myopathy; **ITIM** = immunoreceptor tyrosine-based inhibitory motif; **LDH** = lactate dehydrogenase; **MHC** = major histocompatibility complex; **MMT-8** = Manual Muscle Testing-8; **NK** = natural killer; **PBST** = phosphate-buffered solution-Triton; **PM** = polymyositis; **TIF1- γ** = transcriptional intermediary factor 1 γ ; **TIGIT** = T-cell Ig and ITIM domain; **TNF- α** = tumor necrosis factor α .

Idiopathic inflammatory myopathies (IIMs) are a heterogeneous family of autoimmune disorders clinically characterized by weakness and low endurance of the skeletal muscle and are histopathologically characterized by pathologic myofibers and inflammatory infiltrates in muscle tissues.^{1,2} IIMs are mainly categorized into dermatomyositis (DM), amyopathic DM (ADM), polymyositis (PM), immune-mediated necrotizing myopathy (IMNM), and inclusion body myositis (IBM). The mechanisms involved may vary between the different IIM subtypes. For DM, the membrane attack complex-mediated vascular damage and muscle ischemia are considered as the main immunopathologic mechanism.^{1,3,4} In addition, many studies have confirmed that type 1 interferon play a role in DM.² Intriguingly, the immune response to transcriptional intermediary factor 1 γ (TIF1 γ), a DM-specific autoantigen, has been demonstrated to induce experimental myositis, presenting as muscle necrosis/atrophy in mice.⁵ Furthermore, transfer of TIF1 γ -specific CD8⁺ T cells, but not CD4⁺ T cells or IgG, into naïve recipient mice led to myositis,⁵ thus providing novel insights into DM pathogenesis. In PM and IBM, the primary mechanism underlying muscle damage is mediated by cytotoxic T cells and major histocompatibility complex (MHC)-I antigen-expressing muscle fibers.^{1,3,4} IMNM, a separate entity of IIMs, is characterized by the presence of scattered necrotic fibers in muscle tissue,⁶ and autoantibodies against signal recognition particle and hydroxymethylglutaryl-coenzyme A reductase have shown a pathogenic role in IMNM.^{7,8} Macrophages were considered to be a dominant factor in immune infiltration in IMNM; however, a cohort of patients with IMNM showed significant CD3⁺ T-cell infiltration levels, similar to those in other IIMs.^{9,10} Therefore, despite that the pathophysiology varies within subcategories, muscle inflammation is a common histologic feature across all conditions, and muscle-infiltrating autoreactive T cells are believed to play a role in IIM.¹¹

In recent years, an increasing number of studies have focused on the specific surface molecules of infiltrating cells to identify cell subsets with potential pathogenic roles in IIMs. For example, interactions between inducible co-stimulatory molecule (ICOS⁺) CD8⁺ T cells and ICOS-L-expressing myofibers are critical in initiating or perpetuating T cell-mediated cytotoxicity.¹² High frequencies of CD28null T cells have been found in muscle infiltrates of IIMs, and CD28null T cells have higher levels of inflammatory factor secretion and cytotoxicity than their CD28⁺ counterparts.¹³⁻¹⁶ Recently, enriched programmed cell death protein 1 (PD1)⁺CD3 T cells have been shown to be involved in IMNM and IBM, and their accumulation in muscle tissues could

contribute to T-cell exhaustion through PD-1 and the ligand of programmed cell death protein 1 (PD-L1)/PD-L2 interactions.¹⁷ Further investigations of special subtypes of T cells and their downstream events are required to improve the understanding of the mechanistic role of infiltrating CD4⁺/CD8⁺ T cells in muscle tissues and uncover potential therapeutic targets for IIMs.

The CD155-CD226/T-cell Ig and ITIM domain (TIGIT) immune checkpoint pathway has attracted increased research attention because it plays an important role in regulating immune cell responses. TIGIT serves as a coinhibitory receptor and CD226 functions as a costimulatory receptor. CD226 and TIGIT compete for the same ligand (CD155), and their interactions lead to different outcomes; activation of CD226 promotes T-cell immune responses, whereas activation of TIGIT inhibits T-cell activity.^{18,19} Similar to an “accelerator” and “brake,” respectively, CD226 and TIGIT finely regulate the activity of immune cells, and an imbalance in this regulation may be involved in the development of diseases. In the field of tumor immunology where CD155 is widely expressed on tumor cells and TIGIT is overexpressed on tumor-infiltrating cells, the TIGIT-CD155 pathway has been believed to promote tumoral immune escape and is attracting attention as a promising anti-tumor target to improve immune responses to tumors.^{20,21}

Our previous study found an imbalance of TIGIT/CD226 on the peripheral T cells in DM.²² However, the level of TIGIT and CD226 coexpression in muscle-infiltrating immune cells within the muscle microenvironment in IIMs is currently unknown. Of interest, CD155 has been found at the end plate regions of normal muscle fibers, and denervated and regenerating muscle fibers, suggesting that the expression of CD155 in muscles serves as a route of entry for viruses into motor neurons, increasing the susceptibility to the poliovirus infection.²³⁻²⁵ However, the expression of CD155 in the muscle of IIM remains to be fully clarified. Thus, a thorough examination of CD155 expression and the balance between CD226 and TIGIT in inflamed muscle tissues in IIMs is needed. In this study, we sought to assess the expression profiles of CD155 and its receptors, CD226 and TIGIT, in inflamed muscle tissues and to investigate their potential roles in patients with IIMs.

Methods

Patients and Data Collection

From August 2019 to March 2023, a total of 30 patients with confirmed DM, 10 patients with amyopathic DM (ADM),

and 20 patients with IMNM from China-Japan Friendship Hospital were enrolled in this study. Demographic, clinical, and laboratory data are summarized in Table 1. The diagnosis of DM and ADM was based on the 2017 European League Against Rheumatism/American College of Rheumatology classification criteria.²⁶ The diagnosis of IMNM was based on the European Neuro Muscular Center criteria.⁶ Upon first admission to our department, muscle biopsy was performed in all patients with IIM. In addition, 5 muscle specimens from

patients with dysferlinopathy were collected as disease controls (2 female individuals/3 male individuals, the average age was 54 years). Surgical muscle specimens from 4 trauma patients without myofibropathy were collected as healthy controls (2 female individuals/2 male individuals, the average age was 52 years).

Standard Protocol Approvals, Registrations, and Patient Consents

This study was approved by the Ethics Committee of China-Japan Friendship Hospital (2019-25-K19). All patients provided written informed consent.

Histologic Review and Pathology Scoring

Immunohistochemical and histochemical staining for routine diagnostic purposes were performed, and all individuals were examined using a pathology scoring tool for juvenile DM with some modifications.²⁷ Muscle fiber domain scoring was determined by the presence and extent of perifascicular atrophy, necrosis, and MHC-I expression on the sarcolemma. Inflammatory domain scoring was determined by the number of infiltrated CD4⁺ T, CD8⁺ T, CD20⁺ B, and CD68⁺ cells in the endomysium, perimysium, and perivascular regions. The expression of myxovirus-resistance protein A (MxA) in muscle fibers was scored with values from 0 to 3 (0 = no MxA staining; 1 = weak; 2 = moderate; and 3 = strong).²⁸ The membrane attack complex deposition on capillaries was scored with values from 0 to 2 (0 = no capillary stain; 1 = unequivocal circumferential capillary stain, but focal or weak; and 2 = patchy or diffused, strong circumferential capillary stain).²⁹

Immunohistochemical Staining

Frozen 8- μ m thick muscle sections were fixed with 95% ethanol for 10 minutes and then rinsed thrice in phosphate-buffered solution Triton (PBST) (0.025%). The sections were incubated in H₂O₂ (0.3%) to block any endogenous peroxidase activity for 10 minutes and then washed and blocked in fresh sheep serum for 3 hours at 25°C to decrease nonspecific binding. Antihuman CD155 monoclonal antibodies (Cell signaling, clone D3G7H, dilution 1:100), antihuman CD226 monoclonal antibodies (Cell signaling, clone E8L9G, dilution 1:200), and antihuman TIGIT monoclonal antibodies (ebioscience, clone MBSA43, dilution 1:100) were used as primary antibodies and incubated overnight at 4°C. After incubation, the sections were rinsed thrice for 5 minutes with PBST and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 30 minutes at 25°C. The substrate 3,3'-diaminobenzidine was used as chromogen to visualize the proteins.

The tissue sections were viewed separately by 2 experienced pathologists during a blind exercise. A previously described scoring method was applied to evaluate the CD155, CD226, and TIGIT immunohistochemical staining.²⁷ CD155 scores were defined as 0 (no positive muscle fibers), 1 (positive cell percentage ranging 1%–20%), or 2 (positive cell percentage \geq 20%). CD226 and TIGIT scores were defined as

Table 1 Characteristics of Patients With IIM Enrolled in the Study

Characteristics	ADM (n = 10)	DM (n = 30)	IMNM (n = 20)
Female/male ratio	7/3	18/12	13/7
Mean age (y) \pm SD	46.40 \pm 14.38	42.00 \pm 15.66	44.55 \pm 16.57
Time from disease onset to muscle biopsy, mo, median (range)	—	5.5 (0.3–168)	12 (1–120)
Clinical features			
Skin rash (%)	100.0 (10/10)	100.0 (30/30)	0.0 (0/20)
Muscular weakness (%)	0.0 (0/10)	80.0 (24/30)	75.0 (15/20)
Arthralgia (%)	50.0 (5/10)	30.0 (9/30)	10.0 (1/10)
Dysphagia (%)	0.0 (0/10)	40.0 (12/30)	25.0 (5/20)
ILD (%)	70.0 (7/10)	50.0 (15/30)	35.0 (7/20)
Malignancy (%)	0.0 (0/10)	6.7 (2/30)	0.0 (0/20)
Laboratory features			
CK, IU/L, mean \pm SD	68.3 \pm 56.40	1,437 \pm 3,416	3,677 \pm 2,326
LDH, IU/L, mean \pm SD	208.4 \pm 55.70	454.0 \pm 281.4	703.0 \pm 348.1
MSA positive(%)	60.0(6/10)	70.0 (21/30)	80.0 (16/20)
Anti-MDA5+ (%)	40.0 (4/10)	13.3 (4/30)	0.0 (0/20)
Anti-TIF1-γ+ (%)	0.0 (0/10)	30.0 (9/30)	0.0 (0/20)
Anti-NXP2+ (%)	0.0 (0/10)	3.3 (1/30)	0.0 (0/20)
Anti-Mi-2+ (%)	0.0 (0/10)	13.3 (4/30)	0.0 (0/20)
Anti-SAE+ (%)	0.0 (0/10)	3.3 (1/30)	0.0 (0/20)
Anti-PL-7+ (%)	20.0 (2/10)	3.3 (1/30)	0.0 (0/20)
Anti-EJ+ (%)	0.0 (0/10)	3.3(1/30)	0.0 (0/20)
Anti-SRP+ (%)	0.0 (0/10)	0.0 (0/30)	35.0 (7/20)
Anti-HMGCR+ (%)	0.0 (0/10)	0.0 (0/30)	45.0 (9/20)

Abbreviations: ADM = amyopathic DM; CK = creatine kinase; DM = dermatomyositis; EJ = glycyl-tRNA synthetase; HMGCR = hydroxymethylglutaryl-coenzyme A reductase; IIM = idiopathic inflammatory myopathy; ILD = interstitial lung disease; IMNM = immune-mediated necrotizing myopathy; LDH = lactate dehydrogenase; MDA5 = melanoma differentiation-associated protein 5; Mi-2 = complex nucleosome remodeling histone deacetylase; MSA = myositis-specific antibodies; NXP2 = nuclear matrix protein 2; PL-7 = threonyl-tRNA synthetase; SAE = small ubiquitin-like modifier activating enzyme; SRP = signal recognition particle; TIF1- γ = transcriptional intermediary factor 1 γ .

0 (none or <4 positive cells in a ×20 field), 1 (>4 positive cells in a ×20 field and/or 1 cluster, where a cluster is >10 positive cells), or 2 (> clusters in entire biopsy and/or diffused infiltrating cells, i.e., >20 positive cells in a ×20 field).

Western Blot

Total protein extracted from the muscle tissues was loaded onto 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. After blocking with 5% nonfat milk powder and incubation overnight with anti-CD155 monoclonal antibodies (Cell signaling, clone D3G7H, dilution 1:1,000) and anti-glyceraldehyde-3-phosphate dehydrogenase monoclonal antibodies (Immunoway, catalog #YM3029, dilution 1:10,000) as the primary antibodies, the membranes were incubated with appropriate secondary antibodies and then visualized using an enhanced chemiluminescent detection kit (Thermo Scientific, Catalog #34095). Quantitative analyses were performed using Image J software.

Polychromatic Immunofluorescence Staining

Based on the Tyramide signal amplification technique, a 4-color multiple fluorescent immunohistochemical staining kit (Absin, Catalog #abs50012) was used to perform polychromatic immunofluorescence staining. The steps were as follows: 5- μ m thick paraffin sections were dewaxed in xylene, rehydrated through gradients of alcohol, and fixed in neutral formalin immersion (10%) for 10 minutes. The antigen was repaired in sodium citrate buffer (pH = 6.0) using a microwave and cooled to room temperature. After blocking with goat serum for 10 minutes, sections were incubated with the following primary antibodies for 1 hour at room temperature: anti-CD155 (Cell signaling, clone D3G7H, dilution 1:1,000), anti-CD226 (Cell signaling, clone E8L9G, dilution 1:200), antihuman TIGIT (Thermo Fisher, clone BLR047F, dilution 1:200), anti-CD4 (Origene, catalog #ZA-0519, ready-to-use), anti-CD8 (Origene, catalog #ZA-0508, ready-to-use), anti-CD69 (Abcam, catalog #ab233396, dilution 1:500), anti-HLA-DR (Abcam, catalog #ab92511, dilution 1:500), anti-CD244 (Abcam, catalog #ab256370, dilution 1:50), anti-MHC-I (Abcam, catalog #ab52922, dilution 1:500), anti-MHC-II (Santa, catalog #sc-59318, dilution 1:100), and anti-tumor necrosis factor (TNF)- α (Abcam, catalog #ab183218, dilution 1:5,000). After incubation, the sections were washed in PBST. They were then incubated with their respective secondary antibodies for 10 minutes and then incubated with fluorescent dye (diluted by amplification reagent) for 10 minutes. After the microwave repair, the slides were cooled to room temperature. For multiple fluorescent staining, the abovementioned steps were repeated until all antigens were stained. Finally, sections were incubated with 4',6-diamidino-2-phenylindole for 5 minutes and sealed with antifluorescence quencher. For frozen sections, the microwave repair steps were replaced with elution steps. After incubation with primary antibodies, the sections were washed in an eluent solution (Absin, Catalog #abs994) for 10 minutes. Tissue sections were imaged using Panoramic MIDI, and the image data were analyzed using Case Viewer 2.3.

In Vitro Functional Assays of T Cells by Flow Cytometry

To analyze the effector function of T cells with different phenotypes, we examined intracellular interferon γ (IFN- γ) and TNF- α staining of CD4 T cells with different CD226 phenotypes and CD107a staining of CD8 T cells with different CD226 phenotypes by flow cytometry. Approximately 1×10^6 purified peripheral blood mononuclear cells (PBMCs) were stimulated for 4 hours with Leukocyte Activation Cocktail (BD Biosciences) at 37°C before surface marker staining (anti-CD3-APC-cy7, anti-CD8-PE-cy7, anti-CD226-FITC, and anti-CD107a-PE). After fixation and permeabilization using Cytofix/Cytoperm (BD Biosciences, USA), the cells were incubated with anti-IFN- γ -PerCP-Cy 5.5 and anti-TNF- α -APC for 20 minutes at room temperature. All antibodies used for flow cytometry were obtained from BD Bioscience (Franklin Lakes, NJ). Each experiment was performed on a FACSCanto II instrument (Becton Dickinson, San Jose), and the data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad, Inc., La Jolla, CA) and the R programming package “ggplot2.” For continuous variables, the results were presented as the mean \pm SD and compared with paired or unpaired 2-tailed *t* tests. For nonparametric distribution data, the data were presented as the median and range and compared with Mann-Whitney *U* tests. Categorical data were presented as percentages (%). The Spearman rank correlation analysis was used to examine the correlations. *p* Values <0.05 were considered statistically significant.

Data Availability

The authors confirm that data supporting the findings of this study are available within the article. Data not provided in the article because of space limitations can be available from the corresponding author on reasonable request.

Results

CD155 was Highly Expressed on Muscle Fibers in IIMs

First, we examined the expression of CD155 in the muscle tissues of enrolled participants. Immunohistochemistry analysis showed that CD155 was highly expressed in muscle tissue from most of the patients with DM (21/30, 70.0%). The muscle fibers positive for CD155 were preferentially observed in perifascicular regions, although they were sometimes diffusely distributed (Figure 1A). CD155 expression was also detected in a high proportion (18/20, 90%) of patients with IMNM and showed a diffused pattern (Figure 1B). By contrast, muscle tissue showed negative immunoreactivity to CD155 in patients with ADM (0/10, 0.0%), patients with dysferlinopathy (0/5, 0.0%), and healthy controls (0/4, 0.0%) (Figure 1, C and D).

In addition, Western blot analysis showed that CD155 proteins were highly expressed in muscle tissue from patients with DM and IMNM but were lowly expressed in muscle tissues from healthy controls and patients with ADM (Figure 1E).

High Expression of CD226 and Low Expression of TIGIT on Muscle-Infiltrating Cells

Next, we investigated the expression level of receptors to CD155 in muscle tissue from patients with IIM. Notably, in the muscle biopsies of patients with DM and IMNM, muscle-infiltrating inflammatory cells were largely CD226 positive and rarely TIGIT positive (Figure 2A), indicating that CD155-CD226-mediated stimulatory signaling is much stronger than the CD155-TIGIT-mediated coinhibitory signal in the muscle microenvironment of patients with IIM (Figure 2A). No significant expression of CD226 and TIGIT were found in patients with ADM, patients with dysferlinopathy, and healthy

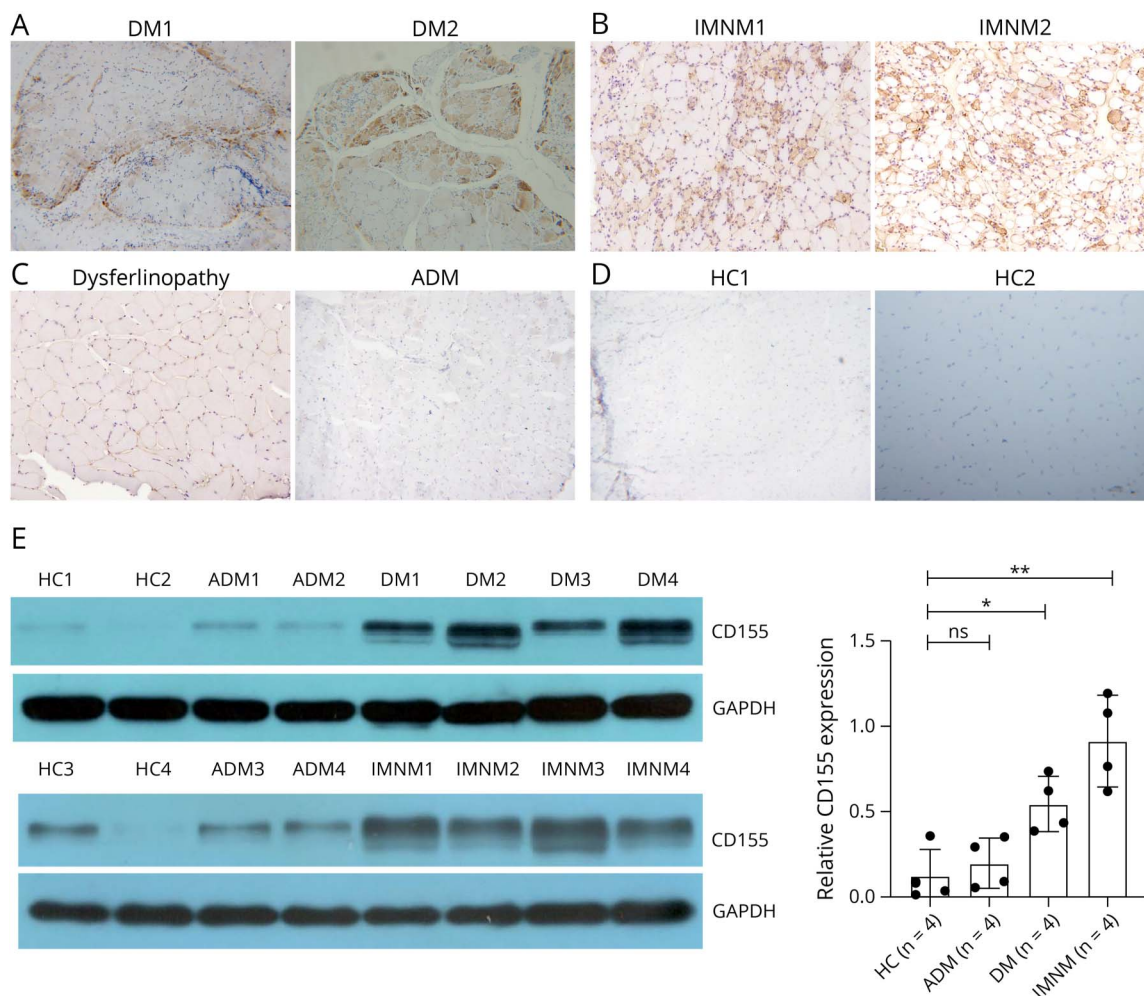
individual (eFigure 1, links.lww.com/NXI/A880). The scores of CD226/TIGIT staining from the entire cohort of patients are presented in Figure 2A.

We further analyzed the relative expression of CD226 and TIGIT in a single muscle biopsy using multiple color fluorescent staining. As shown in Figure 2B, we confirmed a large number of CD226-positive cells and a relatively small number of TIGIT-positive cells in muscle tissue from patients with DM and IMNM.

Muscle-Infiltrating CD226+ Cells are Predominantly Activated Effector CD4⁺ or CD8⁺ T Cells

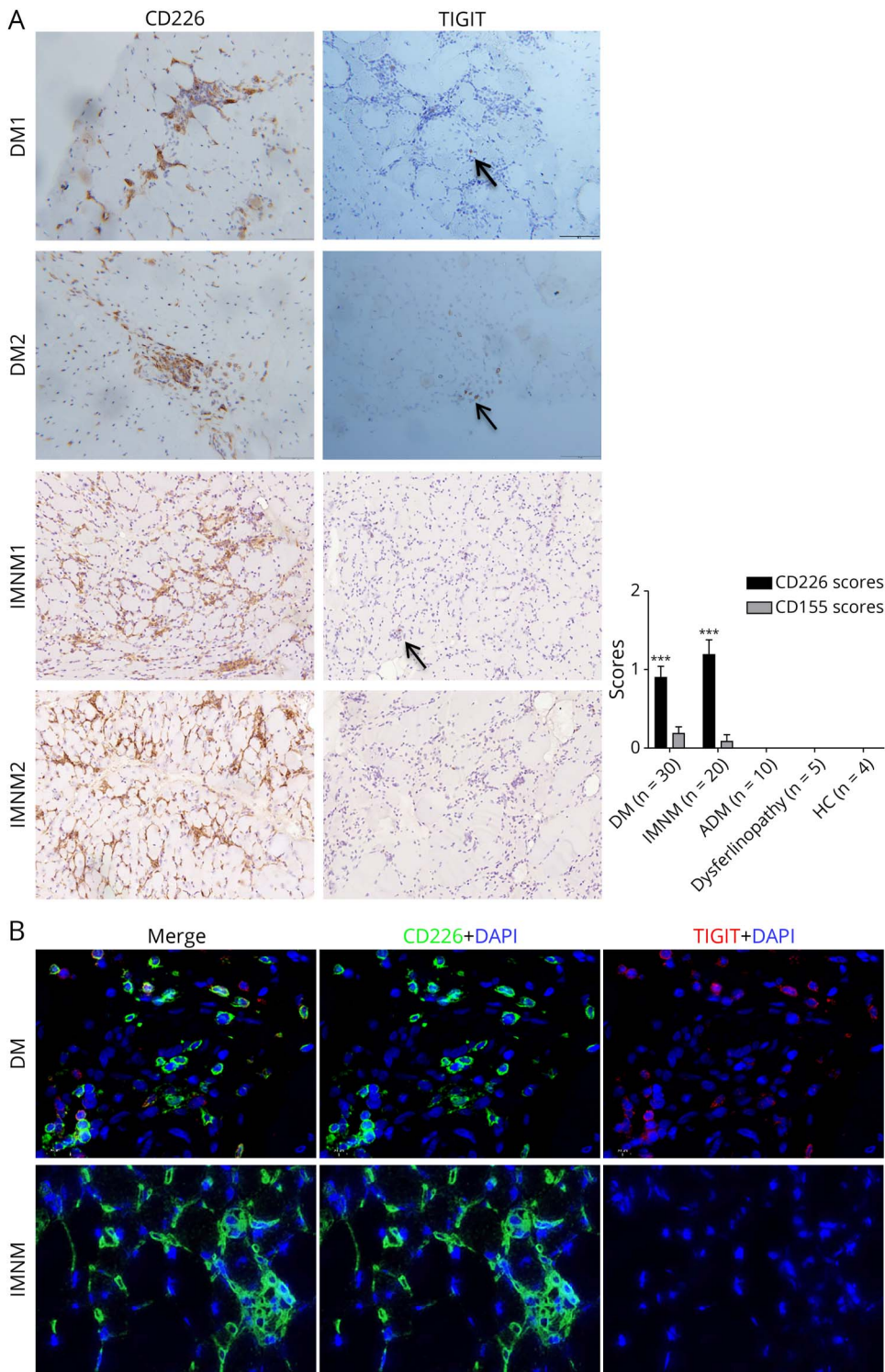
In muscle biopsies from patients with DM and IMNM, CD226+ cells were diffusely distributed or were found in clusters, and they mainly consisted of CD4⁺ T cells, with a small amount of CD8⁺ T cells (Figure 3A). CD69 is an early activation marker that is associated with tissue residence and

Figure 1 Expression of CD155 in the Muscle of Patients With IIM



(A) Robust expression of CD155 was observed preferentially in perifascicular regions in DM. (B) High expression of CD155 in a diffused pattern was found in the muscle of patients with IMNM. (C) and (D) By contrast, this expression was largely negative in patients with ADM and dysferlinopathy and healthy controls. (A-D, original magnification $\times 200$). (E) Western blot analysis revealed higher levels of CD155 in patients with DM and IMNM compared with healthy controls and patients with ADM, and the relative expression levels of CD155 of different groups are shown in the bar graph. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ADM = amyopathic dermatomyositis; DM = dermatomyositis; HC = healthy control; IIMs = idiopathic inflammatory myopathies; IMNM = immune-mediated necrotizing myopathy.

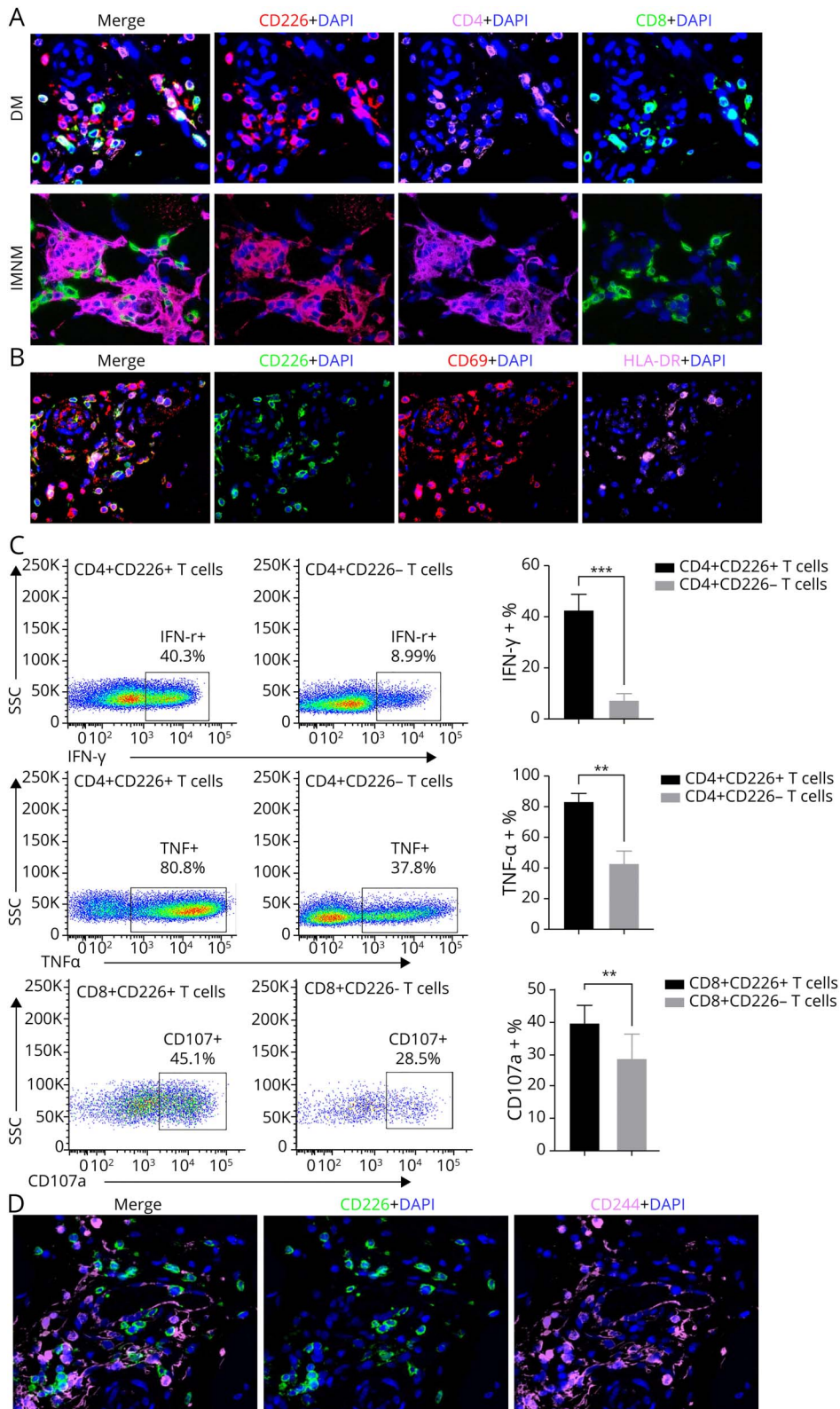
Figure 2 Expression Analysis of CD226 and TIGIT Proteins



chronic refractory inflammation.³⁰ HLA-DR is a classic late activation marker.³¹ Polychromatic immunofluorescence staining showed that most CD226+ cells were CD226+CD69+ or CD226+HLA-DR+ phenotypes (Figure 3B), indicating CD226+ T cells are in an activated state in the muscle tissue of patients with IIM.

We further obtained PBMCs from patients with DM and assessed the effector function of CD4 T cells with different CD226 phenotypes by analyzing their cytokine production capacity, using flow cytometry in vitro. As shown in Figure 3C, the percentages of IFN- γ and TNF- α production from CD4+CD226+ T cells were significantly higher than that

Figure 3 CD226+ Cells Were Mainly Activated Effector CD4+ or CD8+ T Cells



(A) In the biopsies of patients with DM (n = 5) and IMNM (n = 3), CD226+ cells were strong coexpressed CD4 or CD8 cells. (B) A representative staining from a patient with DM showed that most of the CD226+ cells were CD226+CD69⁺ or CD226+HLA-DR⁺ phenotypes. (C) In PBMCs isolated from patients with DM, the production percentages of IFN- γ and TNF- α of CD4⁺CD226⁺ T cells and CD107a expression percentages of CD8⁺CD226⁺ T cells were significantly higher than those of the CD226-negative counterparts in patients with DM (n = 5). (D) Costaining of CD226 and CD244 in the DM sections (n = 3) showed that the CD226+ cells did not colocalize with CD244+ cells. (A, B, and D, original magnification $\times 400$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. DM = dermatomyositis; IFN- γ = intracellular interferon- γ ; IMNM = immune-mediated necrotizing myopathy; TNF- α = tumor necrosis factor α .

from their CD226 counterparts in patients with DM ($45.02 \pm 6.68\%$ vs $7.50 \pm 2.80\%$, $p < 0.001$ for IFN- γ ; and $83.1 \pm 5.48\%$ vs $42.76 \pm 8.38\%$, $p = 0.002$ for TNF- α) (Figure 3C).

Measuring degranulation through CD107a expression has become an advantageous tool for examining the functional capacity of activated cytotoxic CD8 T cells.^{32,33} As shown

in Figure 3C, increased CD107a expression was observed on CD8⁺CD226⁺ T cells when compared with CD8⁺CD226⁻ T cells in patients with DM ($39.54 \pm 6.01\%$ vs $28.26 \pm 8.05\%$, $p = 0.0046$). These results confirmed that CD226⁺ T cells are a significant subset with an enhanced effector function.

Notably, few cells coexpressing CD226 and CD244 were found (Figure 3D) in this study, highlighting CD226-expressing cells in muscle from patients with IIM as a new subset of activated cells distinct from CD28null cells.

CD226⁺ T Cells Were Localized Adjacent to CD155/MHC Coexpressing Muscle Fibers

In muscle biopsies from patients with DM, we found that CD226⁺ cells tended to infiltrate and aggregate near CD155-positive muscle fibers (Figure 4A). By contrast, CD226 was faintly detectable within muscle fascicles that were CD155-negative (Figure 4A). In muscle biopsies from patients with IMNM, a close contact between CD226⁺ cells and CD155-expressing muscle fibers was also observed (Figure 4B). In addition to the colocalized expression, we found a strong positive correlation between the expression levels of CD155 and CD226 in muscles from patients with DM and IMNM ($r = 0.7412$, $p < 0.0001$) (Figure 4C).

We further examined the expressions of MHC molecules in CD155⁺ muscle fibers. As shown in Figure 4D, most CD155⁺ muscle cells coexpressed MHC classes I and II in muscle biopsies from patients with IIM. Notably, polychromatic immunofluorescence staining revealed that a considerable number of CD226⁺ T cells secreted the inflammatory cytokine TNF- α at a location close to the myofibers (Figure 4E). Therefore, we speculate that CD155⁺ myofibers formed immunologic synapses with CD226⁺ T cells.

Expression Levels of CD155 and CD226 Were Associated With the Severity of Muscle Involvement

Next, we examined the clinical relevance of the expression levels of CD155 and CD226 in patients with DM and IMNM. As summarized in Table 2, the CD155 scores were positively correlated with serum creatine kinase (CK) and lactate dehydrogenase (LDH), the muscle fiber domain scores, and the inflammatory domain scores and inversely correlated with Manual Muscle Testing-8 (MMT-8) scores. The CD226 scores also showed a positive correlation with CK and LDH and the muscle histopathology damage scores and an inverse correlation with the MMT-8 scores. These findings suggest that the high expression levels of CD155 and CD226 in the muscle microenvironment of patients with IIM might be associated with the severity of muscle damage. Some statistically significant correlations between CD4/CD8 T cells and clinical indicators were also observed (Table 2). Of note, compared with that of CD4⁺ and CD8⁺ T cells, the expression levels of CD155 and CD226 exhibited superiority in correlation with CK, LDH, and MMT-8.

We further investigated the expression levels of CD155 and CD226 in muscle biopsies by immunohistochemical staining in repeated biopsies from a representative DM (Figure 5A) and an IMNM (Figure 5B) patient who achieved remission after conventional immunosuppressive treatment (glucocorticoids and immunosuppressants). Of interest, CD155 and CD226 levels were drastically decreased after treatment in these patients.

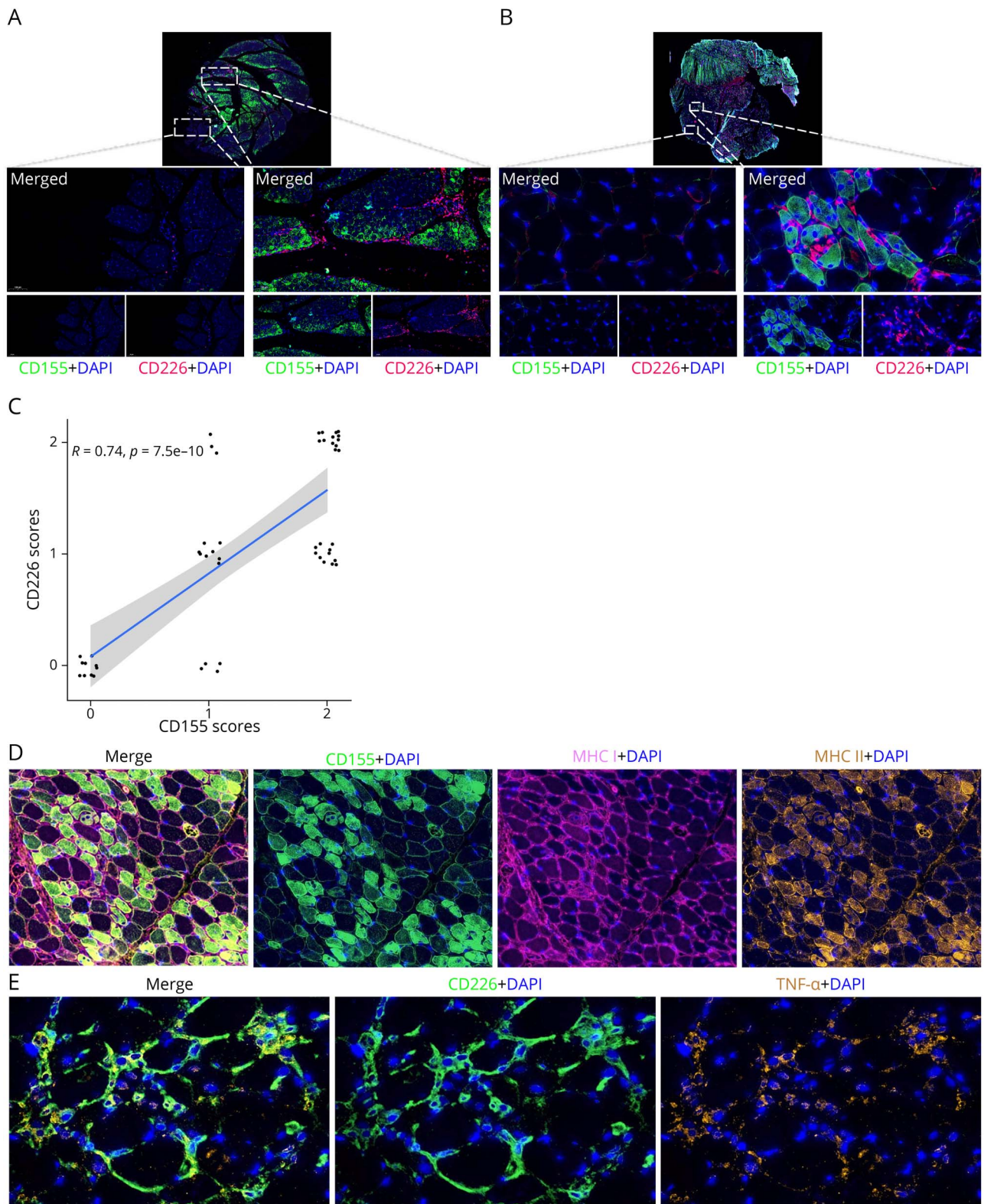
Discussion

In this study, a high expression level of CD155 on muscle fibers and high number of CD226⁺ inflammatory infiltrates were found in patients with IIM. The expression levels of these molecules were significantly associated with the severity of muscle damage, indicating that CD155-CD226-mediated stimulatory signaling may be involved in the pathogenesis of IIMs.

CD155, commonly known as the poliovirus receptor, is a type I transmembrane glycoprotein from the immunoglobulin superfamily.³⁴ Although CD155 can be expressed on normal cells at low levels, including neuronal, epithelial, endothelial, and fibroblastic cells, CD155 is barely detected in most healthy tissues.³⁵ Strong CD155 expression can be detected in a number of human malignancies. It is involved in different processes such as cell adhesion, migration, and proliferation. Moreover, it is correlated with poor prognosis in several human cancers.³⁶⁻³⁹ In this study, we reported that CD155 is highly expressed on muscle fibers in patients with IIM.

As an immunoregulatory molecule, CD155 can bind with the costimulatory receptor CD226 and coinhibitory receptor TIGIT, thereby regulating the immune response.^{18,19} CD226 is an immune-activating receptor that belongs to the Ig superfamily and is mainly expressed on natural killer (NK) and T cells. CD226 interacting with CD155 can initiate intracellular signals that promote cytotoxicity and cytokine production.⁴⁰ Of note, NK and T cells can also express inhibitory receptors that are able to bind to CD155, namely TIGIT. CD155-mediated TIGIT activation can induce inhibitory signals.⁴¹ The balance between the contrasting CD155-CD226 and CD155-TIGIT signals contributes to regulation of the NK/T-cell effector functions.⁴² CD226 downregulation and TIGIT upregulation from tumor-infiltrating NK/T-cell surfaces and concomitant overexpression of CD155 on tumor cells contribute to tumor immune escape.²¹ Of interest, we observed that CD226 was highly expressed on muscle-infiltrating cells, while TIGIT was expressed at low levels in the muscle microenvironment of patients with IIM. Furthermore, we found these CD226-expressing cells were CD69⁺/HLA-DR⁺ activated T cells. In vitro functional assays showed that the production percentages of IFN- γ and TNF- α of CD4⁺CD226⁺ T cells were significantly higher than those of the CD226

Figure 4 CD226+ T Cells Were Strongly Enriched Near CD155/MHC Coexpressing Myofibers



(A) In DM biopsies (n = 5), CD226+ cells localized adjacent to CD155 expressing myofibers. Conversely, CD226 was faintly detectable within the muscle fascicles lacking CD155. (B) In IMNM biopsies (n = 3), a close contact between CD226+ cells and CD155-expressing muscle fibers was observed. (C) There was a strong positive correlation between the CD155 and CD226 scores in patients with DM (n = 30) and IMNM (n = 20). The R language-ggplot2 package was used to create the geom_jitter plot here to prevent hidden points in the same position on the scatter plot. (D) A representative staining from a patient with IMNM showed that CD155+ muscle cells coexpressed MHC class I and/or class II in muscle biopsies. (E) A representative staining from a patient with IMNM showed that TNF- α was detected in some CD226+ T cells at a location close to the myofibers. (A, original magnification $\times 100$; B, original magnification $\times 600$; D and E, original magnification $\times 200$). DM = dermatomyositis; IMNM = immune-mediated necrotizing myopathy; TNF- α = tumor necrosis factor- α .

Table 2 Correlation Between CD155/CD226 Expression Levels and Clinical/Laboratory Parameters in Patients With IIM (30 DM and 20 IMNM)

Scores	Statistical value	CK	LDH	MMT-8	Time from disease onset to muscle biopsy	Pathologic scores			
						Muscle fiber domain scores	Inflammatory domain scores	MxA scores ^a	C5b-9 scores
CD155 scores	Spearman R	0.2955	0.3527	-0.3909	0.1190	0.6574	0.6423	0.5975	0.3744
	<i>p</i> Value	0.0373	0.0120	0.0050	0.4103	<0.0001	<0.0001	0.0005	0.0074
CD226 scores	Spearman R	0.3271	0.3153	-0.3189	0.01832	0.4639	0.6588	0.4633	0.3229
	<i>p</i> Value	0.0204	0.0257	0.0240	0.8995	0.0007	<0.0001	0.0099	0.0222
CD4 scores	Spearman R	0.2941	0.1788	-0.1373	0.0099	0.5779	0.8914	0.5617	0.4708
	<i>p</i> Value	0.0382	0.2141	0.3418	0.9458	<0.0001	<0.0001	0.0012	0.0006
CD8 scores	Spearman R	-0.0761	-0.0872	-0.2339	-0.2113	0.4021	0.8028	0.2447	0.4255
	<i>p</i> Value	0.5995	0.5469	0.1021	0.1408	0.0038	<0.0001	0.1925	0.0021

Abbreviations: CK = creatine kinase; DM = dermatomyositis; IIM = idiopathic inflammatory myopathy; IMNM = immune-mediated necrotizing myopathy; LDH = lactate dehydrogenase; MxA = myxovirus-resistance protein A; MMT-8 = Manual Muscle Testing-8.

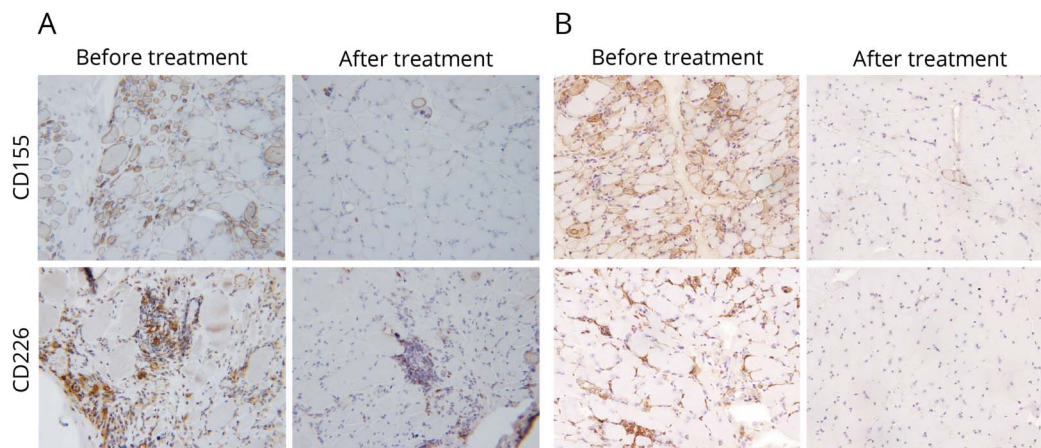
^a Correlation analysis was conducted only in patients with DM.

counterparts in patients with DM. In addition, higher CD107a expression on CD8⁺CD226⁺ T cells was observed when compared with that of CD8⁺CD226⁻ T-cell population. Based on the abovementioned findings, we speculate that, in the muscle microenvironment of patients with IIM, CD155-CD226-mediated costimulatory signaling is much stronger than CD155-TIGIT-mediated coinhibitory signaling in the CD155-CD226/TIGIT pathway, which may lead to pathogenic immune imbalances and drive autoimmune tissue inflammation. Particularly, CD226⁺ cells tended to infiltrate and localize adjacent to muscle fibers coexpressing CD155 and MHC molecules, and CD226⁺ T cells secreted TNF- α at a location close to the myofibers. Thus, the structural elements required for the formation of “immune synapses” seem to have been met. Our

data suggest that the high expression and potential interaction between CD155 and CD226 may be involved in the activation of infiltrating T cells in muscle from patients with IIM.

Furthermore, the importance of CD155-CD226 interaction in IIM is supported by the strong correlation between the CD155/CD226 expression levels and the indicators of muscle involvement (CK, LDH, the scores of muscle histopathology damage, and MMT8 scores), suggesting that CD226 signaling critically aggravates the inflammatory responses in patients with IIM. Of note, compared with CD4⁺ and CD8⁺ T cells, the expression levels of CD155 and CD226 exhibit superiority in correlation with CK, LDH, and MMT-8. Therefore, the overexpression of the CD155-CD226 axis may be a potential

Figure 5 Expression of CD155 and CD226 Before and After Treatment in 2 Patients With IIM



Before treatment, robust expression of CD155 on myofibers and a large number of CD226⁺ infiltrating inflammatory cells were found in muscle sections. However, upon immunosuppressive treatment, the expression levels of CD155 and CD226 were drastically reduced (A, a patient with DM; B, a patient with IMNM). (A and B, original magnification $\times 200$). DM = dermatomyositis; IMNM = immune-mediated necrotizing myopathy.

biomarker for assessing the severity of muscle involvement in patients with IIMs.

Previous studies demonstrated that T-cell infiltrates in the muscles of patients with IIMs are dominated by CD28null (CD244+) T cells, and these cells represent clinically important effector cells.¹³⁻¹⁶ However, the CD226-positive T cells reported here did not seem to be CD28null cells because costaining showed that CD226+ cells were not colocalized with CD244+ cells, suggesting that CD226-positive cells are a different phenotype of effector cells in the muscle tissue of patients with IIM.

As multifunctional molecules, CD155 and CD226 belong to the nectin-like molecule family functioning in cell adhesion and polarization.^{43,44} The interaction of CD155 and CD226 has been confirmed to occur during the diapedesis step of monocyte transendothelial migration process.⁴⁵ In patients with IIM, the high expression of CD155, the increased percentage of CD226+ lymphocytes in skeletal muscle, and a significant decrease of CD226+ T cells in peripheral blood,²² all suggest that CD155-induced CD226 signaling is pronounced in muscle tissues and indicates a systemic leakage of CD226-inducing factor and/or recirculation of CD226+ T cells between the periphery and muscle tissue. Further investigations are required to elucidate whether the muscle microenvironment preferentially locally attracts and retains CD226+ lymphocytes.

Our study has some limitations. First, the sample size was small. To better elucidate the role of CD226-CD155 pathway in IIMs, a follow-up study with a large sample size and using repeated muscle biopsies of patients from different backgrounds (myositis-specific antibody–defined subgroups) and disease states (flare or remission; with or without cancer) is required. Second, although our findings suggest that the CD155-CD226 interaction is involved in the muscle damage of DM, further cytologic experiments are needed to elucidate the specific mechanism of damage. Third, our study did not investigate the subsequent functional changes of muscle fibers after CD155 overexpression.

Collectively, we provided systematic evidence of the presence and relevance of the CD155-CD226 pathway in inflamed muscles of IIM patients in this study. These data suggest that CD155-CD226 signaling may contribute toward the unfavorable inflammatory responses in the muscle tissues and provide novel insights to improve the understanding of the immune pathogenesis of IIMs.

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Disclosure

The authors declare that they have no competing interests. Go to [Neurology.org/NN](https://www.neurology.org/NN) for full disclosures.

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Appendix Authors

Name	Location	Contribution
Wenli Li, PhD	Department of Rheumatology, Key Myositis Laboratories, China-Japan Friendship Hospital, Beijing, China	Including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data
Chuiwen Deng, PhD	Department of Rheumatology and Clinical Immunology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China	Including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data
Hanbo Yang, PhD	Department of Rheumatology, Key Myositis Laboratories, China-Japan Friendship Hospital, Beijing, China	Major role in the acquisition of data; study concept or design; analysis or interpretation of data
Xiaolan Tian, PhD	Department of Rheumatology, Key Myositis Laboratories, China-Japan Friendship Hospital, Beijing, China	Study concept or design; analysis or interpretation of data
Lida Chen, PhD	Department of Blood Transfusion, China-Japan Friendship Hospital, Beijing, People's Republic of China	Analysis or interpretation of data
Qingyan Liu, PhD	Department of Rheumatology, Key Myositis Laboratories, China-Japan Friendship Hospital, Beijing, China	Analysis or interpretation of data
Chang Gao, BS	Department of Rheumatology, Key Myositis Laboratories, China-Japan Friendship Hospital, Beijing, China	Analysis or interpretation of data
Xin Lu, PhD	Department of Rheumatology, Key Myositis Laboratories, China-Japan Friendship Hospital, Beijing, China	Analysis or interpretation of data
Guochun Wang, PhD	Department of Rheumatology, Key Myositis Laboratories, China-Japan Friendship Hospital, Beijing, China	Analysis or interpretation of data
Qinglin Peng, PhD	Department of Rheumatology, Key Myositis Laboratories, China-Japan Friendship Hospital, Beijing, China	Including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data

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