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Research Article

# Toll-like receptors and IL-7 as potential biomarkers for immune-mediated necrotizing myopathies

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**We aimed to verify whether the immune system may represent a source of potential biomarkers for the stratification of immune-mediated necrotizing myopathies (IMNMs) subtypes. A group of 22 patients diagnosed with IMNM [7 with autoantibodies against signal recognition particle (SRP) and 15 against 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR)] and 12 controls were included. A significant preponderance of M1 macrophages was observed in both SRP$^+$ and HMGCR$^+$ muscle samples ($p < 0.0001$ in SRP$^+$ and $p = 0.0316$ for HMGCR$^+$), with higher values for SRP$^+$ ($p = 0.01$). Despite the significant increase observed in the expression of TLR4 and all endosomal Toll-like receptors (TLRs) at protein level in IMNM muscle tissue, only TLR7 has been shown considerably upregulated compared to controls at transcript level ($p = 0.0026$), whereas TLR9 was even decreased ($p = 0.0223$). Within IMNM subgroups, TLR4 ($p = 0.0116$) mRNA was significantly increased in SRP$^+$ compared to HMGCR$^+$ patients. Within IMNM group, only IL-7 was differentially expressed between SRP$^+$ and HMGCR$^+$ patients, with higher values in SRP$^+$ patients ($p = 0.0468$). Overall, innate immunity represents a key player in pathological mechanisms of IMNM. TLR4 and the inflammatory cytokine IL-7 represent potential immune biomarkers able to differentiate between SRP$^+$ and HMGCR$^+$ patients.**

**Keywords:** Cytokines · Immune-mediated necrotizing myopathies (IMNMs) · Innate immunity · Macrophages · Toll-like receptors

Additional supporting information may be found online in the Supporting Information section at the end of the article.

## Introduction

Immune-mediated necrotizing myopathies (IMNMs) are considered a distinct form of idiopathic inflammatory myopathies (IIMs), characterized by prominent necrosis, regeneration, myophagocytosis and a paucity of intramuscular lymphocytes [1, 2]. Clinically, IMNMs show significantly more widespread muscle oedema, atrophy and fatty replacement [3], other than higher serum CK levels at presentation [4] compared with other IIM subgroups.

Depending on serological status, three subtypes of IMNM are currently recognized: anti-SRP$^+$ IMNM, characterized by autoantibodies against components of the endoplasmic reticulum

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(ER)–associated signal recognition particle (SRP) in the protein synthetic pathway, anti-HMGCR$^+$ IMNM, with auto-antibodies against 3-hydroxy-3-methyl-glutaryl–CoA reductase (HMGCR), the rate-controlling enzyme of the mevalonate pathway involved in cholesterol synthesis, and seronegative IMNM [5, 6]. It has been speculated that this latter may be considered an IMNM harbouring as-yet undetected myositis-specific autoantibodies (MSAs) [2].

The pathogenesis of IMNM is presumed to be autoimmune based on in vitro and in vivo passive-transfer data pointing out the pathogenicity of the serum autoantibodies, and it is even suggested by the subacute onset and the clinical response to immunotherapy [7, 8]. Recent reports showed that IMNM is essentially a disease mediated by T lymphocytes (Th1 subtype) and classically activated macrophages (M1), in which high levels of IFN-γ, TNF-α, IL-12 and STAT1 functionally regulate the immune response [9]. On the other hand, Th2-mediated processes seem to play a minor role in this disorder [10]. Serum cytokine and chemokine profiles in patients affected by IMNM have been recently investigated, showing a significant increase in IP-10, MIP-1α, MCP-1, IFN-γ, IL-1Ra and IL-7 and their association with clinical profiles, although their relation with specific IMNM subtypes defined according to the associated antibody has not been analysed [11]. However, other key players of the innate immunity, such as Toll-like receptors (TLRs), have been never investigated in IMNM, although their major role in other IIMs has been well established [12, 13].

In addition to immunological mechanisms, it has been hypothesized that also nonimmune mediators might play an important role in skeletal muscle impairment in IMNM, especially considering that some IMNM patients have poor response to immunosuppressive therapies, even in combination, and inflammation, by definition, is relatively limited in IMNM muscle samples when compared to other IIM [14–16]. Ma et al. recently observed that ER stress correlates with muscle weakness in IMNM and suggested that the antigen–antibody complexes in seropositive IMNM might target the protein translational structures, turning into a potential direct stressor of the ER to induce autophagy [16].

Indeed, IMNM pathomechanisms still need to be completely clarified. Hence, we propose here to investigate the role of innate immunity pathways in the induction and perpetuation of muscle injury in IMNM, focusing on TLR expression and cytokine profiles, even in relation to different antibody profiles.

## Results

### Innate and cell-mediated immune response in IMNM

#### *Difference of C5b-9 expression in IMNM subgroups muscle tissue*

C5b-9 complement complex plays a major role in the IMNM pathogenesis and muscle specimens of all analysed IMNM patients showed its strong expression, without a significant difference between SRP$^+$ and HMGCR$^+$ subgroups (Supporting information Fig. 1).

C5b-9 was strongly expressed at the level of endothelial cells of small and large blood vessels and capillaries, in the sarcoplasm of necrotic muscle fibres and occasionally also of a few non-necrotic myofibres scattered in the muscle tissue (as shown by haematoxylin/eosin and modified Gomori trichrome stain). In some areas, C5b-9 was discontinuously expressed only on the sarcolemma of necrotic fibres, without any immunoreactivity in the sarcoplasm.

Colocalization between C5-b9 and TLR7 or TLR9 positive signals was detected by confocal microscopy (Supporting information Fig. 1).

Overall, data on C5b_9 expression did not show any difference according to the IMNM antibody profile.

#### *Inflammatory immune cells in IMNM subgroups muscle tissue*

Different immune cells are involved in the IMNM pathological mechanisms, with macrophages being the more relevant. The dominant mononuclear cellular infiltrate in both SRP$^+$ and HMGCR$^+$ biopsy specimens was represented by CD68$^+$ macrophages, distributed in the endomysium and perimysium, independently of myophagocytosis (Fig. 1). Characterization of different macrophagic subpopulations identified the inducible nitric oxide synthase positive (iNOS$^+$) classically activated M1 pro-inflammatory macrophages as well as the CD206$^+$ alternatively activated M2 reparative, profibrotic macrophages in both IMNM subgroups. Quantification of the two subpopulations showed a significant preponderance of M1 macrophages in both SRP$^+$ and HMGCR$^+$ muscle samples ($p < 0.0001$ in SRP$^+$ and $p = 0.0316$ for HMGCR$^+$), with higher values for SRP$^+$ ($p = 0.01$) (Fig. 1).

B cells were identified in both SRP$^+$ and HMGCR$^+$ biopsy specimens, without any significant difference between these two groups ($p = 0.8001$; data not shown). Other than being distributed in the endomisium, B cells were localized also within some necrotic muscle fibres in SRP$^+$ muscle tissue.

A few CD3$^+$ T cells were observed in both groups of IMNM, with prevalence in the SRP$^+$ muscle samples ($p = 0.0065$; data not shown). T cells were mainly distributed near large blood vessels and also in proximity of necrotic muscle fibres.

Analysis of the cell types involved in the IMNM inflammatory process pointed out that M1 macrophages and CD3$^+$ T cells were more frequent in the SRP$^+$ subgroup.

### Immunohistochemical analysis of TLR4 and endosomal TLRs in IMNM muscle tissue

With the aim of testing TLR protein expression and localization in the muscle tissue, we specifically evaluated TLR4 and endosomal

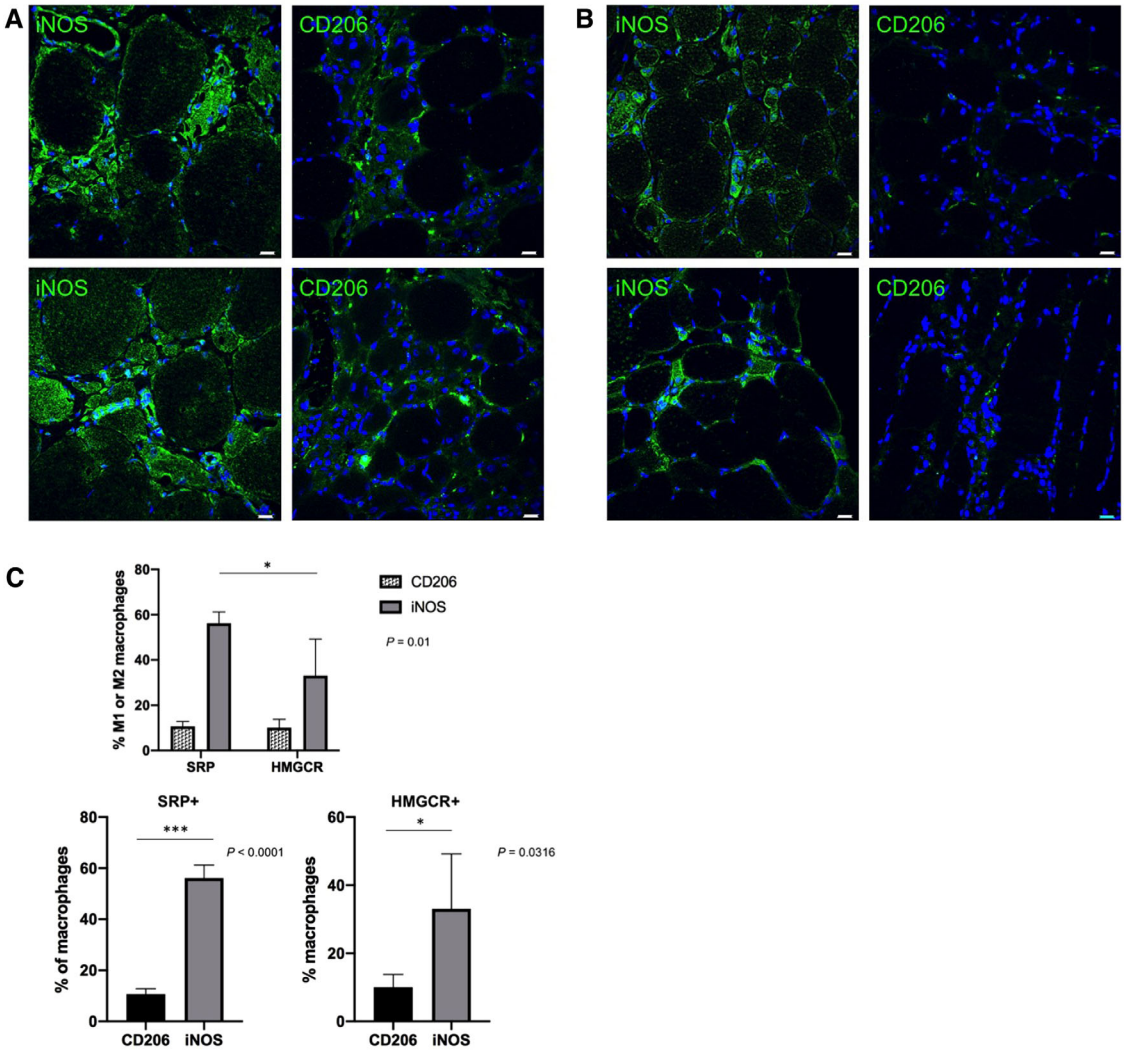


**Figure 1.** Characterization of macrophagic subpopulations in patients with immune-mediated necrotizing myopathies (IMNM). M1 proinflammatory macrophages and M2 reparative macrophages are identified in five SRP$^+$ (A) and five HMGCR$^+$ (B) IMNM patients by immunofluorescence, using anti-iNOS (green) and anti-CD206 (green) antibodies, respectively. Immunofluorescence data are presented as mean ± SD. Original magnification, 40×. Scale bars, 20 μm. (C) Quantification of macrophagic populations in IMNM subgroups highlighted the majority of M1 in both SRP$^+$ (Mann–Whitney *U* test, $p < 0.0001$) and HMGCR$^+$ ($p = 0.0316$) patients, with higher percentage in SRP$^+$ group ($^*p = 0.01$). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

TLRs (TLR3, TLR7 and TLR9) by fluorescence microscopy on muscle samples from patients affected by different forms of IMNM (SRP$^+$, HMGCR$^+$) and controls. In IMNM muscle tissue, TLR3 was mainly localized in the sarcoplasm of some degenerated/necrotic muscle fibres and at the level of some inflammatory cells close to damaged muscle fibres or scattered in the sample (Fig. 2). Occasionally, a positive staining for TLR3 was also observed on the sarcolemma of some myofibres near necrotic areas.

Within IMNM subgroups, SRP$^+$ muscle tissue showed a more intense immunopositivity for TLR3, compared to HMGCR; this is probably due to the presence of a more severe damage to the muscle tissue in this IMNM subgroup (Fig. 2).

TLR4 staining was particularly marked at the level of small sized degenerating/regenerating myofibres, with a more frequent subsarcolemmal localization or randomly distributed within sarcoplasm (Fig. 2). As previously observed in other forms of inflammatory myopathies, mononuclear muscle infiltrating cells showed a positive staining for TLR4.

Unlike TLR3 and TLR4, TLR7 was mainly expressed by inflammatory infiltrating cells, especially in SRP$^+$ IMNM (Fig. 3). The sarcoplasm of necrotic or damaged muscle fibres was weakly positive for this receptor. A similar pattern of expression was found for TLR9 (Fig. 3); moreover, in SRP + IMNM muscle tissue, TLR9 staining was particularly intense at the level of some severely injured myofibres.

In nonmyopathic controls, the staining for all TLRs was absent or rarely detected at the level of small blood vessels.

In conclusion, TLR3, TLR7 and TLR9 were significantly more expressed in muscle fibres or inflammatory infiltrating cells from SRP$^+$ samples.

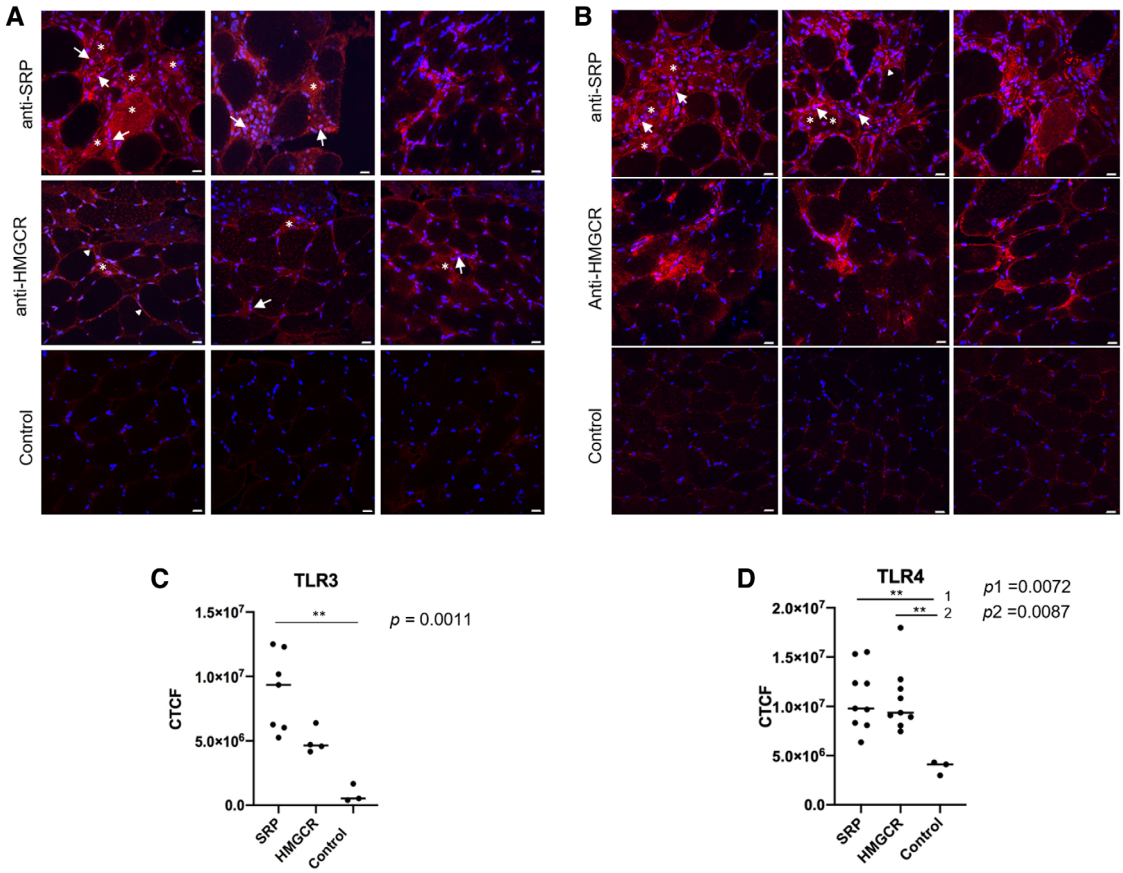


**Figure 2.** Immunofluorescence staining of immune-mediated necrotizing myopathies (IMNM) and control muscle sections for TLR3 (red) and TLR4 (red). (A) TLR3 is expressed in association with mononuclear inflammatory cells (arrows) and occasionally in the sarcoplasm of some degenerated/necrotic muscle fibres (asterisks). (B) In addition to a positive staining at the level of infiltrating cells (arrows), TLR4 is localized within small degenerating/regenerating myofibres, mainly in the subsarcolemmal region or randomly distributed within sarcoplasm (asterisks). Blue staining shows DAPI-positive nuclei. Immunofluorescence data are presented as mean ± SD. Original magnification, 40×. Scale bars, 20 μm. The analysis of the fluorescence intensity for TLR3 (C) and TLR4 (D), performed by the application of the corrected total cell fluorescence (CTCF) measurement (ImageJ software), shows an overexpression for both receptors in IMNM patients compared to controls, with SRP$^+$ patients presenting higher values (Mann–Whitney *U* test, $p = 0.0011$ and $p = 0.0072$, respectively). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

## Impaired TLR7 and TLR9 mRNA expression levels in IMNM compared to control muscles

After the detection of increased expression of specific TLRs through immunohistochemical analysis, we investigated TLR mRNA expression. The transcript levels of TLR4 and endosomal TLRs were assessed by real-time polymerase chain reaction (PCR) in 18 IMNM and 6 control muscle tissues (Fig. 4). At first, we compared the whole IMNM group (anti-SRP$^+$ and anti-HMGCR$^+$) with control muscles. TLR3, TLR4 and TLR7 mRNA expression levels tended to be upregulated in myopathic specimens compared to controls, although only TLR7 mRNA was statistically higher ($p = 0.0026$); conversely, the expression of TLR9 was significantly decreased in IMNM ($p = 0.0223$). When considering separately SRP$^+$ and HMGCR$^+$ subgroups compared to healthy controls, we observed a significant increase in TLR4 and TLR7 mRNAs in anti-SRP myopathic patients (TLR4: $p = 0.0116$ versus HMGCR$^+$ and $p = 0.0122$ versus controls; TLR7: $p = 0.0025$ versus controls) (Fig. 4). The expression levels for TLR3 showed a trend towards the upregulation in SRP$^+$ as well as HMGCR$^+$ muscles, although not statistically significant. TLR9 mRNA decreased in both SRP$^+$ and HMGCR$^+$ patients, even if without any statistical prevalent subgroup (Fig. 4).

Overall, the mRNA expression of TLR7 and TLR9 was increased and reduced, respectively, in IMNM compared to controls, whereas when considering separately SRP$^+$ and HMGCR$^+$ subgroups, only TLR4 mRNAs were higher in SRP$^+$ cases than in HMGCR$^+$.

## TLR9 sequencing in IMNM patients' sera

To better investigate the reduced mRNA expression of TLR9, we performed the sequencing analysis of the TLR9 gene from four patients, which revealed three silence variants p.Thr383Thr, p.Ser509Ser and p.Pro545Pro and one nonsense variant p.Arg863Gln (p.R863Q) (Supporting information Table 2).

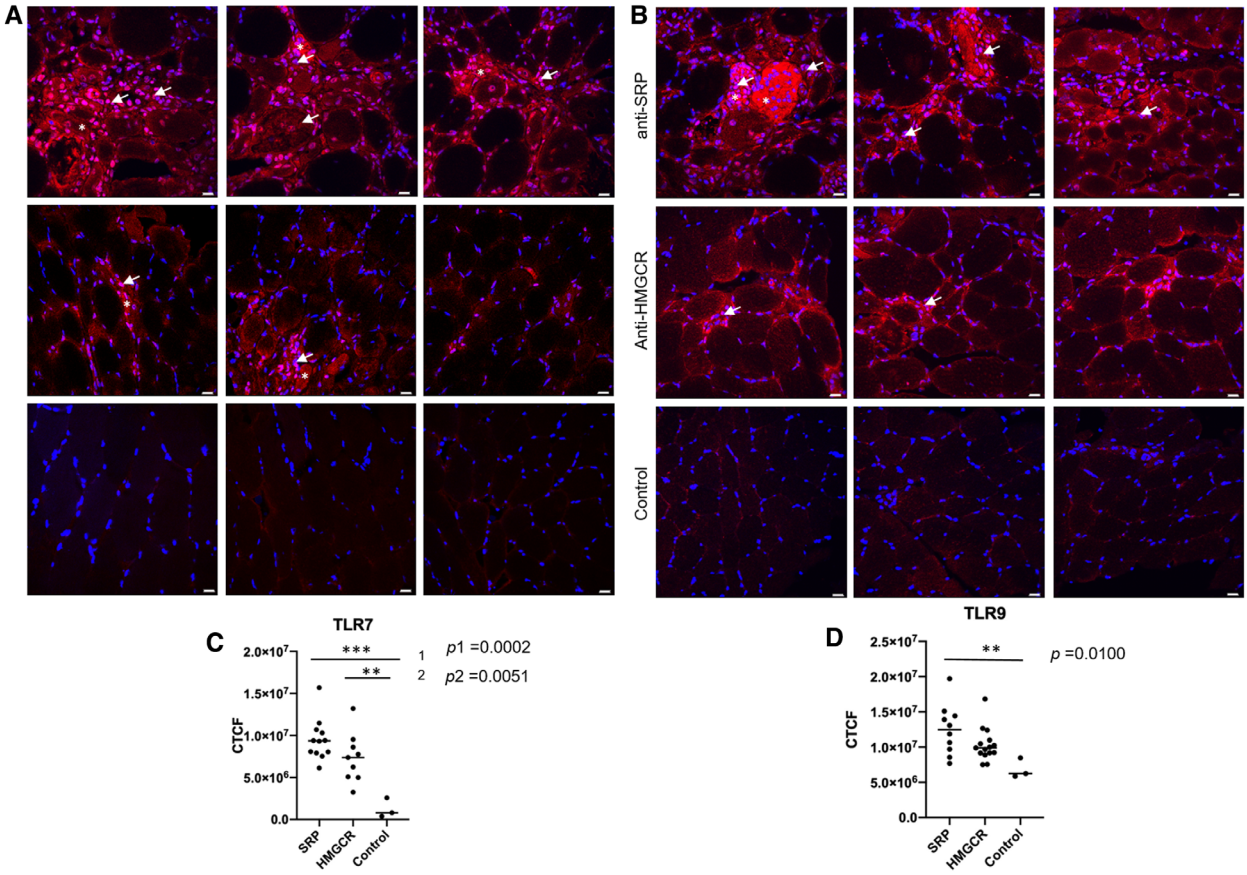


**Figure 3.** Immunofluorescence staining of immune-mediated necrotizing myopathies (IMNM) and control muscle sections for TLR7 (red) and TLR9 (red). Blue staining shows DAPI-positive nuclei. Immunofluorescence data are presented as mean ± SD. Original magnification, 40×. Scale bars, 20 μm. (A) As previously observed for idiopathic inflammatory myopathies (IIMs), also in IMNM muscles TLR7 is mainly distributed at the level of inflammatory cells, whereas a weak staining for the receptor is observed in the sarcoplasm of some degenerated muscle fibres. (B) TLR9 shows a distribution similar to TLR7, with a higher number of damaged muscle fibres in the $SRP^+$ patients' group. Quantification of the fluorescence intensity for TLR7 (C) and TLR9 (D) shows an overexpression for both receptors in IMNM patients compared to controls, with $SRP^+$ patients presenting higher values (Mann–Whitney *U* test, $p = 0.0002$ and $p = 0.0100$, respectively). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

The distribution of these variants in the four analysed patients is reported in Supporting information Table 3. In particular, the c.1635G>A variant was present in two patients in homozygous form (AA), in one in heterozygous form (GA) and in WT (GG) form in the remainder.

### Cytokine profile in IMNM serum samples

To further analyse the immune profile of our cohort, we quantified the serum concentration of 22 different cytokines and chemokines, showing a significant increase in the levels of IL-1Ra ($p = 0.0307$), IFN-α ($p = 0.0005$), VEGF ($p = 0.0261$), CCL-2 (MCP-1) ($p = 0.0007$), CXCL-10 (IP-10) ($p = 0.0073$), CXCL-8 (IL-8) ($p = 0.0086$) and CXCL-1 (GROα) ($p = 0.0005$) in IMNM patients compared to healthy controls (Fig. 5). Within the IMNM group, no significant difference was observed between $SRP^+$ and $HMGCR^+$ patients, except for IL-7 for which a higher concentration level for this cytokine was measured in $SRP^+$ patients ($p = 0.0468$) (Fig. 5).

## Discussion

IMNM pathogenesis has not been completely elucidated. Hence, in our study, we focused on specific key players of the immunity, to better understand their role in IMNM and investigate possible differences between $SRP^+$ and $HMGCR^+$ IMNM subgroups.

Consistent with the previous histological description of IMNM, our data confirm the presence of a mild lymphocytic infiltrate, including T and B cells restricted to the perivascular and endomysial regions, in a few patients. Complement depositions were observed mainly on necrotic myofibres, in a few nonnecrotic myofibres, and at the level of some capillaries. The main effector cells identified in IMNM were represented by macrophages rather than T cells. Characterization of the macrophage population associated with necrotic muscle fibres revealed the coexistence of both classical and alternatively activated cells, although with a significant prevalence of the M1 subtype. Comparison between the two IMNM subgroups showed a significantly higher number of M1 macrophages in $SRP^+$ than in $HMGCR^+$ muscle samples. These findings are in agreement with the recent literature,

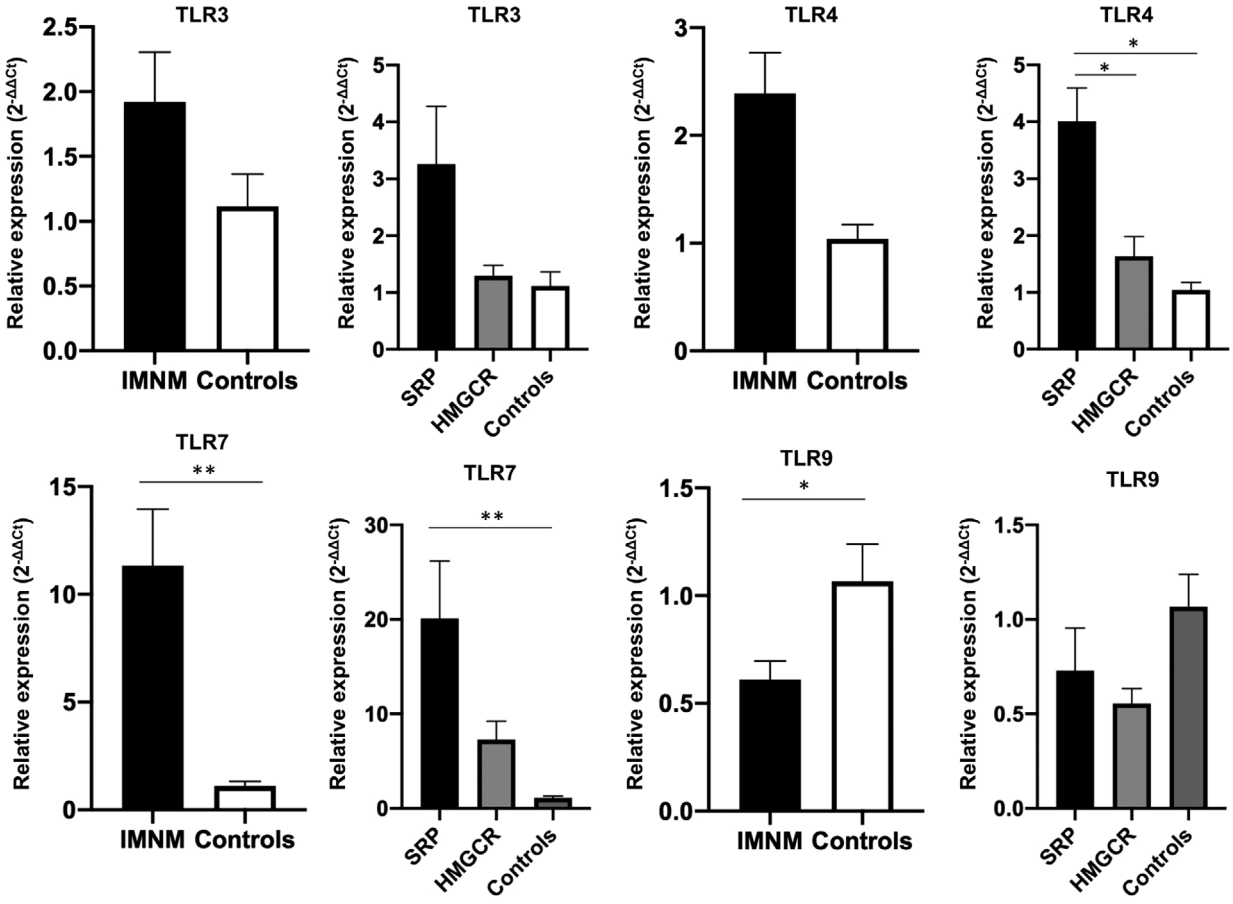


**Figure 4.** Dysregulation of TLR expression in immune-mediated necrotizing myopathies (IMNM). The comparison between IMNM patients and controls shows the upregulation of TLR3, TLR4 and TLR7 mRNAs in myopathic muscles, with a statistical significant difference only for TLR7 ($p = 0.0026$). Expression data are presented as mean ± SD. The expression levels of TLR9 are significantly decreased in IMNM (Kruskal–Wallis test followed by Dunn's post hoc test, $p = 0.0223$). Within IMNM group, only TLR4 discriminates between $SRP^+$ and $HMGCR^+$ patients (Kruskal–Wallis test followed by Dunn's post hoc test, $p = 0.0116$ versus $HMGCR^+$). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

establishing that IMNMs may be considered a Th1–M1-mediated disease [10].

Of the signalling pathways that modulate macrophage function, TLRs constitute a key system. The pivotal role of TLRs in the pathogenetic mechanisms of IIMs has just been extensively demonstrated, although never investigated in IMNM [12, 13]. TLRs are the trans-membrane receptors expressed on immune and nonimmune cells that recognize pathogens as well as self-molecules [17]. Cell surface TLRs are mainly involved in the recognition of membrane components of the microorganisms, such as lipids, lipoproteins and proteins, whereas intracellular TLRs are able to recognize nucleic acids derived from pathogens or self-nucleic acids in a disease condition [18]. Myonecrosis, scattered in the muscle tissue and at different stages, might represent an important source of damage-associated molecular patterns (DAMPs), contributing to the activation of an innate immune response by the host. TLRs exert a prominent role in the response to endogenous DAMPs [19, 20]. Fatty acids, lipoproteins, proteoglycans, glycosaminoglycans, proteins and peptides such as β-defensins, heat shock proteins, alarmins, fibrinogen, antiphospholipid antibodies as well as nucleic acids such as double- or single-stranded RNA and IgG-chromatin complexes, are all well-known endogenous TLR activators [21]. As observed in other forms of IIMs [22, 23], TLRs, particularly TLR4 and endosomal TLR3 and TLR7, were significantly upregulated in our IMNM samples. The increased TLR expression was mainly observed at the level of inflammatory cells scattered in the IMNM muscle tissue or localized close to damaged muscle fibres, emphasizing the readiness of the innate immune system to respond to muscle injury. Furthermore, positive staining for endosomal TLRs was also reported in the sarcoplasm of some degenerated/necrotic muscle fibres and, occasionally, on the sarcolemma of some nonnecrotic myofibres near necrotic areas. This finding once again underlines how muscle is not just a passive target of immune reactions. Indeed, muscle cells or myoblasts can express various cytokines, chemokines, adhesion molecules and costimulatory molecules, upon immunological challenge, hence actively participating in immune reactions, for example, as antigen-presenting cells.

DAMP-mediated activation of TLR triggers several transcription factors regulating the expression of genes that mediate

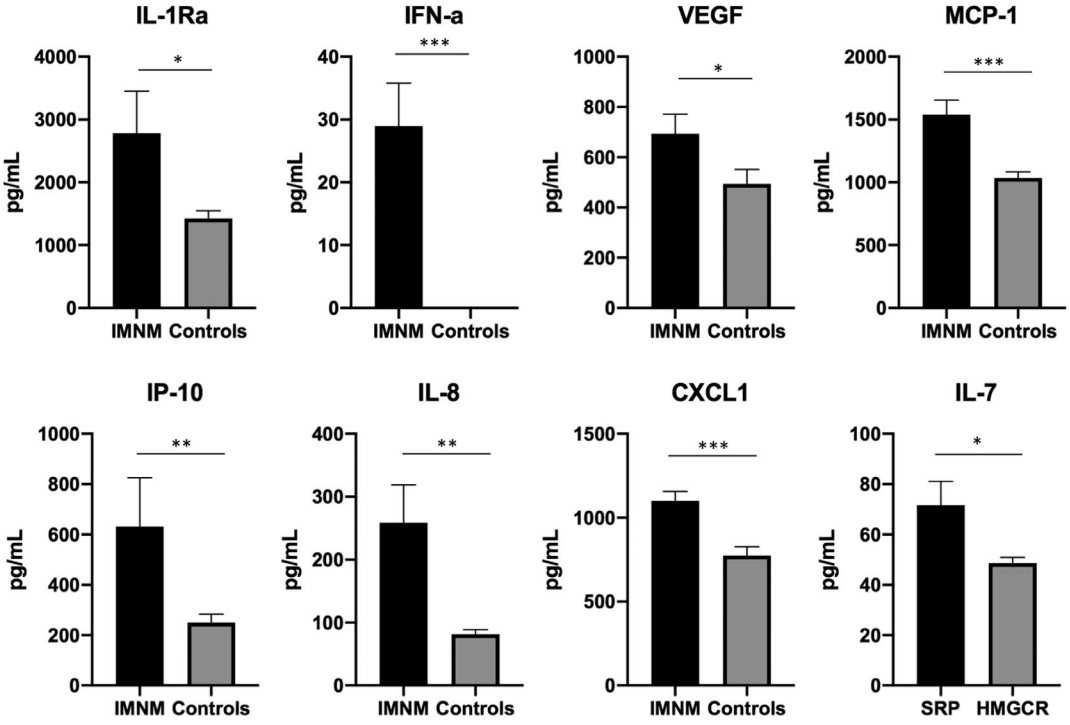


**Figure 5.** The immune mediator expression in serum samples from immune-mediated necrotizing myopathies (IMNM) patients and healthy controls. Concentration levels are presented as mean ± SD. Among the 22 immune mediators analysed, the serum concentration levels of seven molecules [interleukin-1Ra (IL-1Ra), $p = 0.0307$; IFN-α, $p = 0.0005$; VEGF, $p = 0.0261$; CCL-2 (MCP-1), $p = 0.0007$; CXCL-10 (IP-10), $p = 0.0073$; CXCL-8 (IL-8), $p = 0.0086$ and CXCL-1 (GROα), $p = 0.0005$] are significantly higher in the IMNM patients than in healthy controls. Within the IMNM group, a higher concentration level for IL-7 is observed in $SRP^+$ patients (Mann–Whitney *U* test, $p = 0.0468$). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

inflammatory and immune responses. Molecules synthesized following TLR signalling activation include inflammatory cytokines, such as IL-1, IL-6, TNF-α, IL-12, IFNs, chemokines, adhesion molecules, costimulatory molecules, growth factors, tissue-degrading enzymes, such as metalloproteinases, and enzymes that generate inflammatory mediators such as cyclo-oxygenase 2 and iNOS. All these factors contribute to downstream processes that facilitate degeneration and regeneration in skeletal muscle.

Despite the significant increase observed in the expression of TLR4 and all endosomal TLRs at protein level in IMNM muscle tissue, only TLR7 has been shown considerably upregulated compared to controls at transcript level, whereas TLR9 was decreased. This finding is in contrast to what was reported for the other subtypes of IIMs, in which the upregulation of all TLR mRNAs was described, even though with differences among polymyositis (PM), dermatomyositis (DM) and sporadic inclusion body myositis [13, 22–24]. Furthermore, TLR4 transcript levels were significantly higher in $SRP^+$ than in $HMGCR^+$ IMNMs, representing a potential discriminating factor for these two IMNM subtypes.

Particularly interesting was the downregulation of TLR9: This finding has never been observed in the other IIM subtypes; thus, it seems to represent a specific feature of IMNMs. The explanations for this discordance between protein and transcript levels might be different. For instance, it is known that on average proteins are about 1850 times more abundant than their respective mRNAs [25], and proteins are characterized by longer half-lives than their mRNAs. MicroRNAs might be produced during specific conditions, such as stress or injury that might recognize an RNA transcript and inhibit its translation. Otherwise, single-nucleotide polymorphisms (SNPs) might be responsible for a decrease or arrest of mRNA transcription [26, 27]. In order to verify this hypothesis, we sequenced *TLR9* gene in a small group of IMNM patients (two with anti-SRP and two with anti-HMGCR auto-antibodies) using the Sanger method. Sequencing of the TLR9 gene showed one variant c.1635G>A (p.P545P, rs352140) (Supporting information Table 3) with a genotype distribution in this study group of 25% heterozygotes GA, 25% homozygotes AA and 50% homozygotes GG. The rs352140 polymorphism was already notified in several disorders, such as primary immune thrombocytopenia [28], systemic lupus erythematosus [29], vasculitis [30] and HCV [31], highlighting a primary role of this polymorphism in the dysregulation of immune response in susceptible individuals. Therefore, in our study, the identification of specific SNPs seems not to correlate with an imbalance between TLR9 protein and transcript level in IMNM, but they are probably involved in the modulation of immune response promoting necrotizing myopathies. Certainly, future studies on larger samples should shed light on the significance of the TLR9 rs352140 polymorphism and its association with the IMNMs.

As recently reported [9, 10], macrophages abundantly infiltrate IMNM muscle tissue, regardless of the autoantibody pattern. Predominance of macrophages and the hyperactivation of endo-

somal TLRs might be responsible for the cytokine profile identified in IMNM. Comparing IMNM with controls, we observed a substantial increase in the serum concentration of a group of cytokines/chemokines, including IL-1Ra, IFN-α, VEGF, CCL-2 (MCP-1), CXCL-10 (IP-10), CXCL-8 (IL-8) and CXCL-1 (GROα), in IMNM patients, in agreement with data recently reported by Oda et al. [11] and studies on IIMs [9, 10, 32, 33]. Analysing serum profile within IMNM group, we observed that only IL-7 was differentially expressed between $SRP^+$ and $HMGCR^+$ patients, with a higher concentration in $SRP^+$ patients.

IL-7 is well known for its strong immunostimulatory properties, in particular for modulating T-cell homeostasis in humans. IL-7 may promote antigen-independent activation of T cells leading to the enhanced proliferation and production of proinflammatory cytokines [34]. Furthermore, this cytokine has been shown to prime human naive $CD4^+$ T cells, in the absence of accessory cells, for proinflammatory (IFN-γ, IL2) and anti-inflammatory cytokines (IL4). IL-7 can also stimulate proliferation and, under certain conditions, cytokine secretion of memory T cells [35]. A major increase of IL-7 in $SRP^+$ compared to $HMGCR^+$ IMNM might be due to the pathological features of this IMNM subtype; indeed, $SRP^+$ myopathy is considered the most disabling auto-immune myopathies, with more severe muscle weakness, muscle tissue degeneration/regeneration and inflammatory response compared to $HMGCR^+$, often associated with poor muscle recovery after treatment [36]. In view of these considerations, IL-7 appeared to be able to discriminate between these two forms of IMNMs.

This study highlights for the first time the involvement of specific immune mediators in IMNMs, and their potential in discriminating between the two major serotypes. No sufficient clinical data were available to investigate potential relations among immune mediator expression levels and disease severity, except for CK for which no significant correlations have been detected, even stratifying by anti-SRP and anti-HMGCR serotypes (data not shown). Further work is needed to clarify the role of the investigated molecules as diagnostic, prognostic and pharmacodynamic biomarkers of the disease.

In conclusion, innate immunity represents a key player in pathological mechanisms of IMNMs. In particular, here we demonstrated that TLR4 might be considered a potential diagnostic biomarker in the discrimination between $SRP^+$ and $HMGCR^+$ patients, whereas TLR7 and TLR9 might help in distinguishing between IMNM and control individuals. Among several cytokines and chemokines induced by TLR activation, IL-7 might represent a useful discriminating biomarker between $SRP^+$ and $HMGCR^+$ IMNMs.

## Materials and methods

### Patients

We included a total of 22 patients diagnosed with IMNM, characterized by subacute, symmetric and predominantly proximal muscle weakness and a muscle biopsy featuring prominent muscle fibre necrosis with sparse to no inflammatory infiltrates, based on the European Neuromuscular Centre criteria, 224th ENMC international workshop (14–16 October 2016) [1]. Exclusion criteria included a family history of muscle disease and histopathologic findings consistent with other IIM, such as polymyositis and dermatomyositis, or muscular dystrophies.

The following MSAs were assessed by a commercial semiquantitative line blot essay (EUROIMMUN, Lubeck, Germany): anti-Mi2 α and β, anti-transcriptional intermediary factor 1γ (TIF-1γ), anti-melanoma differentiation-associated protein 5 (MDA5), antinuclear matrix protein 2 (NXP2), anti-small ubiquitin-like modifier activating enzyme 1 (SAE1), anti-histidyl-tRNA synthetase (Jo1), anti-SRP, anti-threonyl-tRNA synthetase (PL7), anti-alanyl-tRNA synthetase (PL12), anti-glycyl-tRNA synthetase (EJ) and anti-isoleucyl-tRNA synthetase (OJ). On the same line blot assay, the following myositis-associated autoantibodies (MAAs) were assessed: anti-Ku, anti-polymyositis-scleroderma 100 protein (PMScl100), anti-polymyositis-scleroderma 75 protein (PMScl75) and anti-Ro52.13 A negative or a weak titre (1+) were considered negative, whereas moderately elevated (2+) and high titres (3+) were considered positive. In addition, anti-HMGCR antibodies (IgG) were assessed by a commercial semiquantitative ELISA (Inova Diagnostics, San Diego, CA) in the IMNM patient sera. A level of anti-HMGCR Abs of 20 units/mL or higher was considered positive. Patients were subsequently serologically classified as follows: (1) anti-$HMGCR^+$ IMNM, further subclassified into statin-associated and statin-naive disease; (2) anti-SRP + IMNM; (3) seronegative IMNMs.

Twelve controls, which underwent biopsy for diagnostic purposes but without myopathy confirmation, were included in the study. Clinical and molecular features of patients and controls are shown in Table 1.

No significant differences were detected by sex, age at onset and CK levels between anti-HMGCR positive and anti-SRP positive patients (data not shown).

Muscle tissues were obtained by open or needle biopsy, immediately frozen in precooled isopentane and stored in liquid nitrogen pending use.

### Standard protocol approvals, registrations and patient consents

This study was approved by the institutional review board of Fondazione IRCCS Istituto Neurologico Carlo Besta (08-1-12102022). Each individual provided written informed consent to muscle biopsy for diagnosis and research purposes.

### Immunofluorescence

Six micrometre–thick muscle cryosections from 10 IMNM (5 $SRP^+$, 5 $HMGCR^+$) to 5 controls (fixed for 10 min with 4% paraformaldehyde and permeabilized for 10 min with 0.1% Triton

**Table 1.** Patients' characteristics.

| Patient no. | Diagnosis | Age (years) at biopsy | Age (years) at onset | Gender | Autoantibody (MAA/MSA) | CK levels | Statin association | Myositis-related neoplasia | Inflammatory infiltrates |
|---|---|---|---|---|---|---|---|---|---|
| 1 | IMNM | 61 | 59 | M | SRP | 8504 | Yes | No | ? |
| 2 | IMNM | 51 | 50 | F | SRP | 4100 | No | No | – |
| 3 | IMNM | 57 | 57 | M | SRP | 4900 | No | No | + |
| 4 | IMNM | 74 | 74 | F | SRP | 6000 | Yes | No | ++ |
| 5 | IMNM | 73 | 72 | F | SRP | 1538 | No | Yes: timoma B1/2, basal cell carcinoma, giant lipomas | No |
| 6 | IMNM | 26 | 26 | F | SRP | 6000 | No | No | ++ |
| 7 | IMNM | 65 | 64 | F | SRP | 2000 | Yes | No | ++ |
| 8 | IMNM | 72 | 72 | M | HMGCR | 3600 | Yes | No | – |
| 9 | IMNM | 70 | 70 | F | HMGCR | 6000 | Yes | No | + |
| 10 | IMNM | 57 | 55 | F | HMGCR | 16000 | Yes | No | – |
| 11 | IMNM | 67 | 66 | M | HMGCR | 6030 | Yes | No | ? |
| 12 | IMNM | 47 | 45 | M | HMGCR | 3429 | No | No | ++ |
| 13 | IMNM | 67 | 56 | M | HMGCR | 240 | Yes | Yes | ++ |
| 14 | IMNM | 62 | 60 | F | HMGCR | N.A. | No | No | ++ |
| 15 | IMNM | 39 | 31 | F | HMGCR | 1900 | No | No | + |
| 16 | INMN | 69 | 69 | F | HMGCR | 4887 | No | No | ++ |
| 17 | IMNM | 61 | 60 | M | HMGCR | 9675 | Yes | No | + |
| 18 | IMNM | 69 | 69 | M | HMGCR | 500 | Yes | No | ++ |
| 19 | IMNM | 55 | 54 | F | HMGCR | 6000 | Yes | No | ++ |
| 20 | IMNM | 87 | 87 | M | HMGCR | 2059 | No | No | + |
| 21 | IMNM | 73 | 73 | F | HMGCR | 2706 | Yes | No | ++ |
| 22 | IMNM | 67 | 66 | M | HMGCR | 3000 | Yes | Yes (melanoma) | ++ |
| 23 | Control | 60 | – | M | – | – | – | – | – |
| 24 | Control | 60 | – | F | – | – | – | – | – |
| 25 | Control | 61 | – | M | – | – | – | – | – |
| 26 | Control | 34 | – | M | – | – | – | – | – |
| 27 | Control | 49 | – | F | – | – | – | – | – |
| 28 | Control | 60 | – | M | – | – | – | – | – |
| 29 | Control | 58 | – | M | – | – | – | – | – |
| 30 | Control | 60 | – | F | – | – | – | – | – |
| 31 | Control | 63 | – | M | – | – | – | – | – |
| 32 | Control | 40 | – | M | – | – | – | – | – |
| 33 | Control | 45 | – | M | – | – | – | – | – |
| 34 | Control | 55 | – | M | – | – | – | – | – |

Abbreviations: HMGCR, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; IMNM, immune-mediated necrotizing myopathy; MAA, myositis-associated autoantibody; MSA, myositis-specific autoantibody; SRP, signal recognition particle.

X-100) were incubated with 5% BSA and PBS for 30 min at room temperature to quench nonspecific binding. The slides were then incubated overnight at 4°C with various combinations of two primary antibodies diluted in 1% BSA and PBS at the following dilutions: 1:20 for anti-TLR4 (RRID: AB_2240715), anti-TLR3 (RRID: AB_2204855), anti-TLR9 (RRID: AB_2287377), all from Santa Cruz Biotechnology (Dallas, TX, USA), 1:20 for anti-TLR7 (RRID: AB_2052699, from Enzo Life Sciences, New York, NY, USA), 1:100 for anti-HLA-ABC (RRID: AB_2220942, clone W6/32, mouse monoclonal), 1:25 for anti HLA-DR (clone TAL 1B5, mouse monoclonal), 1:10 for anti-CD3 (clone UCHT1, mouse monoclonal), 1:50 for anti MAC/C5-b9 (clone aE11, mouse monoclonal), 1:100 for anti-CD68 (clone KP1, mouse monoclonal), 1:100 for anti-CD20cy (clone L26, mouse monoclonal) all from Dako Agilent (Santa Clara, CA, USA), 1:50 for anti-myosin heavy chain-developmental (MHCdev; mouse monoclonal RNMy2/9D2, NCL-MHCd) from Novocastra/Leica Biosystems (Nussloch, Germany), 1:20 for anti-iNOS (rabbit polyclonal), 1:500 for anti-CD206 (rabbit polyclonal) both from Novus Biologicals (Littleton, CO, USA).

After three washes with PBS, the slides were incubated with two secondary antibodies [Alexa Fluor 488 goat anti-mouse ready probe reagent, RRID: AB_2556548, dilution 2 drops/mL; Alexa Fluor 546 goat anti-rabbit IgG (heavy + light chains) antibody, RRID: AB_2534093, dilution 1:600; all from Thermo Fisher Scientific (Waltham, MA, USA)] for 1 h at room temperature. DAPI solution (62248, dilution 1:1000; Thermo Fisher Scientific) was used as a nuclear marker. As a negative control, sections were incubated with isotype-specific nonimmune IgG (Agilent Technologies) and rabbit serum (from our laboratory, at the same dilution of the corresponding primary antibodies). The slides were mounted with FluorSave Reagent (Merck, Kenilworth, NJ, USA), sealed and dried for 1 h at room temperature. Images were captured with a Nikon Eclipse TE2000-E confocal laser-scanning microscope and analysed with EZ-C1 3.70 imaging software (both from Nikon, Tokyo, Japan). Positive fluorescence staining was quantified by applying the corrected total cell fluorescence (CTCF) measurement by ImageJ software.

Connective tissue and necrotic myofibres were identified by a standard staining protocol using Harris' haematoxylin and Gömöri trichrome stain (both from Bio-Optica, Milan, Italy).

## RNA extraction and cDNA synthesis

Total RNA was extracted from 10 to 20 mg of frozen muscle tissue from 18 IMNM patients (including 7 $SRP^+$, 11 $HMGCR^+$) and 10 controls using TRIzol Reagent (Invitrogen, Thermo Fisher Scientific, #15596018), followed by DNA-free DNA Removal kit (Ambion, Thermo Fisher Scientific, #AM1906). For mRNA amplification, random-primed cDNA was prepared using the SuperScript VILO cDNA Synthesis kit (Invitrogen, Thermo Fisher Scientific, #11754-050) following the manufacturer's instructions and stored at −20°C pending PCR amplification.

## Real-time quantitative PCR

cDNA amplification and data acquisition were performed with a ViiA 7 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific), using predesigned functionally tested assays: TLR3: Hs00152933_m1; TLR4: Hs00152939_m1; TLR7: Hs00152971_m1; TLR9: Hs00152973_m1 (all from Applied Biosystems, Thermo Fisher Scientific, #4331182). All the reactions were done in triplicate and normalized to the reference gene GAPDH (Hs99999905_m1) (Applied Biosystems, Thermo Fisher Scientific, #4331182).

## Sequence analysis of TLR9 gene (RefSeq NM_017442)

Genomic DNA from four IMNM patients (two $SRP^+$ and two $HMGCR^+$) was extracted from peripheral blood on Freedom EVO 100 (Tecan, Männedorf, Switzerland) by NucleoSpin blood Kit following the manufacturer's instructions (Macherey-Nagel, Düren, Germany). DNA quality and quantity were analysed by NanoDrop (Thermo Fisher), gel electrophoresis and fluorescence absorbance (Qubit 2.0 Fluorometer; Thermo Fisher). The two exons of TLR9 were amplified by PCR using intronic oligonucleotide primers newly designed using Primer3web (version 4.1.0) reported in Supporting information Table 1. The PCR products were purified by EuroSAP PCR Enzymatic Clean-up kit (EuroClone, Pero, Milan, Italy) and sequenced by bidirectional sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher), on an Applied Biosystems 3130xl Genetic Analyzer (Thermo Fisher). The obtained sequences were analysed with SeqScape v.3.0 software (Thermo Fisher Scientific) and compared with reference WT sequence (GenBank accession number: NM_017442).

## Cytokine, chemokine and growth factor quantification

A Human chemokine magnetic Luminex performance assay 8-plex fixed panel which includes CCL2/JE/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CCL11/Eotaxin, CXCL1/GROα/KC/CINC-1, CXCL10/IP-10/CRG-2, IL-8/CXCL8 and a Human cytokine magnetic Luminex performance assay 14-plex fixed panel, including IFN-α, IFN-γ, IL-1α/IL-1F1, IL-1β/IL-1F2, IL-1ra/IL-1F3, IL-2, IL-3, IL-4, IL-6, IL-7, IL-10, IL-15, IL-33 and VEGF (both from R&D Systems, Minneapolis, MN, USA), were used to measure the serum concentration of pro- and antiinflammatory cytokines, chemokines and growth factors in the serum of ten IMNM patients (six $SRP^+$ and four $HMGCR^+$) and ten age-matched healthy controls. The assays were performed according to manufacturer's guidelines, and the plates were read on the BioPlex 200 system (Bio-Rad, Hercules, CA, USA), powered by Luminex xMAP technology.

Each patient's and healthy control's serum was diluted 1:2 and tested in duplicate. Data are expressed as concentration (pg/mL). The concentration of analyte bound to each bead is proportional

to the median fluorescence intensity of reporter signal and is corrected by the standards provided in the kit (R&D Systems).

### Statistical analysis

The Kruskal–Wallis nonparametric test, with Dunn's nonparametric comparison post hoc test, was used to assess differences in mRNA levels in disease groups and controls, as they were not normally distributed. The Mann–Whitney *U* test was used to evaluate differences between IMNM group and controls. Differences were considered significant at $p < 0.05$. The analyses were performed with GraphPad Prism software version 8.0 (GraphPad Software, San Diego, CA) for Macintosh.

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*Abbreviation:* **CTCF**: corrected total cell fluorescence · **DAMP**: damage-associated molecular pattern · **DM**: dermatomyositis · **HMGCR**: 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase · **IIMs**: idiopathic inflammatory myopathies · **IMNM**: immune-mediated necrotizing myopathy · **MAAs**: myositis-associated autoantibodies · **MSA**: myositis-specific autoantibody · **PM**: polymyositis · **SNP**: single-nucleotide polymorphism · **SRP**: signal recognition particle

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