MUSCULOSKELETAL PATHOLOGY

Dermatomyositis With or Without Anti-Melanoma Differentiation-Associated Gene 5 Antibodies

Common Interferon Signature but Distinct NOS2 Expression

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The anti-melanoma differentiation-associated gene 5 (MDA5) autoantibody is specifically associated with dermatomyositis (DM). Nevertheless, anti—MDA5^{+}-patients experience characteristic symptoms distinct from classic DM, including severe signs of extramuscular involvement; however, the clinical signs of myopathy are mild or even absent. The morphological and immunological features are not yet described in adulthood. Data concerning the pathophysiology of anti-MDA5 DM are sparse; however, the importance of the interferon (IFN) type I pathway involved in DM has been shown. Our aim was to define morphological alterations of the skeletal muscle and the intrinsic immune response of anti—MDA5-positive DM patients. Immunohistological and RT-PCR analysis of muscle biopsy specimens from anti-MDA5 and classic DM were compared. Those with anti-MDA5 DM did not present the classic features of perifascicular fiber atrophy and major histocompatibility complex class I expression. They did not show significant signs of capillary loss; tubuloreticular formations were observed less frequently. Inflammation was focal, clustering around single vessels but significantly less intense. Expression of IFN-stimulated genes was up-regulated in anti-MDA5 DM; however, the IFN score was significantly lower. Characteristic features were observed in anti-MDA5 DM and not in classic DM patients. Only anti-MDA5 DM showed numerous nitric oxide synthase 2—positive muscle fibers with sarcoplasmic colocalization of markers of regeneration and cell stress. Anti—MDA5-positive patients demonstrate a morphological pattern distinct from classic DM. *(Am J Pathol 2016, 186: 691—700; http://dx.doi.org/10.1016/j.ajpath.2015.11.010)*

Dermatomyositis (DM) is a group of idiopathic inflammatory myopathies defined by skin rash, proximal weakness, and specific pathological muscle features.[1] Characteristically, these encompass the following: perivascular inflammation in the perimysium, perifascicular fiber atrophy, sarcolemmal major histocompatibility complex (MHC) class I staining with a perifascicular enhancement, and microvascular pathology, including capillary enlargement,

capillary loss, deposition of membrane attack complex (C5b-9) on vascular endothelial cells, and tubuloreticular inclusions in endothelial cells.[1,2] The pathophysiology of the disease is not fully understood. Hence, on the basis of results of transcriptome analysis of skeletal muscle, it has been emphasized that interferon (IFN) pathways seem to play an important role.[3,4] Also, a specific vasculopathy associated with molecular mechanisms of hypoxia has been described in detail.[5]

Nevertheless, DM encompasses an inhomogeneous group of disorders because a large spectrum of different extramuscular manifestations can be observed, and the muscle involvement is variable, even absent sometimes: the so-called amyopathic DM.[6]

Different DM-specific autoantibodies have been reported so far, and it has been postulated that their presence is associated with homogeneous clinical subgroups of DM.[7] Anti-melanoma differentiation-associated gene 5 (MDA5; official symbol *IFIH1*) is one of them, and the presence of anti-MDA5 antibodies (MDA5^{+}) delineates a clinical syndrome characterized by mild or absent muscle weakness [with mild or no increase of creatine kinase levels (ie, the prototype of amyopathic DM)]. Extramuscular manifestations are predominant, including severe interstitial lung disease and atypical but characteristic skin lesions with skin ulcers, palmar papules, and/or mechanic's hands.[7] Thus, MDA5^{+} patients harbor a characteristic clinical phenotype, at variance from the classic form of DM, raising the question of the role of anti-MDA5 antibodies, and the involvement of separate underlying molecular mechanisms. It appeared that there are ethnic variations in frequency of anti-MDA5 positivity because most of the cases have been described from Asian populations; however, the associated clinical phenotype in different ethnicities has to be further explored.[8]

To date, muscle pathology of 11 MDA5^{+} children has been analyzed in comparison to MDA5^{−} juvenile DM patients,[9] but extensive illustrations have not been reported in adult patients, nor have molecular pathways, including immune parameters, been analyzed.

Herein, we hypothesized that specific morphological features and specific molecular pathways occur in MDA5^{+} patients' skeletal muscles, in accordance with the unique phenotype of MDA5^{+} patients, which is at variance from classic DM patients. We demonstrate that none of the MDA5^{+} patients presented the characteristic morphology of classic DM (perifascicular fiber atrophy, characteristic vasculopathy, or important perivascular inflammation). The expression of IFN-stimulated genes was up-regulated, but at lower levels compared with DM patients. In addition, only MDA5^{+} patients showed focal nitric oxide synthase 2 (NOS2) positive fibers. NOS2 expression colocalized with markers of regeneration and markers of cell stress, suggesting a protective mechanism in the skeletal muscle.

Materials and Methods

Patients and Samples

Ten muscle biopsy specimens from MDA5^{+} patients ($n = 9$; one patient had two muscle biopsy specimens) were identified in four French university hospitals involved in this retrospective study. Patients were diagnosed on the basis of the positivity of qualitative commercial dot blot assay, performed according to the manufacturer's recommendations (D-tek, Mons, Belgium). Muscle biopsy specimens ($n = 7$) from DM patients ($n = 7$), diagnosed on the basis of morphological and clinical European Neuromuscular Centre criteria,[1] were included as controls. The serum of the classic DM controls was tested negative for anti-MDA5 antibodies.

Furthermore, the serum of all patients was also tested for presence of Jo-1, anti−PL-7, anti−PL-12, anti−signal recognition particle, and anti-PM/Scl and anti−Mi-2 autoantibodies using commercial kits (D-tek).

For quantitative real-time PCR, gene expression in DM samples was normalized to results obtained in normal muscle [ie, skeletal muscle biopsy specimens without any light microscopic or ultrastructural abnormalities ($n = 7$)]. The following patients' characteristics were documented: epidemiological characteristics, presence of muscle weakness, creatine kinase level, presence of typical skin rash of DM (heliotrope periorbital edema; violaceous papules or macules, scaly if chronic, at metacarpopharyngeal and interpharyngeal joints, and other bony prominences; and erythema of chest, neck, and upper back),[1] and presence of interstitial lung disease.[10]

Histological and Immunohistochemical Analysis

All frozen samples were analyzed in the Department of Neuropathology (Charité-Universtitätsmedizin, Berlin, Germany), following standardized procedures. Cryostat sections (7 μm thick) of muscle biopsy specimens were used. The following stains were performed: hematoxylin and eosin, periodic acid−Schiff, acid phosphatase, nonspecific esterase, and Gömöri trichrome. Immunohistochemical analysis included using the following antibodies: anti-MHC class I (mouse, clone W6/32; Dako, Glostrup, Denmark), anti-MHC class II (mouse, clone C3/43; Dako), anti-CD3 (rabbit, polyclonal; Dako), anti-CD4 (rabbit, clone SP35; Zytomed, Berlin, Germany), anti-CD8 (mouse, clone C8/144B; Dako), anti-CD68 (mouse, clone EBM11; Dako), anti-CD79a (rabbit, polyclonal; GeneTex, Irvine, CA), anti-CD20 (clone 144B; Dako), anti−C5b-9 complex (mouse, clone aE11; Dako), anti−IFN-stimulated gene 15 (ISG15; rabbit, polyclonal; Abcam, Cambridge, UK), retinoic acid−inducible gene 1 (RIG-I; mouse, 2M6F10; Thermo Scientific, Rockford, IL), anti-neural cell adhesion molecule (clone ERIC-1; AbD Serotec, Kidlington, UK), anti-NOS2 (rabbit, polyclonal; GeneTex), anti-heat shock protein

(HSP) 70 (mouse, clone 3A3; Abcam), and anti-blood dendritic cell antigen 2 (mouse, clone 10E6.1; Millipore, Schwalbach, Germany). An automated slide staining system (BenchMark XT; Ventana Medical Systems, Tucson, AZ) was used. Utrophin (mouse, clone DRP3/20C5; Leica Microsystems, Wetzlar, Germany) staining was used to visualize endomysial capillaries.

Electron Microscopy

Ultrastructural analysis was performed after fixation in 2.5% glutaraldehyde for 48 hours at 4°C, post fixation in 1% osmium tetroxide, and embedding of the muscle tissue in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate. A P902 electron microscope (Zeiss, Ober-kochem, Germany) was used to analyze the specimens.

Histological Quantification

All slides were retrospectively analyzed blinded to the antibody status by three myopathologists (W.S., Y.A., H.-H.G.). The presence or absence of each item is mentioned in the study. For quantitative analysis, slides were digitized using Pannoramic 250 (3DHistech, Budapest, Hungary) at a resolution of 0.244 μm per pixel. Whole slide images were processed using a virtual microscope on the basis of ImageJ software version 6.1.1 (NIH, Bethesda, MD; http://imagej.nih.gov/ij).[11] The density of immune cells was calculated after counting positive cells from the whole area of the biopsy specimen. For capillary analysis, a sufficient number of randomly selected detail images at ×100 magnification allowed coverage of 50% to 60% of the whole cross section of the biopsy. For each sample, the number of capillaries and the minimum Feret diameters were measured automatically. The number of entire cross sections of muscle fibers was counted manually. These data permitted us to calculate the capillary/fiber ratio and capillary density.[12]

Real-Time Quantitative PCR

For RNA extraction from the muscle tissue samples, RT-PCR and quantitative PCR were used with the same methods as described before.[13] Briefly, the expression levels of gene transcripts were analyzed by quantitative real-time RT-PCR using the 5′-nuclease technology on an ABI PRISM 7900HT Sequence Detection System, and the human TaqMan predeveloped assay reagents (both from Applied Biosystems, Darmstadt, Germany). The assay identification probes are as follows: 2′-5′-oligo-adenylate synthetase 1 (OAS1), Hs00973637_m1; OAS3, Hs00196324_m1; MDA5, Hs01070332_m1; myxovirus (influenza virus) resistance 1 (MX1), Hs00895608_m1; RIG-I, Hs00204833_m1; IFN-γ (IFNG), Hs00989291_m1; and STAT1, Hs01013989_m1. The relative abundance of target transcripts was normalized to the expression levels of the endogenous control gene: phosphoglycerate kinase 1 (PGK1), Hs99999906_m1. For each probe, individual (MDA5$^+$ and classic DM patients) data were expressed as relative quantification value. The relative quantification value is equal to $2^{-\Delta\Delta Ct}$ (ie, normalized fold-change relative to the mean expression for the seven healthy controls combined). As previously described, the median fold-change in expression of six IFN-stimulated genes (ISGs) was given for each patient's biopsy specimen, defining an IFN score.[14]

Immunofluorescence Analysis

Immunofluorescence analysis was performed as previously described.[13] Briefly, sections were blocked with the appropriate serum and incubated with the previously mentioned primary antibodies. The secondary antibody was added for 1 hour. The same protocol was repeated with a second primary antibody and an appropriate secondary antibody for double immunofluorescence staining. Images were taken with the Zeiss Observer.Z1 microscope (immunofluorescence), Axiovision software version 4 (Zeiss), or the Olympus (Hamburg, Germany) BX50 microscope, the digital camera DP25, and cell D software version 5.1.

Agreement from the French Ministry of Research (AC 2008-87) was obtained for the collection, analysis, storage, and reuse. All samples were taken for diagnostic purposes. We conducted the research after consideration and approval of the research ethics committee of the Pitié-Salpêtrière Hospital (Paris, France).

Statistical Analysis

Categorical variables are reported as numbers and/or percentages and were compared using a Fisher's exact test. Quantitative variables are reported as means ± SD and compared using the Mann-Whitney test. $P < 0.05$ was considered significant. Kruskal-Wallis one-way analysis of variance was applied to analyze quantitative differences of mRNA transcripts, using Bonferroni correction of the post hoc tests. GraphPad Prism software version 6.00 (GraphPad Software, La Jolla, CA) was used for analysis.

Table 1 Patient Characteristics

Criterion	MDA5$^+$	Classic DM	P values
Age, years*	44 ± 17.7	47 ± 12.1	0.6
Female, %	77	85	1
Muscle weakness	28	100	0.003
CK level, IU/L*	517 ± 856	2693 ± 3446	0.03
Interstitial lung disease, %	67	0	0.01
Atypical skin lesions, %	90	0	0.001

The atypical skin lesions correspond to presence of palmar papules, mechanic's hands, and/or skin ulcers.

*Data presented as means ± SD.

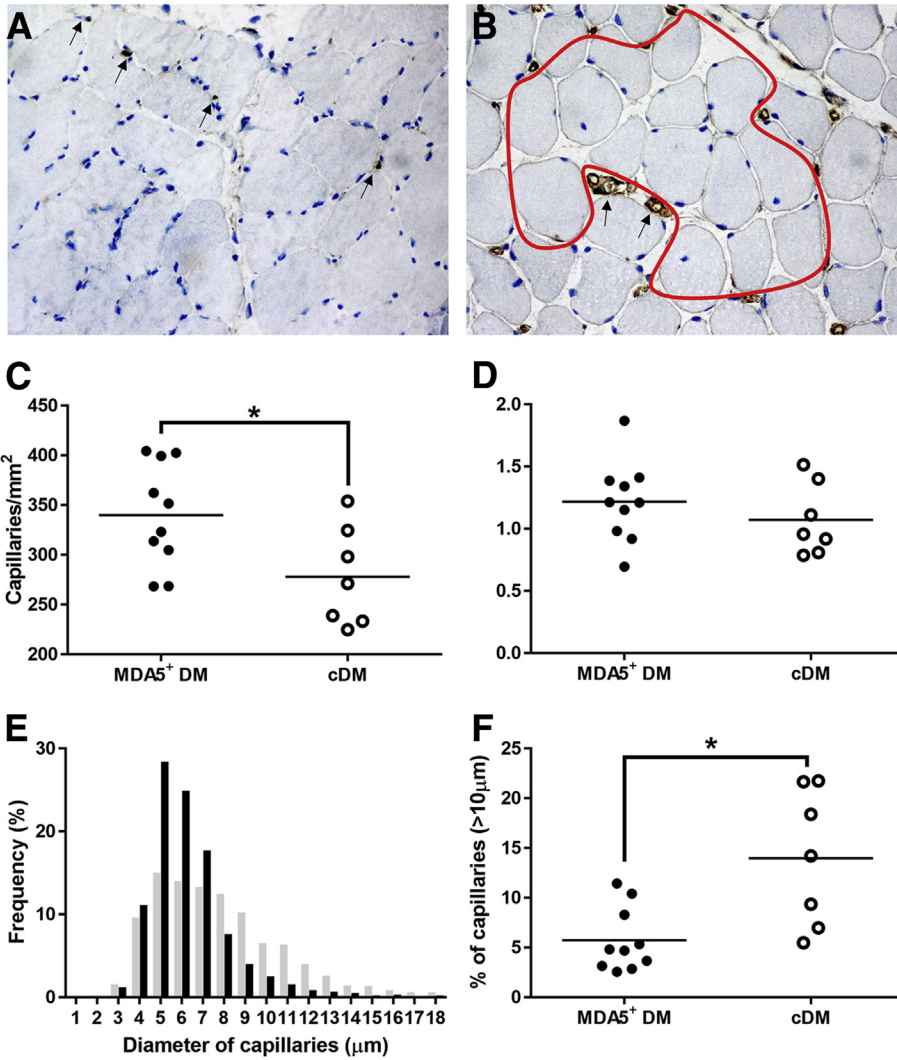CK, creatine kinase; DM, dermatomyositis; MDA, melanoma differentiation-associated protein.

Figure 1 Vasculopathy in MDA5+ patients. **A:** Images of muscle fibers from a MDA5+ patient showing mild membrane attack complex deposition on few capillaries (**arrows**); C5b-9 was used. **B:** Rare capillary loss in a MDA5+ patient, delineated by the **red outline**. Presence of few enlarged capillaries (**arrows**); utrophin was used to stain the capillaries. **C:** Significantly lower capillary density in cDM patients. **D:** No significant difference in capillary/fiber ratios in MDA5+ and cDM patients. **E:** Quantitative distribution of capillaries depending on their diameter in one representative MDA5+ patient (black bars) and one representative cDM patient (gray bars). For each case, >1000 capillaries were counted. **F:** Dot plot representing the percentages of enlarged capillaries. **G:** Ultrastructural evidence of tubuloreticular inclusion in an endothelial cell of a MDA5+ DM patient. *$P < 0.05$. Original magnifications: ×400 (**A** and **B**); ×12,000 (**G**). cDM, classic dermatomyositis; MDA5, melanoma differentiation-associated gene 5.

Results

Characteristics of MDA5+ and Classic DM Patients

Patients' characteristics are summarized in Table 1. Patients were mainly female and were aged 40 to 49 years when the muscle biopsy specimen was obtained. Age and sex ratio were not different in MDA5+ versus classic DM groups. The extramuscular phenotype was different because most MDA5+ patients experienced interstitial lung disease, whereas interstitial lung disease was absent in controls. In addition, signs of myopathy were more frequent and/or more intense in classic DM patients. Only one quarter of MDA5+ patients experienced verifiable muscle weakness, and only 55% of them had increased creatine kinase levels. All DM patients presented typical skin manifestations, whereas most MDA5+ DM patients experienced characteristic skin lesions uncommon in classic DM (none of the classic DM patients presented atypical skin lesions, such as palmar papules, mechanic's hands, and/or skin ulcers) (Table 1). None of the DM patients harbored anti-synthetase syndrome, two classic DM patients were anti−Mi-2 antibody positive, and none of

MDA5+ patients had myosis-specific antibodies other than anti-MDA5.

Vasculopathy in MDA5+ Patients

One of the main characteristics of DM pathology is the presence of a characteristic vasculopathy. To assess signs of vasculopathy, we first tested the presence of C5b-9 deposition on endomysial capillaries (Figure 1A). Characteristic C5b-9 deposition was regularly (85%) detected in classic DM, but this was not the case for MDA5+ patients (30%, $P = 0.049$).

Capillary rarefaction is a further feature of vasculopathy in DM. Indeed, capillary density was low in classic DM patients (277.9 ± 49.5 per mm^2), and it was significantly higher (340 ± 52.3 per mm^2, $P = 0.05$) in MDA5+ patients (Figure 1, B and C). Focal capillary rarefaction was only rarely noted in MDA5+ patients (Figure 1B). The capillary/fiber ratio was slightly elevated in MDA5+ patients (1.2 ± 0.3) compared with classic DM (1.1 ± 0.2); however, it did not reach statistical significance ($P = 0.36$) (Figure 1D). Enlarged capillaries, another sign of

vasculopathy, were commonly observed in classic DM patients; however, they could only occasionally be observed in MDA5$^+$ patients (Figure 1B). Indeed, the mean percentage of enlarged capillaries (>10 μm diameter) was high in classic DM patients (13.9% ± 6.8%) and was only 5.7% ± 3.2% in MDA5$^+$ patients ($P = 0.006$) (Figure 1, E and F). In addition, all classic DM patients had tubuloreticular inclusions within vascular endothelial cells (Figure 1G), whereas they were detected in barely 50% of MDA5$^+$ patients ($P = 0.04$). Together, these results show that typical vasculopathy, as found in DM, is not a characteristic feature of MDA5$^+$ patients.

Skeletal Muscular Fiber Abnormalities in MDA5$^+$ Patients

The presence of perifascicular fiber atrophy is one of the most characteristic signs of DM. This pattern was not observed in MDA5$^+$ patient biopsy specimens ($P = 0.003$) (Figure 2A), except in one case in which a focal mild perifascicular atrophy was noted next to a perivascular infiltrate (data not shown). In contrast, all but one classic DM patient showed the typical perifascicular atrophy ($P = 0.003$) (Figure 2B). In the same line, in MDA5$^+$ patients, MHC-I expression was essentially focal or absent (Figure 2C), only one patient showed a mild diffuse expression. On the contrary, a diffuse MHC class I expression with reinforcement in perifascicular regions, which is a characteristic feature of DM, was regularly met in classic DM ($P = 0.004$) (Figure 2D).

Inflammation in MDA5$^+$ Patients

By definition, DM is a myopathy with inflammatory infiltrates mainly occurring in the perivascular and perimysial areas. All but one MDA5$^+$ patient harbored inflammatory infiltrates; however, the topographic distribution of these infiltrates differed considerably from classic DM patients. In MDA5$^+$ patients, infiltrates were clustering focally in the perimysium near a single vessel (Figure 3A), whereas in classic DM, as expected, inflammation was detected multifocally, leading to a more homogeneous inflammatory aspect (Figure 3B). Macrophages were the predominant immune cells in MDA5$^+$ patients and essentially accumulated only focally in the perimysium as well (Figure 3, C and D). Along that line, cellular density of T cells (CD3$^+$ cells) was significantly elevated in classic DM (35.8 ± 30 cells per mm^2), whereas T cells were rare in MDA5$^+$ patients (3.6 ± 3.1 cells per mm^2; $P < 0.0001$) (Figure 3E). Among T cells, CD8$^+$ T cells represented 7.7 ± 7.1 cells per mm^2 in classic DM patients, and were significantly lower in MDA5$^+$ patients (1.3 ± 2.5 cells per mm^2; $P < 0.01$). CD4$^+$ T cells were detectable more frequently in classic DM (22.4 ± 5.7 cells per mm^2 versus 2.9 ± 2.9 cells per mm^2; $P < 0.001$) (Figure 3, F and G). CD20$^+$ B-cell density was elevated in classic DM (8.2 ± 6.3 cells per mm^2), but it was significantly lower in MDA5$^+$ patients (2.1 ± 2.8 cells per mm^2; $P < 0.02$) (Figure 3H). Few plasma cells were present in MDA5$^+$ and classic DM patients, with no significant difference (0.3 ± 0.6 and 1.0 ± 1.2 cells per mm^2; $P = 0.1$; data not shown).

IFN Signature in Skeletal Muscle of MDA5$^+$ Patients

We next compared immune pathways in MDA5$^+$ and classic DM patients and first tested the IFN pathway. We analyzed the expression of six ISGs: *OAS1*, *ISG15*, *OAS3*, *MX1*, *RIG-I*, and *IFIH1*. All these genes were considerably up-regulated in both groups compared with normal
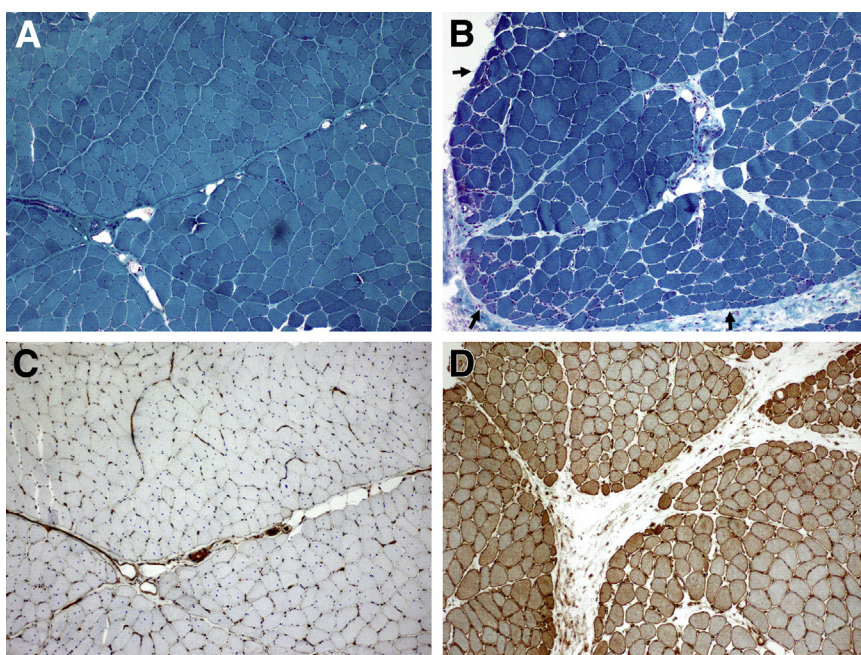

Figure 2 Characteristics of inflammatory myopathy in MDA5$^+$ patients. **A:** No significant muscle atrophy in a MDA5$^+$ patient. **B:** Myofiber atrophy in the perifascicular region (**arrows**) in a classic DM patient. **A** and **B:** Gömöri trichrome staining was used. **C:** No sarcolemmal MHC- class I positive immunoreactivity on muscle fibers in a MDA5$^+$ patient; physiological staining was on endothelial cells. **D:** Diffuse and strong MHC class I positive immunoreactivity on muscle fibers with a perifascicular reinforcement in a classic DM patient. Original magnification, ×100 (**A–D**). DM, dermatomyositis; MDA5, melanoma differentiation-associated gene 5; MHC, major histocompatibility complex.
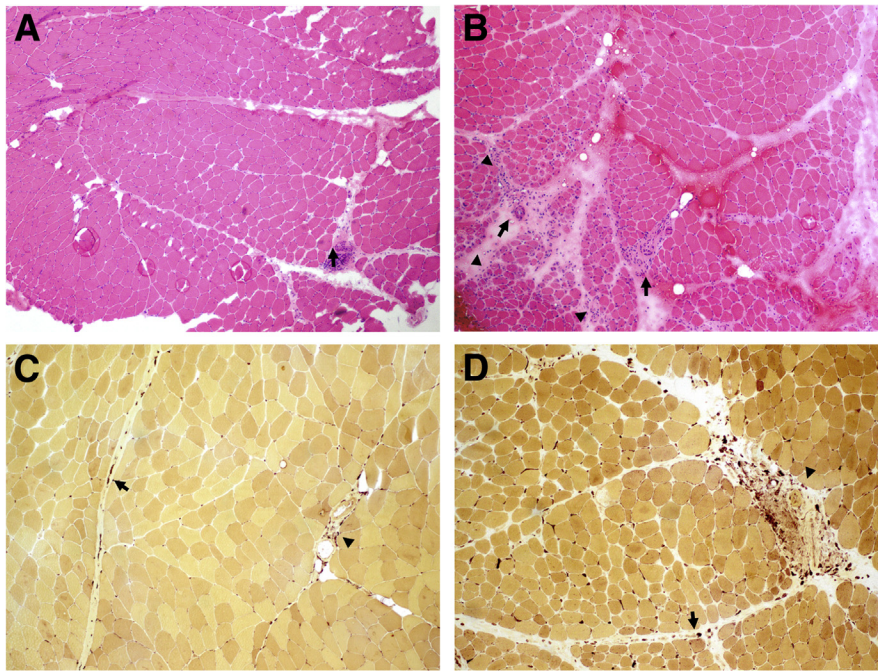
Figure 3 Inflammation in skeletal muscle of MDA5+ patients. **A** and **B:** Focal unique inflammatory infiltrate (**arrow**) clustering around a vessel in the biopsy specimen of skeletal muscle from a MDA5+ patient (**A**). Multifocal inflammatory infiltrates (**arrows**) in perivascular regions, associated with perimysial inflammation with extension to the endomysium (**arrowheads**) in a cDM patient (**B**). Hematoxylin and eosin staining was used. **C:** Mild inflammatory infiltrate in a MDA5+ patient composed of macrophages (nonspecific esterase positive cells in dark brown) around a vessel (**arrowhead**) or in a perifascicular region (**arrow**). **D:** Significant amount of macrophages in perifascicular (**arrows**) and perivascular (**arrowheads**) areas in a cDM patient. **E—H:** Significantly lower T-cell density (**E**), encompassing CD4+ (**F**) and CD8+ (**G**) T-cell subpopulations and CD20+ B cells (**H**) as well in MDA5+ DM patients compared with cDM patients. *$P < 0.05$. Original magnifications: ×80 (**A** and **B**); ×100 (**C** and **D**). cDM classic dermatomyositis; DM dermatomyositis; MDA5, melanoma differentiation-associated gene 5.
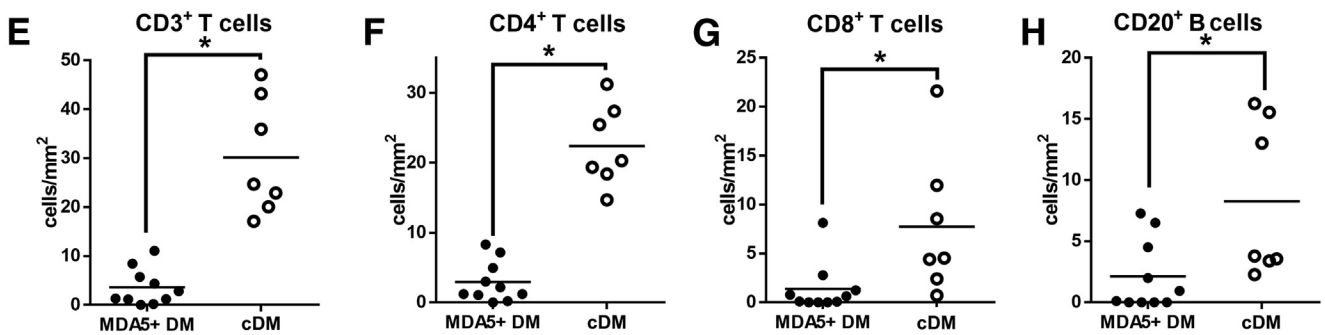
muscle with high relative quantification values (Figure 4A). Nevertheless, the up-regulation of ISGs was less important in MDA5+ patients than in classic DM, and the IFN score was significantly lower in MDA5+ patients (165.8 ± 138.2 versus 590.2 ± 141.5; $P < 0.004$) (Figure 4B).

To identify cells involved in the IFN pathway, we first searched for the presence of plasmacytoid dendritic cells (pDCs; stained by anti-blood dendritic cell antigen 2), which are known to secrete a large amount of IFN-α. Only occasional pDCs were observed in a minority of MDA5+ and classic DM patients (Supplemental Figure S1). On the other hand, we observed that muscle fibers expressed proteins of the ISG group at different levels. RIG-I and ISG15 were regularly detectable in classic DM, on skeletal muscle fibers in the perifascicular region (Figure 4, C and E). On the contrary, only 50% of MDA5+ patients showed a variable degree of RIG-I staining (Figure 4D), and none of the MDA5+ patients exhibited detectable ISG15 immunoreactivity in muscle fibers (Figure 4F); however, some immune cells showed a positive immunostaining (Figure 4F). Together, these results show that the IFN pathway was also

activated to a lesser degree in MDA5+ patients compared with classic DM patients.

Role of NOS2 Expression and Th1 Immunity in MDA5+ Patients

The IFN-associated cytokines are considered strong inducers of STAT1, which is required for type 1 helper T-cell (Th1) activation as part of the adaptive immune response.[15] We tested *STAT1* gene expression and found increased levels of *STAT1* transcription in MDA5+ specimens but less important compared with classic DM patient biopsy specimens (Figure 5A). These results are consistent with the increase of gene expression of the Th1 cytokine *IFNG* that we observed in both groups, which again was less pronounced in specimens from MDA5+ patients (Figure 5B). To further investigate Th1 immunity, we also tested *NOS2* expression characterizing macrophage activation in a Th1 environment.[16] We detected numerous NOS2+ mononuclear infiltrates in both groups, but they were more abundant in the classic DM patients (Figure 5, B and C). Strikingly, however, specimens from MDA5+ patients showed a strong NOS2+ immune reaction on the
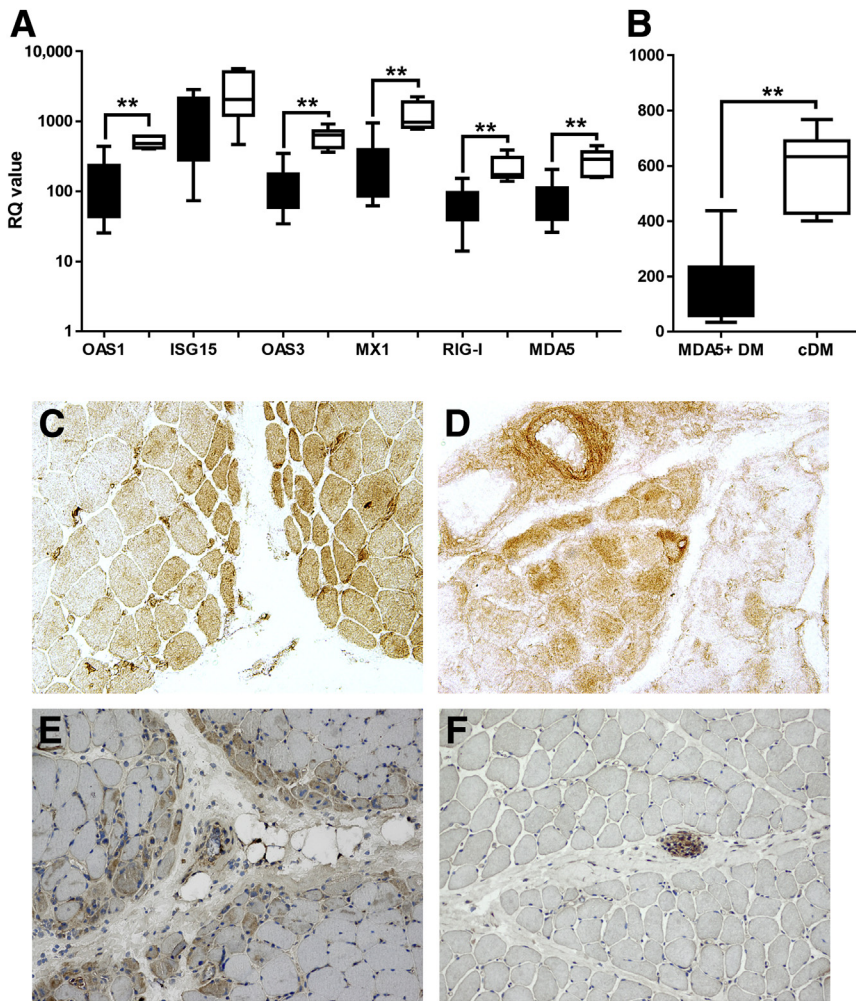
Figure 4 IFN signature in MDA5$^+$ DM patients. **A:** Box-and-whiskers graph showing RQ values for a panel of six ISGs [2′-5′-oligoadenylate synthetase (*OAS*)*1*, *ISG15*, *OAS3*, myxovirus (influenza virus) resistance (*MX*)*1*, retinoic acid–inducible gene (*RIG*)*1*, and *IFIH1*] measured in skeletal muscle tissue from MDA5$^+$ (black boxes) and cDM (white boxes) patients. **B:** IFN score calculated from the median fold-change of the RQ value for the panel of six ISGs in MDA5$^+$ (black boxes) and cDM (white boxes) patients. **C** and **D:** RIG-I–positive fibers were detected in both cDM and MDA5$^+$ DM biopsy specimens. **E** and **F:** ISG15-positive myofibers were detectable in the perifascicular region on atrophic fibers in cDM biopsy specimens (**E**), whereas ISG15 was not detectable in MDA5$^+$ DM biopsy specimens, but only on inflammatory infiltrates (**F**). *******P* ≤ 0.01. cDM, classic dermatomyositis; DM, dermatomyositis; IFN, interferon; ISG, IFN-stimulated genes; MDA5, melanoma differentiation-associated gene 5; RQ, relative quantification.

sarcoplasm of myofibers; conversely, classic DM patients did not (*P* < 0.003) (Figure 5B). In MDA5$^+$ patients, numerous NOS2$^+$ fibers were detectable in fascicles adjacent to perimysial vessels with inflammatory infiltrates (Figure 5D).

Because NOS2 positivity was a striking feature in MDA5 patients' biopsy specimens only, we hypothesized a protective, and not a detrimental, effect of NOS2 in the skeletal muscle. Therefore, we tested for the presence of chaperone proteins associated with protection. Indeed, NOS2$^+$ fibers in MDA5$^+$ patient biopsy specimens strongly colocalized with HSP70 (Figure 5E). In addition, colocalization with neural cell adhesion molecule was also observed (Figure 5F); as mentioned above, classic DM (MDA5$^-$) patients did not show NOS2-positive fibers (no colocalization, data not shown). In classic DM, however, only single fibers were NOS2 positive, whereas fibers in the perifascicular region were HSP70 positive and no colocalization was detectable (Figure 5G).

Discussion

In this study, addressing morphological and immunological aspects in MDA5$^+$ patients, we show that muscle pathology

and the local immune response differ from classic DM patients. The main finding of this study is that we observed a characteristic specific NOS2 expression on the sarcoplasm of HSP70-positive muscle fibers in MDA5$^+$ DM patients, which was absent in classic DM patients. Conversely, the IFN pathway was activated in both groups. We analyzed these observations in the light of the intriguing fact that MDA5$^+$ patients show mild signs of myositis but a severe systemic disease.

To our knowledge, there is no comprehensive report documenting muscle pathology in adult MDA5$^+$ patients. Indeed, the MDA5 antibody was the first one specifically associated with clinically amyopathic DM.[17] Interestingly, Tansley et al[9] have also described a reduced severity score in juvenile MDA5$^+$ patients compared with other MDA5$^-$ juvenile DM biopsy specimens.

There are only few reports documenting morphological findings of skeletal muscle biopsy specimens in clinically amyopathic DM patients.[5,18,19] They postulate that clinically amyopathic DM may serve as a paradigm of vasculopathy, because clinically amyopathic DM muscle biopsy specimens show severe capillary loss.[5,19] However, in our study,
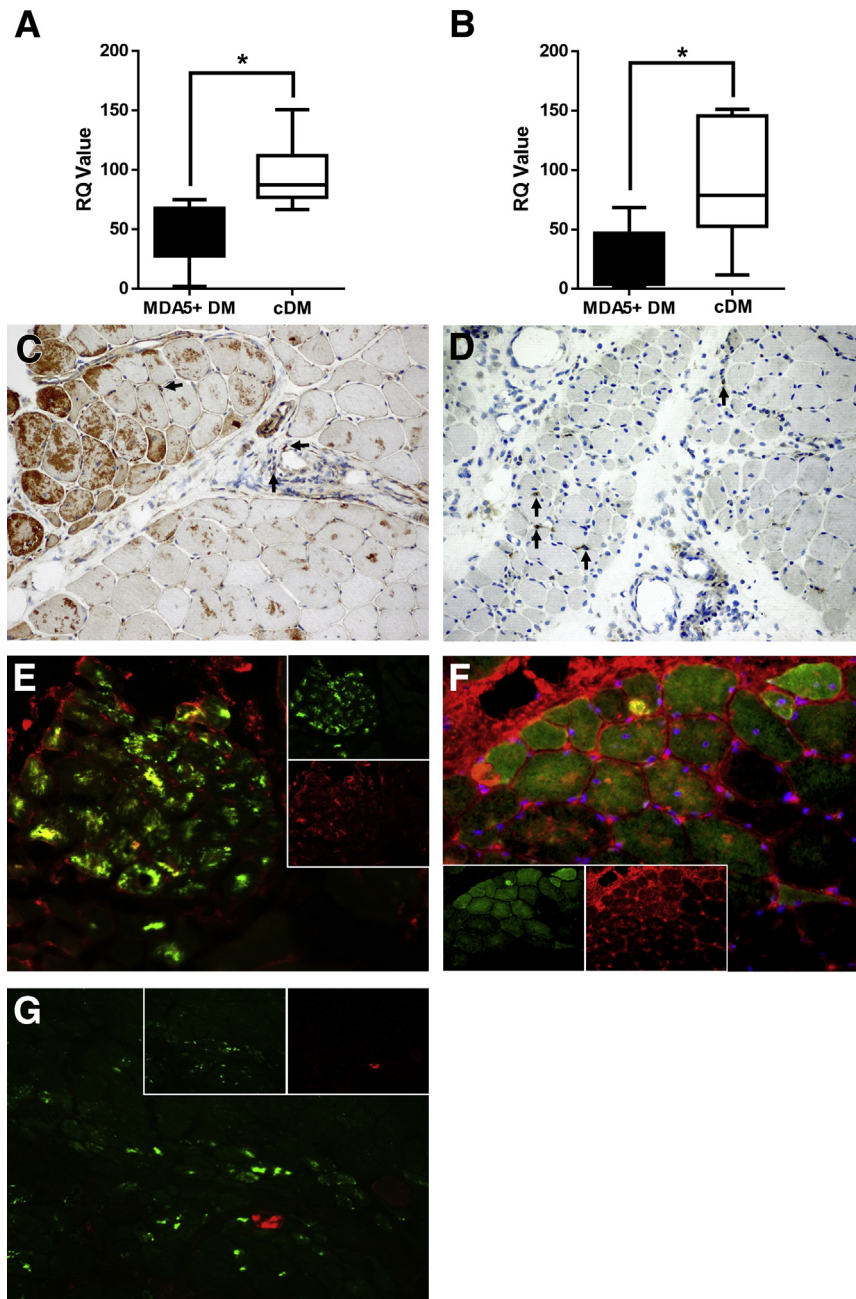
Figure 5 Type 1 helper T-cell immunity and expression of NOS2 in muscle fibers of MDA5+ patients. Box-and-whiskers graph showing RQ values for gene expression of *STAT1* (**A**) and *IFNG* (**B**) genes measured in muscle tissue from MDA5+ (black boxes) and cDM patients (white boxes). **C:** NOS2-positive immune cells corresponding to activated M1 macrophages (**arrows**), and strong immunoreactivity with NOS2 on numerous muscle fibers in a MDA5+ DM patient. Positive fibers were close to a vessel, and some were surrounded by inflammatory infiltrates. **D:** NOS2-positive immune cells (**arrows**) in a cDM patient. No immuno-staining on sarcolemma or sarcoplasm of fibers. **E:** Colocalization of NOS2 (red) positive myofibers with heat shock protein 70 (green) positive myofibers in an MDA5+ patient illustrated by immunofluorescence. **F:** Colocalization of NOS2 (green) positive myofibers with neural cell adhesion molecule (red) positive myofibers in an MDA5+ patient illustrated by immunofluorescence. **G:** One single fiber shows NOS2 positivity (red) and does not colocalize with perimysially localized atrophic fibers in a cDM patient biopsy specimen. *$P < 0.05$. Original magnification, ×200 (**C**−**G**). cDM, classic dermatomyositis; MDA5, melanoma differentiation-associated gene 5; NOS, nitric oxide synthase; RQ, relative quantification.

vasculopathy was less pronounced or absent in comparison to biopsy specimens from classic DM patients without MDA5 antibodies.[20] Electron microscopy, which has never been reported, revealed that half of the specimens showed tubuloreticular inclusions in endothelial cells, whereas all DM patients showed this feature, as previously reported.[2]

In contrast to classic DM, inflammatory infiltrates were essentially detected focally, clustering around single vessels. This mild quantitative presence of inflammatory cells fits to the mild muscle phenotype in general, including low CK levels. All patients showed immune inflammatory abnormalities, even if clinical signs of myopathy were completely absent.

The IFN signature was highlighted to be of paramount importance in the pathophysiology of DM.[3] On the basis of recent publications confirming these results,[3,4,21] six ISGs were selected and found up-regulated; however, this occurred more strongly in classic DM than in MDA5+ patients. The IFN pathways can be activated by IFN-α, a cytokine mainly produced by pDCs; however, we were not able to identify a significant amount of pDCs in both groups, in accordance with what was described by Eloranta et al.[22] However, others reported significant amounts of pDCs in classic DM.[3] Consistent with previous studies showing expression of IFN-dependent genes (*ISG15*, *MX1*, or *RIGI*) at the molecular level in muscle fibers of DM patients,[3,4,21] we were able to illustrate expression of RIG-I and ISG15 on muscle fibers by immunohistochemistry in MDA5+ patients. This illustrates that IFN

pathways also involve muscle fibers per se in addition to immune cells.

The common IFN signature we observed in both groups is not a conflicting result regarding the differences in extramuscular phenotypes and the muscle pathology in DM and MDA5^{+} patients. Indeed, differences in the quality or quantity of the IFN response, and/or the existence of specific protective mechanisms in the skeletal muscle, may occur. As an example, type I interferonopathies because of genetically manifest gain of function lead to different clinical phenotypes, depending on their specific mutation, whereas the IFN signature is common.[23,24]

Along that line, expression of NOS2 in muscle fibers of MDA5^{+} DM patients was widespread and strong, largely confined to regenerating fibers in contrast to classic DM patients. Nitric oxide produced by NOS2 comprises two opposing effects of the immune system (namely, regulatory and effector functions).[25] The significant NOS2 expression we observed in MDA5^{+} DM patients suggests that NOS2 and nitric oxide production may have protective and homeostatic effects. In this context, it was reported that nitric oxide can down-regulate immune responses,[25,26] and NOS2 is especially required for muscle regeneration after damage.[27] Interestingly, research on myocardial ischemia has highlighted the ability of cardiac myofibers to adapt to stress. Ischemic preconditioning is a biphasic phenomenon,[28] which is protective against myocardial infarction and stunning.[29] NOS2 and HSP70 are responsible for conferring protection during a subsequent ischemic challenge *in vivo* and *in vitro*.[30−34] Thus, we hypothesize that NOS2 and HSP70 in MDA5^{+} patients may elicit reduced generation of reactive oxygen species (eg, in mitochondria) and enhanced cellular protection. Furthermore, we found that NOS2 colocalized with neural cell adhesion molecule in MDA5^{+} DM myofibers, which was not the case for classic DM patients. Neural cell adhesion molecule is mostly expressed by regenerating fibers in inflammatory myopathies.[35] Thus, our results suggest that NOS2 expression is associated with regenerating effects.

To conclude, we show that muscle pathology in MDA5^{+} DM does not merely represent an attenuated form of classic DM on the basis of the morphological picture, distribution, and phenotyping of immune cells and signs of vasculopathy as well IFN signature. Contrarily, we document a striking colocalization of NOS2 and HSP70 muscle fibers, which we interpret as protective because this association was shown to be involved in tissue homeostasis.

Acknowledgments

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2015.11.010*.

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