TRIM63 and Atrogin-1 are key drivers of systemic and muscle inflammation in patients with idiopathic inflammatory myopathies

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Abstract

Objective

The ubiquitin proteasome system is the main mediator of inflammation-induced muscle atrophy through the expression of TRIM63 and Atrogin-1. The aim of this study was to address the expression of these ubiquitin ligases and their relationship with inflammatory and atrophy parameters of patients with idiopathic inflammatory myopathies (IIM).

Methods

We recruited 37 adult IIM patients, and 10 age- and sex-matched healthy donors. We assessed the proportion of different peripheral blood mononuclear cells (PBMC) subsets expressing TRIM63 and Atrogin-1 and the serum amount of theses ubiquitin ligases, cytokines, and chemokines, using multiparametric flow-cytometry, ELISA and luminometry, respectively. The muscle expression of TRIM63 and Atrogin-1 was assessed by confocal microscopy. We compared the quantitative variables with the Mann-Whitney U-test and assessed the correlations with Spearman Rho.

Results

IIM patients had a higher proportion of TRIM63+ CD4+ T cells (24.56 (7.71-53.23) vs. 2.55 (0.42-4.51), p<0.0001), TRIM63+ CD8+ T cells (15.1 (3.22-37.40) vs. 1.06 (0.83-2.45), p=0.0002), TRIM63+ monocytes (14.09 (3.25-29.80) vs. 1.97 (0.59-7.64), p=0.011), Atrogin-1+ CD4+ T cells (27.30 (6.61-64.19) vs. 2.55 (0.42-4.51), p<0.0001), Atrogin-1+ CD8+ T cells (14.88 (5.99-34.30) vs. 2.33 (0.60-8.01), p=0.001), and Atrogin1+ monocytes (17.38 (8.93-47.37) vs. 1.41 (0.79-3.77), p<0.0001). Muscle from IIM patients had a higher expression of TRIM63 and Atrogin-1. TRIM63+ CD8+ T cells mainly correlated with serum IL-2, IL-4, IL-8, IL-10, G-CSF, and TNF-α.

Conclusion

TRIM63 and Atrogin-1 are expressed in PBMC and muscle from patients with IIM and correlate with serum cytokines, and chemokines. This mechanism may contribute to the inflammation-induced muscle atrophy in IIM.

Key words

myositis, idiopathic, TRIM63, Atrogin-1, muscular atropy

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Introduction

Idiopathic inflammatory myopathies (IIM) are a group of systemic autoimmune diseases that represent an important cause of disability and early mortality (1). Regardless of the adequacy of the immunosuppressive therapy, most patients develop persistent weakness due to muscle atrophy, which is the main feature of damage accrual in subjects with IIM (2, 3). Muscle atrophy causes disability in 74-84% of IIM patients and decreases significantly the quality of life (QoL) (1, 2). Approximately 42% of subjects with an IIM diagnosis cannot work at some point throughout their disease and up to 70% remain mildly or moderately disabled due to muscle atrophy even during periods of inactive disease (3).

Muscle atrophy is the consequence of an increased myofilament degradation and decreased muscle regeneration by satellite cells (4). Through the activation of two ubiquitin ligases called muscle RING finger-1 protein (MuRF-1/TRIM63) and muscle atrophy F-box protein (MAFbx/Atrogin-1) (5), the ubiquitin-proteasome system is the primary mediator of muscle atrophy (6). The expression of these ligases is the result of the transcription of the atrogenes (7), which are over-expressed in catabolic states, return to normal during anabolic stimuli (8) and have been described in over 25 models of muscle atrophy (7). In IIM, previous studies have depicted the increased expression of atrogenes after the *in vitro* stimulation of human muscle cells with anti-3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMGCR) and anti-signal recognition particle (SRP) antibodies (9). Besides, chronic inflammation, immobilisation and ischaemia are part of the pathogenesis of IIM and are key drivers of the expression of these ubiquitin ligases in other diseases (10). Notwithstanding the relevance of muscle atrophy in IIM, a thorough assessment of the expression of TRIM63 and Atrogin-1 in these patients is still lacking. The aim of this study was to address the expression of these ubiquitin ligases in serum, peripheral blood mononuclear cells (PBMC) subsets and muscle of patients with IIM and to assess their relationship with clinical, inflammatory and muscle atrophy parameters.

Materials and methods

This cross-sectional study was conducted from March 2020 to March 2023 in the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, a tertiary care centre for patients with IIM in Mexico. Thirty-seven adults classified as patients with IIM according to the Bohan and Peter and/or ACR/EULAR 2017 criteria (11) were recruited. Patients with anti-synthetase syndrome were classified according to Connor's criteria (12). We excluded subjects with diabetes mellitus, denervation, chronic obstructive pulmonary disease, cancer, sepsis and chronic heart failure, since these conditions are known to stimulate the expression of TRIM63 and Atrogin-1 (7). In addition, 10 healthy controls without signs, symptoms or family history of autoimmune diseases adjusted for age and gender were recruited. The study was approved by the institutional Ethics and Research Committee (REF: 2711) in accordance with the Helsinki declaration and all patients and healthy controls signed an informed consent to participate.

The following disease activity and damage accrual parameters were evaluated by the same rheumatologists (DGM and JTR): manual muscle testing 8 (MMT8), patient's and physician's global disease activity with a visual analog scale (VAS), cutaneous dermatomyositis disease area and severity index (CDASI), myositis disease activity assessment tool (MDAAT), myositis intention to treat index (MITAX), myositis damage index (MDI) and the health assessment questionnaire disability index (HAQ-DI) (13). Additionally, we assessed the following parameters:

1. *Evaluation of the TRIM63 and Atrogin-1 expression in serum, peripheral blood mononuclear cells (PBMC), CD4$^+$, CD8$^+$ and CD14$^+$ cells:* PBMC were obtained by blood separation with density gradients after centrifugation with Ficoll-Paque (GE Healthcare Life Sciences, Illinois, USA). The cells were washed twice with PBS and then, we incu-

bated them with the Zombie aqua viability marker (Biolegend, San Diego, California, USA) and the following fluorochrome-coupled antibodies after blocking the Fc receptors with the human TruStain FcX (Biolegend, San Diego, California, USA): CD3-APC/Fire 750, CD4-Alexa Fluor 488, CD8-PE-Dazzle594, CD14-PerCP (all from Biolegend, San Diego, California, USA). For the TRIM63 and Atrogin-1 intracytoplasmic staining, the cells were fixed and permeabilized using the cytofix/cytoperm kit (BD Biosciences, Franklin Lakes, New Jersey, USA). Afterwards, the cells were incubated for 30 minutes at 4°C with the following primary antibodies: 1: 100 goat anti human TRIM63 (R&D systems, Minneapolis, Minnesota, USA) and 1: 100 goat anti human atrogin-1 (Abcam, Cambridge, United Kingdom). Subsequently, the cells were washed twice with the perm wash solution from the aforementioned kit and incubated with 100 microliters of 1:1000 donkey anti goat Alexa Fluor 647 (Thermofisher, Waltham, Massachusetts, USA) for 30 min at 4°C. Cells solely stained with the secondary antibody were used as an isotype control. We acquired the samples on an LSRFortessa flow cytometer, and we analyzed the data using the FlowJo software V10.6. The used gating strategy is shown in Supplementary Figure S1. We assessed the proportion of total PBMCs, CD4, CD8 and CD14 cells expressing the ubiquitin ligases. Also, the expression intensity of the ubiquitin ligases was expressed as the mean fluorescence intensity (MFI) of Alexa Fluor 647. The serum levels of TRIM63 and Atrogin-1 were assessed by ELISA (MyBioSource, San Diego, California, USA) according to the manufacturer's instructions.

2. *Evaluation of serum cytokines, chemokines, the myositis-specific autoantibodies (MSA) and the myositis associated autoantibodies (MAA):* We analysed the following serum cytokines and chemokines by luminometry using the Bio-Plex Pro Human Cytokine 17-Plex (Bio-Rad, Hercules. California, USA): granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage stimulating factor (GM-CSF), interferon (IFN)-, interleukin (IL)-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17A, monocyte-chemoattractant protein 1 (MCP-1), macrophage inflammatory protein b (MIP-1b), and tumour necrosis factor a (TNF-α). The MSA and MAA were assessed by Immunoblot using the kit from EUROIMMUN (Lübeck, Germany).
3. *Assessment of TRIM63, Atrogin-1 and atrophy-related parameters in muscle biopsies:* A left deltoid biopsy was taken from patients with IIM and healthy donors. The biopsies were immediately snap frozen in liquid nitrogen-precooled isopentane (Sigma, Burlington, Massachusetts, USA). Transversal 7 microns sections were mounted on electrically charged slides (Ward's Science's, Rochester, New York, USA). After blocking for 1 hour with 10% bovine serum albumin (BSA), the samples were incubated overnight at 4ºC with the following primary antibodies diluted in 5% BSA: 1:50 mouse anti human TRIM63 (Santa Cruz Biotechnology, Dallas, Texas, USA), 1:200 rabbit anti human Atrogin-1 (Abcam, Cambridge, United Kingdom), and 1:200 rat anti human laminin gamma (Millipore, Burlington, Massachusetts, USA). After three washes with PBS, the samples were incubated at room temperature with the following secondary antibodies diluted in 5% BSA for one hour: 1:250 donkey anti goat Alexa Fluor 647, 1:500 donkey anti rabbit Alexa Fluor 555, 1:500 donkey anti mouse Alexa Fluor 488 (all from Thermofisher, Waltham, Massachusetts, USA). The images were acquired in a Nikon N-STORM confocal microscope (Minato City, Japan) and were analysed using the ImageJ software. The mean fluorescence intensity of the mentioned parameters was measured in six 20X fields. The variability coefficient and the atrophy factor were calculated in at least 300 transversal fibres of each biopsy as previously described (9). The diagnosis of atrophy was made using the following parameters of the muscle biopsy: variability index higher than 250 and an atrophy factor higher than 110 in women and of 150 in men as previously described (14).
4. *Statistical analysis:* The sample size was calculated to detect a correlation between the atrophy factor and the expression of TRIM63 in muscle biopsies. With a Spearman Rho of 0.5, a significance level of 0.05 and a power of 80%, the sample size corresponded to 37 patients. Quantitative variables were expressed as medians and interquartile range (IQR) and were compared with the Mann-Whitney U-test. Correlations between quantitative variables, including the expression of the ubiquitin ligases in muscle and PBMC subsets with the clinical features of IIM patients were addressed using the Spearman Rho. All analyses were corrected for multiple comparisons with the Benjamini-Hochberg correction. The sample size and statistical analysis were performed using the R project (R Core Team (2021, R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/) and the GraphPad Prism v. 9.3.1 software.

Results

Most patients were women (n=21, 67.7%). The median (IQR) of age and the time since disease diagnosis were 42 (27–54) years and 6 (3–10.5) months respectively. The most frequent diagnosis was dermatomyositis (N=24, 77.4%). Five patients (16.1%) had antisynthetase syndrome and 2 were classified as anti-Ku and anti PM/Scl myopathies (6.5%). The three most frequent MSA were anti-MDA5 (n=10, 27%), anti-Mi2 (n=7, 18%), and anti-NXP2 (n=5, 13%). The most frequent MAA were anti-Ro52 (n=9, 24%), and anti-PM/Scl (n=3, 8%). Patients had a median (IQR) of MMT8 was 124 (12-150). The laboratory features, and immunosuppressive therapy are depicted in Supplementary Table S1. The median

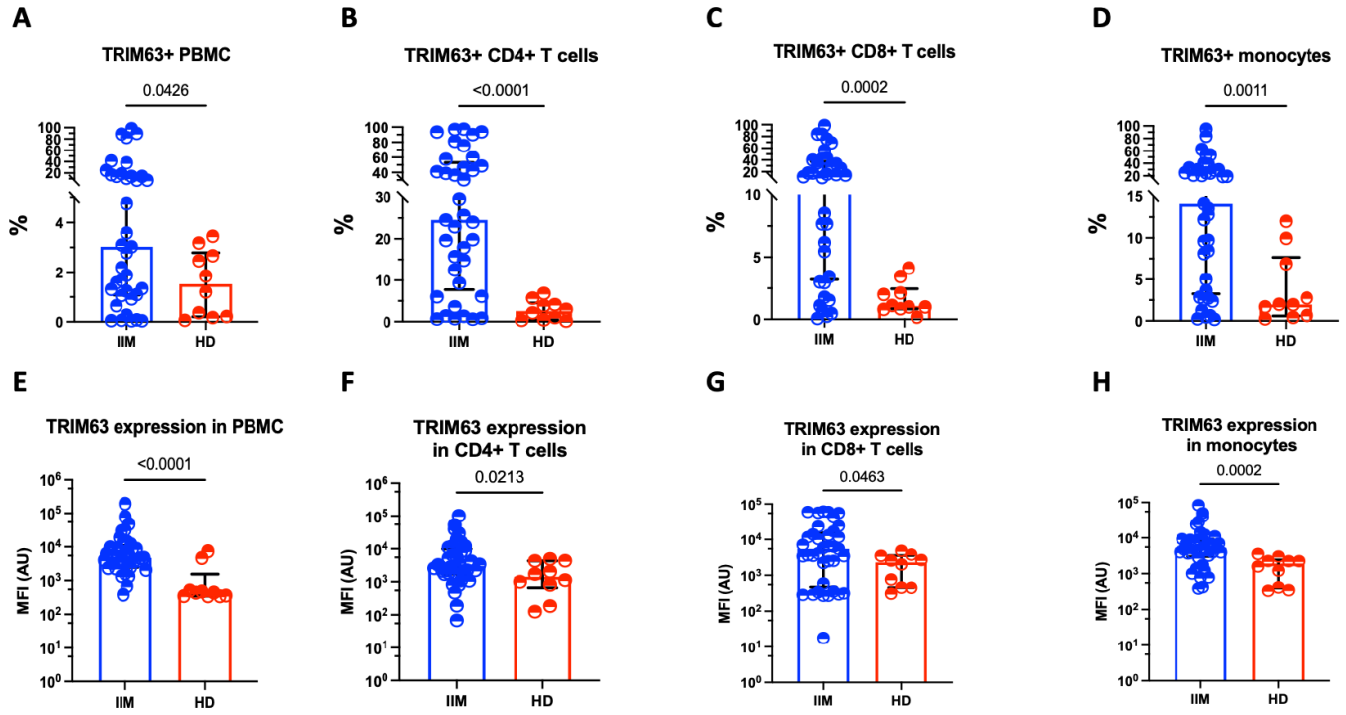

Fig. 1. TRIM63 expression in PBMC, CD4+, CD8+ T cells and monocytes of patients with IIM and healthy donors. Quantitative variables were compared using the Mann-Whitney U-test.

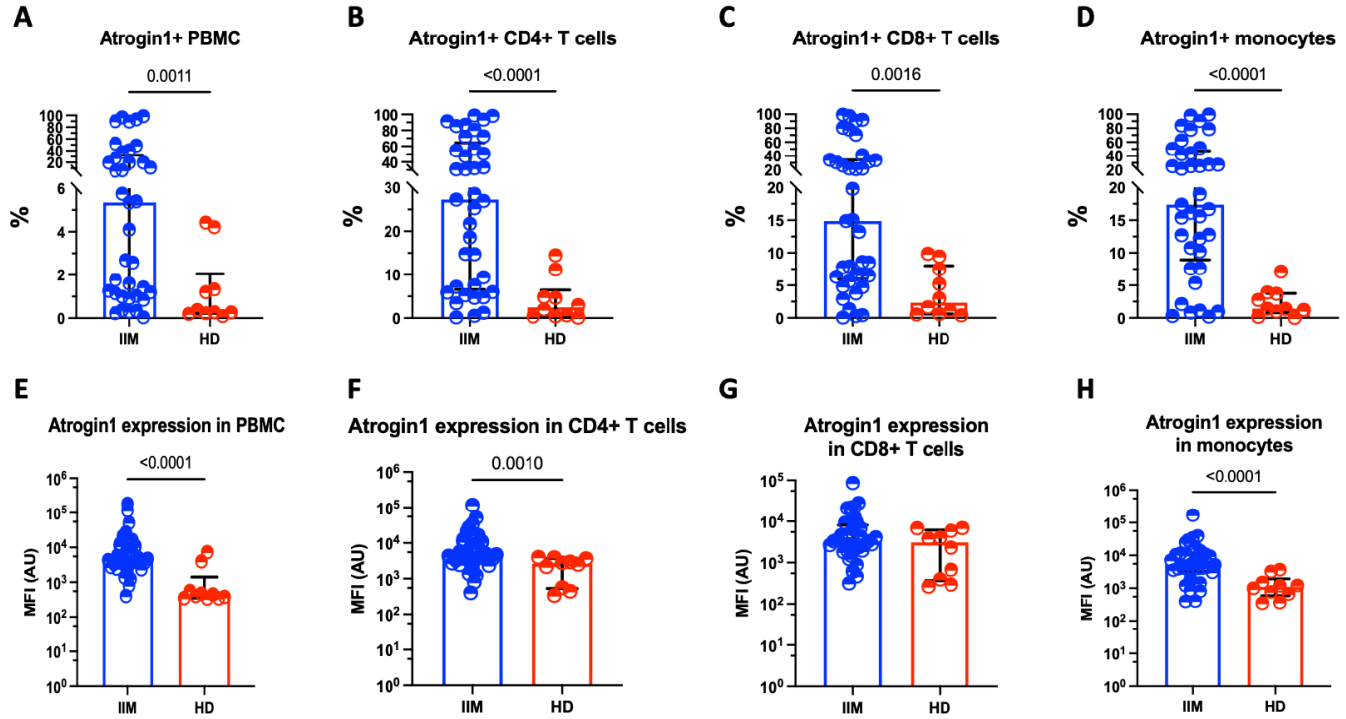

Fig. 2. Atrogin-1 expression in PBMC, CD4+, CD8+ T cells and monocytes of patients with IIM and healthy donors. Quantitative variables were compared using the Mann-Whitney U-test.

(IQR) of CPK in patients with IIM was 483 (83-3404). At recruitment, 27 patients (72.9%) were treated with prednisone, 10 (27%) with methotrexate, 6 (16%) with azathioprine, 5 (13.5%) with mycophenolate mofetil, 2 (5.4%) with cyclophosphamide, and 12 (32.4%) with antimalarials. The disease activity and damage accrual parameters at recruitment are described in Supplementary Table S2.

As shown in Figure 1, the proportion of cells expressing TRIM63 was higher in PBMC, CD4+, CD8+ and CD14+ cells from patients with IIM in comparison to healthy donors. The expression of TRIM63 as measured by MFI of Alexa Fluor 647 was higher in all cell subsets

Table I. Expression of TRIM63 and Atrogin-1 in PBMC, T lymphocytes and monocytes.

Variable	IIM n=37 Median (IQR)	Healthy donors n=10 Median (IQR)	*p*-value
Percentage of TRIM63+ PBMC	3.03 (1.08-16.65)	1.52 (0.20-2.79)	0.042
TRIM63 expression in PBMC (AU)	4752 (2477-10854)	444 (350-1549)	<0.0001
Percentage of TRIM63+ CD4⁺ T cells	24.56 (7.71-53.23)	2.55 (0.42-4.51)	<0.0001
TRIM63 expression in CD4⁺ T cells (AU)	3039 (1719-9859)	1413 (659-4337)	0.021
Percentage of TRIM63+ CD8⁺ T cells	15.1 (3.22-37.40)	1.06 (0.83-2.45)	0.0002
TRIM63 expression in CD8⁺ T cells (AU)	5488 (472-15496)	2298 (454-3581)	0.046
Percentage of TRIM63+ monocytes	14.09 (3.25-29.80)	1.97 (0.59-7.64)	0.001
TRIM63 expression in monocytes (AU)	5272 (3011-9202)	1919 (397-2447)	0.0002
Percentage of Atrogin-1+ PBMC	5.35 (1.09-32.60)	0.33 (0.22-2.05)	0.011
Atrogin-1 expression in PBMC (AU)	2695 (1700-6811)	242 (199-810)	<0.001
Percentage of Atrogin-1+ CD4⁺ T cells	27.30 (6.61-64.19)	2.48 (0.40-6.46)	<0.0001
Atrogin-1 expression in CD4⁺ T cells (AU)	4716 (2975-11920)	423 (349-1418)	<0.0001
Percentage of Atrogin-1+ CD8⁺ T cells	14.88 (5.99-34.30)	2.33 (0.60-8.01)	0.001
Atrogin-1 expression in CD8⁺ T cells (AU)	3290 (1963-8109)	3068 (369-6163)	0.125
Percentage of Atrogin-1+ monocytes	17.38 (8.93-47.37)	1.41 (0.79-3.77)	<0.0001
Atrogin-1 expression in monocytes (AU)	5229 (3303-11342)	1097 (591-1933)	<0.0001

AU: arbitrary units.

from patients with IIM in comparison to healthy donors (Fig. 1).

The proportion of all PBMC subsets expressing Atrogin-1 was higher in patients with IIM in comparison to healthy donors, and the expression of Atrogin-1 was higher in all PBMC subsets except CD8⁺ T cells (Fig. 2).

The cumulative statistic of the expression of both ubiquitin ligases in different PBMC subsets is summarised in Table I. We did not find a correlation between the expression of the ubiquitin ligases in the different PBMC subsets and the time of disease diagnosis in patients with IIM.

We did not find a statistically significant difference in the serum amount of TRIM63 in IIM *vs*. healthy donors (146.9 pg/mL (49.3–223.0) *vs*. 97.1 pg/mL (56.1–127.8), p=0.15). There was a trend towards an increased serum concentration of Atrogin-1 in patients with IIM in comparison to healthy donors 1.68 pg/mL (0.95–2.35) *vs*. 1.27 pg/mL (0.99–1.40), p=0.07).

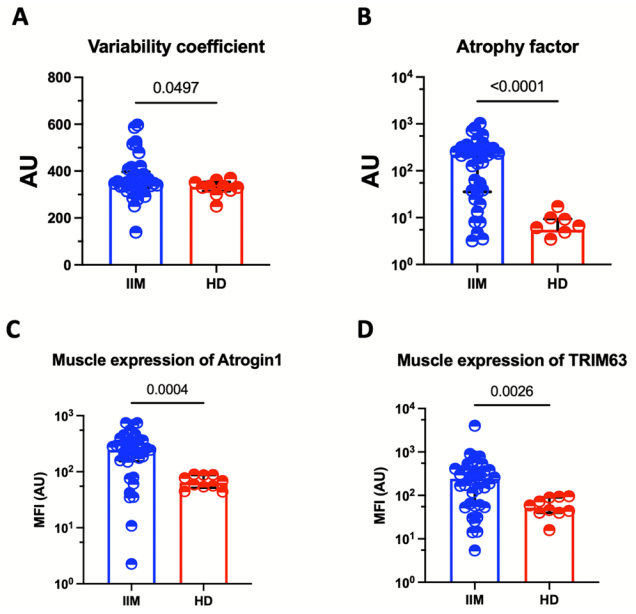

Fig. 3. Muscle atrophy parameters (**A-B**), and expression of the ubiquitin ligases (**C-D**) in muscle biopsies from patients with IIM in comparison to healthy donors. Quantitative variables were compared using the Mann-Whitney U-test.

As shown in Figure 3, muscle biopsies of patients with IIM had a higher variability index (347 AU (328–396) *vs*. 332 AU (314–353), p=0.049) and an increased atrophy factor (221 AU (35–303) *vs*. 5 (0–9.43), p<0.0001). Furthermore, all muscle biopsies from patients with IIM fulfilled the histological criteria for atrophy as defined in Methods.

As shown in Figure 4, the muscle biopsies of patients with IIM had a higher expression of TRIM63 (MFI 242 (58–398) AU *vs*. 52 (39–90) AU, p=0.002) and Atrogin-1 (MFI 243 (154–358) AU *vs*. 64 (51–87) AU, p=0.0004) in comparison to healthy donors. We found an inverse correlation between the expression of TRIM63 in muscle biopsies and the CPK levels in patients with IIM (Spearman Rho = -0.48, p=0.017), but a correlation between the expression of the ubiquitin ligases in the muscle and the PBMC subsets was not found.

Finally, we analysed the correlation between inflammatory markers and parameters of atrophy in peripheral blood and in muscle biopsies. The results are depicted as a correlation matrix in Figure 5. The proportion of TRIM63⁺ CD8⁺ T cells, Atrogin-1⁺ CD4⁺ T cells and Atrogin-1+ monocytes correlated with the serum levels of IL-2, IL-4, IL-5, IL-8, IL-10, G-CSF, GM-CSF, and TNF-α. The serum levels of TRIM63 correlated as well with the serum concentrations of IL-6, IL-8, IL-10 and IFN-γ. We did not find a correlation between the variability coefficient, the atrophy factor, and the ubiquitin ligases.

Discussion

In this study, we aimed to explore the expression of TRIM63 and Atrogin-1 as part of the pathogenesis of inflammation-induced atrophy in patients with IIM. These ubiquitin ligases are important mediators of muscle atrophy since their substrates are both structural proteins and skeletal muscle differentiation factors, such as MyoD1, Myogenin, eIF3-f (15), Desmin, Vimentin, myosin binding peptide (MyBP)-C, myosin light chain (MyLC)1, MyLC2, and myosin heavy chain (MyHC) (16). Therefore, TRIM63 and Atrogin-1 reduce both the size and differentiation

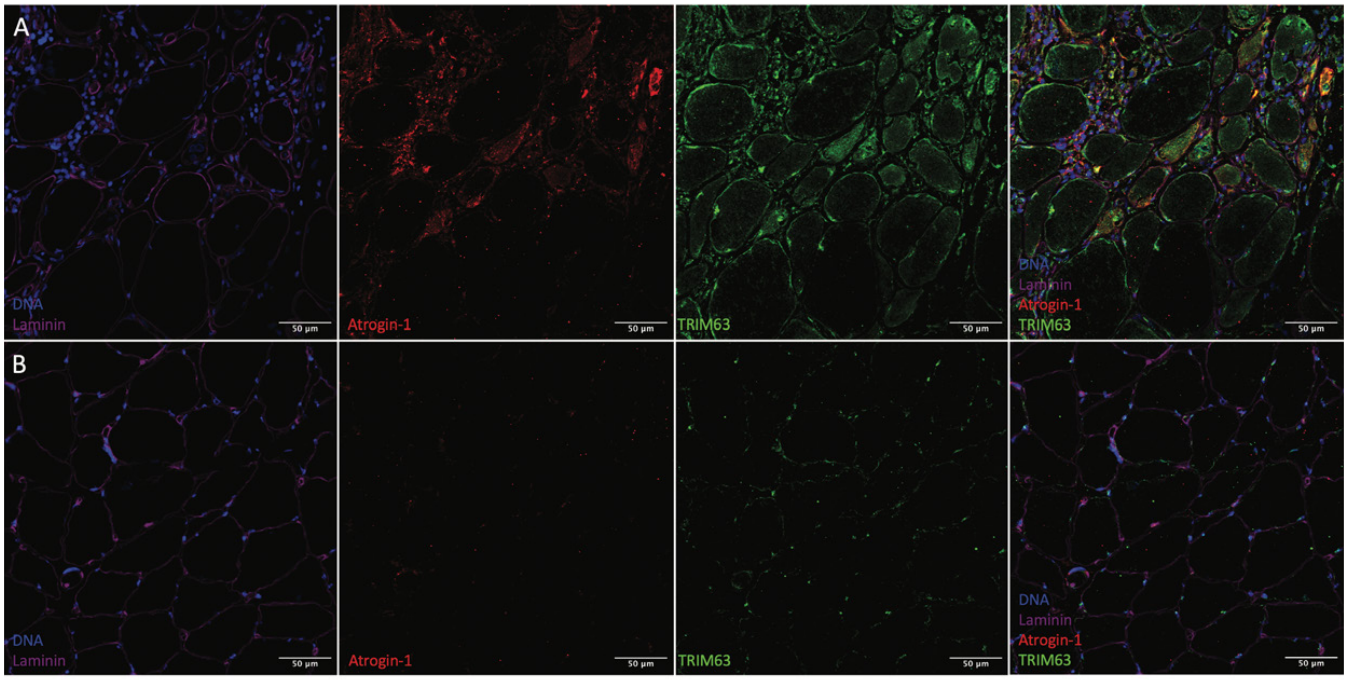

Fig. 4. Representative confocal microscopy image of the expression of TRIM63 and Atrogin-1 in patients with IIM (**A**) and healthy donors (**B**).

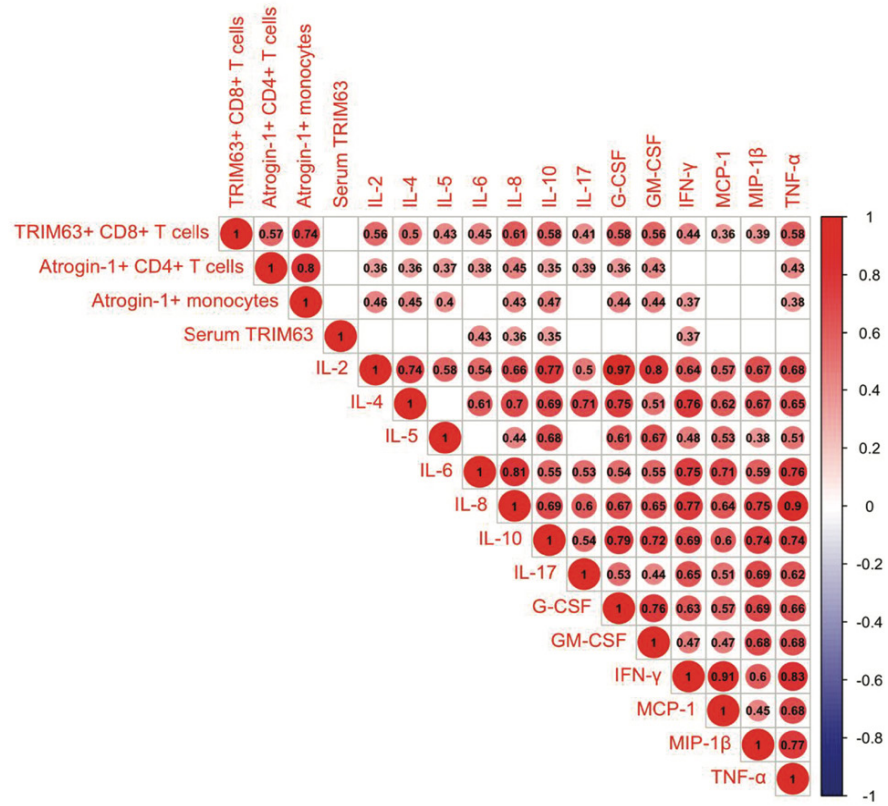

Fig. 5. Correlations between the expression of TRIM63 and Atrogin-1 in peripheral blood and muscle of patients with IIM. The statistically significant correlations are summarised in a correlation matrix. Correlations between quantitative variables were addressed using the Spearman Rho with the Benjamini-Hochberg correction for multiple comparisons.

of muscle cells (17). The expression of TRIM63 and Atrogin-1 is regulated by the transcription factors NF-κB (18) and FOXO (19) in response to different stimuli such as reactive oxygen species (ROS) (20), glucocorticoids (21), myostatin (22), TNF-α (23), IFN-γ (24) and IL-1β (25).

Previous studies have confirmed the dynamic expression of Atrogin-1 in PBMCs (26), which in our study may explain the differential expression of these ubiquitin ligases in IIM patients in comparison to healthy donors. IIM are systemic autoimmune diseases characterised by an increased expression of IL-1β, IL-2, IL-4, IL-6, IL-15, IL-17, IL-18, TNF-α, TGF-β, and interferons in peripheral blood and muscle (27). Our data suggest that this pro-inflammatory microenvironment may induce the expression of TRIM63 and Atrogin-1 in peripheral blood, as shown by the correlation between these ubiquitin ligases and many key pro-inflammatory cytokines in patients with IIM. On the other hand, the potential role of Atrogin-1 in inflammation-induced muscle atrophy has been previously suggested in patients with inclusion body myositis (IBM). Similar to our results, in muscle biopsies from these patients, an overexpression of Atrogin-1 was observed in both muscle cells, $CD4^+$ and $CD8^+$ lymphocytes (28).

Previous studies have demonstrated the local expression of IFN-γ, TNF-α, IL-1β, and IL-6 in muscle of patients with IIM (29). All these factors are induc-

ers of the expression of TRIM63 and Atrogin-1 (30). Since we found a higher expression of TRIM63 and Atrogin-1 in muscle biopsies from patients with IIM, our results suggest that these proinflammatory cytokines contribute to the inflammation-induced muscle atrophy observed even in early stages of the disease. Nonetheless, our results are in contrast with those of Mahoudeau *et al*., who showed a decreased expression of myostatin, TRIM63 and Atrogin-1 in muscle from 18 patients with IIM by RNA sequencing (31). These differences may be related to the clinical phenotype of patients with IIM, since most of our patients had DM and AS, whilst Mahoudeau *et al*. included only 6 patients with DM, and the rest of the subjects had immune mediated necrotizing myopathy and inclusion body myositis (31). Therefore, if TRIM63 and Atrogin-1 contributes to inflammation-induced muscle atrophy in all types of IIM requires further research.

Interestingly, we found an inverse correlation between the muscle expression of TRIM63 and the serum CPK in patients with IIM which is according to previous studies showing that patients with muscle atrophy as a sign of damage accrual have reduced CPK levels (32), reinforcing the role of these ubiquitin ligases as an inducer of muscle atrophy in inflammatory myopathies. Besides, the lack of correlation between the muscle expression of the ubiquitin ligases and the time since disease diagnosis is consistent with a recent study showing that the main risk factor for the development of sarcopenia in IIM patients is a high disease activity regardless of the time since disease diagnosis (33).

To the best of our knowledge this is the first study to address the expression of TRIM63, Atrogin-1 and their relationship to atrophy, and inflammatory parameters of patients with IIM. Nonetheless, we acknowledge its limitations including its cross-sectional design with a small sample size composed exclusively by Hispanic patients. Besides, we did not evaluate the functionality of the ubiquitin ligases and therefore, our data solely indicate an association between TRIM63, Atrogin-1 and inflammation, but not a casual effect.

In summary, we found an overexpression of TRIM63 and Atrogin-1 in peripheral blood and muscle biopsies from patients with IIM, which is correlated with serum proinflammatory cytokines and chemokines. This suggests that TRIM63 and Atrogin-1, as part of the ubiquitin proteasome system, contribute to the inflammation-induced muscle atrophy even in early stages of the disease.

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