Immune-Mediated Necrotizing Myopathy Is Characterized by a Specific Th1-M1 Polarized Immune Profile

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Immune-mediated necrotizing myopathy (IMNM) is considered one of the idiopathic inflammatory myopathies, comprising dermatomyositis, polymyositis, and inclusion body myositis. The heterogeneous group of necrotizing myopathies shows a varying amount of necrotic muscle fibers, myophagocytosis, and a sparse inflammatory infiltrate. The underlying immune response in necrotizing myopathy has not yet been addressed in detail. Affected muscle tissue, obtained from 16 patients with IMNM, was analyzed compared with eight non-IMNM (nIMNM) tissues. Inflammatory cells were characterized by IHC, and immune mediators were assessed by quantitative real-time PCR. We demonstrate that immune- and non-immune-mediated disease can be distinguished by a specific immune profile with significantly more prominent major histocompatibility complex class I expression and complement deposition and a conspicuous inflammatory infiltrate. In addition, patients with IMNM exhibit a strong type 1 helper T cell (Th1)/classically activated macrophage M1 response, with detection of elevated interferon- γ , tumor necrosis factor- α , IL-12, and STAT1 levels in the muscle tissue, which may serve as biomarkers and aid in diagnostic decisions. Furthermore, B cells and high expression of the chemoattractant CXCL13 were identified in a subgroup of patients with defined autoantibodies. Taken together, we propose a diagnostic armamentarium that allows for clear differentiation between IMNM and nIMNM. In addition, we have characterized a Th1-driven, M1-mediated immune response in most of the autoimmune necrotizing myopathies, which may guide

therapeutic options in the future. (*Am J Pathol* 2012, 181: 2161–2171; <http://dx.doi.org/10.1016/j.ajpath.2012.08.033>)

Immune-mediated inflammatory myopathies (IIMs) comprise the classic inflammatory myopathies, dermatomyositis (DM), and polymyositis (PM). However, some researchers have expressed a critical view on PM as an entity,^{1,2} inclusion body myositis (IBM), and immune-mediated necrotizing myopathy (IMNM),³ recently also termed necrotizing autoimmune myositis.^{4,5} These diseases are defined as immune-mediated inflammatory myopathies,⁵ outnumbering other rare inflammatory myopathies, such as eosinophilic myofasciitis, granulomatous myositis, or macrophagic myofasciitis. They all share the classic symptoms of myalgia and muscle weakness, with each of them being characterized by specific clinical and morphological features.^{5,6} DM and PM show a relatively good responsiveness to immunosuppressive therapies, whereas IBM, in general, displays a slower development of symptoms and shows no substantial response to immunosuppressive therapy.^{7–13} In addition to the widely accepted clinical classification, a new one has been presented recently that is based on pathological criteria of immune and inflammatory myopathies. In this classification, IMNM is described as immune polymyopathy containing myofiber necrosis, regeneration, and a poor mononuclear cell infiltrate with varying trans-sarcolemmal major histocompatibility complex (MHC) class I positivity.^{1,14,15}

Patients with subacute skeletal muscle weakness, myalgia, elevated serum creatine kinase levels, and an elec-

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tromyogram showing signs of muscle irritability may display NM on a muscle biopsy specimen. NM, be it IMNM or non-IMNM (nIMNM), can be encountered in a large variety of etiopathological contexts.^{16,17} Within the heterogeneous group of NMs, the immune-mediated ones are of particular interest, because they can potentially be amenable to immunosuppressive/immunomodulatory treatment.^{4,7,18} Clinically, patients with IMNM, in addition to the myalgia, proximal weakness, and high creatine kinase levels previously mentioned, manifest with additional symptoms, such as fever, cardiac or pulmonary involvement, and weight loss; these symptoms are generally not encountered in patients with nIMNM.

To our knowledge, the underlying molecular mechanisms of the immune response in IMNM have not been established. For the design of potential therapeutic approaches, it is of utmost importance to identify and understand the underlying pathogenic mechanisms. Corticosteroids are administered on a first-line basis by many clinicians, but their long-term use is problematic and evidence for more specific therapeutic approaches is lacking.^{3,12,19–21} Several new therapeutic attempts have been described in individual patients or small patient series.^{19,20,22,23}

Similar to DM and PM, in which so-called myositis-specific antibodies and myositis-associated antibodies are important parts of the diagnostic approach,^{24,25} interesting specific autoantibodies have recently been described in IMNM.^{24–27} These autoantibodies compose the signal recognition particle (SRP), a ribonucleoprotein involved in the protein transport to the endoplasmic reticulum,^{27,28} and the HMG-CoA antibody identified in a subgroup of immune-mediated statin-induced NMs,²⁹ which recently have been termed statin-induced necrotizing autoimmune myositis.⁴ Furthermore, patients with antibodies against antisynthetase antibodies (eg, Jo1, PL7, and PL12) and patients harboring autoantibodies associated with tumors, thus defining paraneoplasia (eg, anti-Ro or anti-Mi-2), appear to also show the classic pathomorphological picture of IMNM.^{24,30–34} There is evidence that involvement of the muscle is a secondary event to a primary autoimmune response against, for example, a tumor.³⁵

In contrast to patients with IMNM, a toxic cause may also produce necrotizing myopathy, which, however, does not show additional clinical or morphological signs of a significant immune contribution. The latter cases need to be evaluated carefully (eg, toxicological screening and medical history) because the removal or cessation of the triggering agent will be mandatory for successful treatment.^{18,36} Also, a careful medical history about the duration of symptoms is of paramount significance.

Because immunosuppression/immunomodulation is useful in IMNM,^{3,27} the definition of a pathological concept may help to decide about putative therapeutic approaches and escalation in therapy.

In the present study, we aimed at identifying cellular and molecular characteristics of IMNM as opposed to those of nIMNM, to propose diagnostic tools, and molecular markers and mediators underlying the pathogenesis of IMNM and nIMNM.

Materials and Methods

Patients and Biopsy Specimens

Biopsy specimens of muscle tissue from 16 patients with IMNM, eight muscle samples from patients with nIMNM, and five skeletal muscle biopsy specimens from healthy control individuals were analyzed retrospectively. Based on the following different clinical parameters, we assigned patients with necrotizing myopathies to one of these groups. Assignment to the immune-mediated subgroup was based on at least one of the following parameters: myositis-specific autoantibodies, myositis-associated autoantibodies, autoantibodies associated with paraneoplastic disease, and/or defined autoimmune disease. In contrast, the non-immune-mediated subgroup did not display any autoantibodies or autoimmune disease or neoplasia. By definition, a causative toxic agent was identified in each patient with nIMNM, whereas none of the patients with IMNM received statins before the muscle biopsy was performed. Other conditions, such as muscular dystrophy (especially dystrophinopathy or dysferlinopathy), have been carefully excluded. Healthy controls constitute muscle biopsy specimens derived from neurologically healthy patients without any histological or ultrastructural abnormalities, including the absence of inflammatory infiltrates or MHC class I up-regulation and the absence of all relevant parameters associated with myositis (eg, autoantibodies and signs of systemic inflammation). Biopsy specimens were obtained from healthy control individuals because of diffuse myalgia. All procedures performed were single biopsies, and the analyzed area did not significantly differ between both groups [measured with an Olympus BX50 microscope and cell D software version 2.8 (Olympus, Hamburg, Germany); see Supplemental Figure S1 at <http://ajp.amjpathol.org>]. The entire section of each biopsy specimen was analyzed. The available clinical information is given in Table 1 and Supplemental Table S1 (available at <http://ajp.amjpathol.org>). Informed consent was obtained from all patients, and the Charité ethics committee has approved the study (approval number EA1/204/11). All muscle biopsy specimens were cryopreserved immediately after removal at -80°C before diagnostic workup.

Histological, Enzyme Histochemical, and IHC Studies

The cryostat sections ($7\ \mu\text{m}$ thick) were stained with routine H&E, modified Gömöri trichrome, Elastica van Gieson, PAS, PAS after diastase digestion, ATPase at pH 4.3, 4.6, and 9.4, acid phosphatase and nonspecific esterase, NADH tetrazolium reductase, succinic dehydrogenase, cytochrome c oxidase, and alkaline phosphatase. The immunohistochemical (IHC) demonstration of cells expressing CD4, CD8, CD11b, CD19, CD20, CD31, CD45/leukocyte common antigen, CD68, CD79a, CD138, deposition of complement C5b9 [membrane attack complex (MAC)], endothelial staining of factor VIII (von-Willebrand factor), expression of MHC class I on the sarcolemma, and IHC presentation of CD56 (neural cell

Table 1. Clinical Findings of 24 Patients with Necrotizing Myopathy and Five Healthy Patients as Normal Controls

Patient no.	Age (years)	Sex	Time since onset of symptoms (months)	CK level (times fold)	Autoantibody (MAA/MSA)	Variable*
IMNM Group						
1	57	F	ND	14	ANA 1:1280, PL7, PL12, Ro, Ku, Ro52	+
2	74	M	1	5	—	+
3	80	F	24	10	ND	+
4	36	F	13	46	Ro52	—
5	66	M	ND	41	SRP	+
6	57	M	1	ND	ASMA	+
7	19	F	2	78	SRP	—
8	55	M	>12	47	ND	+
9	63	F	4	ND	SRP	+
10	37	F	22	ND	ANA 1:2560, Ro52	—
11	64	F	ND	35	Jo1	+
12	79	M	ND	2.4	AChR	+
13	68	F	1	71	Jo1	+
14	37	F	1.5	51	Jo1	+
15	71	M	0.75	45	—	+
16	47	M	1.5	5	—	+
nIMNM						
17	52	F	ND	33	ND	Malarone
18	62	M	1	90	—	Statins, cyclosporine, voriconazole
19	55	F	12	15	—	Statins
20	76	M	0.25	900	ND	Acute renal failure, dialysis, diabetes
21	53	F	12	50	—	Quetiapine [†]
22	75	F	~2	300	—	Statins
23	33	M	2	36	ND	Alcohol excess
24	70	F	1	93	ND	Statins
Healthy Control Group						
25	47	M	—	Normal	—	Diffuse myalgia and arthralgia
26	41	F	—	Normal	—	Myalgia
27	2	M	—	Normal	—	+
28	57	F	—	Normal	—	Myalgia
29	55	M	—	Normal	—	Myalgia and collagenosis

Grouped for immune-mediated (16 patients), non-immune-mediated (8 patients), and control (5 patients). For further specified information about the systemic disease and treatment, see [Supplemental Table S1](#) (available at <http://ajp.amjpathol.org>).

*For IMNM, systemic disease (autoimmune or paraneoplastic) is listed as present or absent; for nIMNM, toxic agent/clinical aspect is listed; and for healthy controls, symptoms are listed.

[†]Recent change in antipsychotic treatment concomitant with onset of muscle symptoms.

[‡]Mother with a history of malignant hyperthermia; therefore, due to an upcoming operation of the heart in her child, the risk for malignant hyperthermia in the child was excluded, among other means by doing a muscle biopsy which was entirely normal.

F, female; M, male; AChR, acetylcholine receptor; ANA, antinuclear antibodies; ASMA, α -smooth muscle actin; MAA, myositis-associated antibody; MSA, myositis-specific antibody; ND, no data available; +, present; —, absent.

adhesion molecule), MHC neonatal, and α B crystallin (Table 2) was performed by using the iView-Ventana DAB Detection Kit (Ventana, Tucson, AZ). Appropriate biotinylated secondary antibodies and diaminobenzidine visualization of the peroxidase reaction product on a Benchmark XT immunostainer (Ventana) in a standardized manner were used. Omission of primary antibodies in control sections resulted in absence of any cellular labeling.

For immunofluorescence, the sections were blocked with the appropriate serum (1:10 in PBS), dependent on the source of the secondary antibody and incubated with the previously mentioned primary antibodies overnight at 4°C. After a washing step, the secondary antibody was added for 1 hour. For double immune staining, the same protocol was repeated with a second primary antibody and an ap-

propriate secondary antibody. After a final washing step, the sections were mounted and stored at 4°C. Photomicrographs were taken with the Zeiss Observer.Z1 Microscope (immunofluorescence), Axiovision software version 4 (Zeiss, Oberkochen, Germany), or the Olympus BX50 microscope, the digital camera DP25, and cell D software.

Semiquantitative Scale and Cell Counts

We defined a semiquantitative scale to compare IMNM with nIMNM. All cases were graded from 0 (none) to 4 (maximum), based on different histological stainings, and characterized two times in a blinded manner by three independent myopathologists (W.S., H.H.G., C.P.). The mean values from both scaling rounds are displayed.

Table 2. Source of Primary Antibodies Used for IHC and Immunofluorescent Studies

Antibody	Type, species	Clone	Company	Dilution
α B-crystallin	Monoclonal, mouse	G2JF	Novocastra (Wetzlar, Germany)	1:50
CD4	Monoclonal, rabbit	SP35	Zytomed Systems (Berlin, Germany)	Undiluted
CD8	Monoclonal, mouse	C8/144B	Dako (Hamburg, Germany)	1:100
CD11b	Monoclonal, mouse	SP2/0	Novocastra	1:20
CD19	Monoclonal, mouse	LE-CD19	Zytomed	1:100
CD20	Monoclonal, mouse	L26	Dako	1:400
CD31	Monoclonal, mouse	JC70A	Dako	Undiluted
CD45	Monoclonal, mouse	2B11 and PD7/26	Dako	1:400
CD79a	Monoclonal, mouse	JCB117	Dako	1:100
CD206	Monoclonal, mouse	7-450	Acris Antibodies (Herford, Germany)	1:100
CD56 (NCAM)	Monoclonal, mouse	ERIC-1	AbD Serotec (Düsseldorf, Germany)	1:200
CD68	Monoclonal, mouse	KP1	Dako	1:10,000
MHC class I	Monoclonal, mouse	W6/32	Dako	1:1000
MHC neonatal	Monoclonal, mouse	WB-MHC neonatal	Novocastra	1:20
CD138	Monoclonal, mouse	MI15	Dako	1:30
C5b9	Monoclonal, mouse	aE11	Dako	1:200
Factor VIII	Polyclonal, rabbit	NA	Dako	1:1000

NA, not applicable.

The scale for MHC class I expression was defined as follows: 0, no sarcolemmal staining; 1, >10% of fibers with sarcolemmal staining; 2, >30% of fibers with sarcolemmal staining; 3, >60% of fibers with sarcolemmal staining and scant sarcoplasmic staining; and 4, >90% of fibers with sarcolemmal and strong sarcoplasmic staining (see Supplemental Figure S2 at <http://ajp.amjpathol.org>).

The scale for C5b9 complement (MAC) deposition was defined as follows: 0, no capillaries stained; 1, >10% of capillaries (some or few); 2, >30% of capillaries (focal); 3, >60% of capillaries (large areas); and 4, >90% of capillaries show MAC deposition.

CD56- and MHC neonatal-positive fibers were scored as follows: 0, no fibers stained; 1, >10% of fibers stained; 2, >30% of fibers stained; 3, >60% of fibers stained; and 4, >90% of fibers stained.

For quantification of CD4⁺ or CD8⁺ T cells, CD20⁺ or CD79a⁺ B cells, CD138⁺ plasma cells, and CD68⁺ macrophages, we performed cell counts of 10 high-power fields (one high-power field is defined as 0.16 mm²) of each biopsy specimen. The average number of cells per high-power field per case is displayed.

Electron Microscopy

Ultrastructural analysis of all muscle specimens was performed routinely after fixation in 2.5% glutaraldehyde for 48 hours at 4°C, post fixation in 1% osmium tetroxide, and embedding in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate, after three to five blocks were generated from each sample. Electron microscopy was performed with a Zeiss P902 electron microscope.

Real-Time qPCR

Total RNA was extracted from the muscle tissue samples using the TRIzol/chloroform method, according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). Thereafter, RNA was resuspended in 60 μ L of diethylpyrocarbonate-treated water and the concentration of

total RNA was photometrically determined with a TECAN fluorescence plate reader (Tecan, Männedorf, Switzerland). The RNA was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol, by using 1 or 2 μ g of total RNA per sample, as described.³⁷

The expression levels of gene transcripts and of the endogenous control gene, phosphoglycerate kinase 1 (PGK1), were analyzed in the specimens 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). The expression level of the housekeeping gene *PGK1* was similar in all analyzed samples and unaffected by duration of the diseases. The assay identification numbers are as follows: IL-4, Hs00929862_m1; IL-4 receptor, Hs00166237_m1; IL-13, Hs99999038_m1; CD206, Hs00267207_m1; inducible nitric oxide synthase (iNOS), Hs01075529_m1; interferon (IFN)- γ , Hs00989291_m1; tumor necrosis factor (TNF)- α , Hs00174128_m1; IL-12p40, Hs01011518_m1; cyclooxygenase 2, Hs00153133_m1; CXCL13, Hs00757930_m1; STAT1, Hs01013989_m1; and PGK, Hs99999906_m1.

The quantitative PCR (qPCR) reactions were prepared in a final volume of 20 μ L, with the TaqMan Fast Universal PCR Master Mix (Applied Biosystems), containing 20 ng of cDNA. All analyses were performed in triplicate, and the C_T was determined. Data display the reciprocal Δ C_T to show gene expression of the respective markers of the two groups of NM compared with healthy controls.

Statistics

For analysis of the semiquantitative score of histological stainings, we used the *U*-test. Furthermore, the Kruskal-Wallis one-way analysis of variance with Bonferroni correction was applied for analysis of quantitative differences of mRNA transcripts. It was not possible to perform statistics with the Kruskal-Wallis test for the expression of

Figure 1. Morphological characterization of both subgroups with necrotizing myopathy by Gömöri trichrome staining of IMNM and nIMNM shows myophagocytosis and necrosis of muscle fibers (A and B). Significantly more CD68⁺ macrophages (C and D), CD4⁺ cells (E and F), and CD8⁺ T cells (G and H) are found in IMNM (A, C, E, and G) versus nIMNM (B, D, F, and H). Arrows, immunoreactive cells of the respective subtype; asterisk, necrotic muscle fiber. Original magnification, $\times 400$.

IL-12, IL-4, and IL-13, because the respective cytokines were not expressed in healthy controls. Data are presented as means with SDs. The level of significance was set at $P < 0.05$. Statistics were calculated with GraphPad Prism software version 5.02 (GraphPad Software, Inc., San Diego, CA).

Results

Analysis of Clinical Data

To differentiate IMNM from nIMNM, we analyzed the clinical data of the patients enrolled in the study to identify similarities or differences between the two groups (Table 1). By definition, a causative toxic agent was identified in each patient with nIMNM; in some cases, it was likely that statins had induced the disease. None of these cases showed signs of an autoimmune-mediated process, because MHC class I up-regulation or complement deposition, criteria defined by Christopher-Stine et al,²⁶ were not present.

There was no significant difference in the average age of the patients in both groups. In IMNM, the mean age was

56.9 ± 16.9 years; and in nIMNM, 59.5 ± 13.4 years. In addition, the female/male ratio was not different between IMNM and nIMNM (both, 60:40). Furthermore, we observed higher creatine kinase levels and a shorter duration between admission to the hospital and onset of disease in patients with nIMNM compared with IMNM (Table 1).

Patients with IMNM Exhibit a Prominent Expression of MHC Class I Molecules and Conspicuous Deposition of Complement on Capillaries in Skeletal Muscle

It may be difficult to distinguish IMNM from nIMNM by routine histochemical staining (Figure 1, A and B). Therefore, additional IHC and ultrastructural analyses were of substantial help.

In the past, conflicting results regarding the expression of MHC class I molecules in muscle biopsy specimens derived from patients with necrotizing myopathies were reported. Although some studies showed no signs of MHC class I expression,²⁷ others described at least some immunoreactivity on myofibers.^{19,26,28,38} In the present series, we demonstrated that skeletal muscle tissue from patients with IMNM displayed ample and

Figure 2. Illustration of a semiquantitative scale based on histological staining and cell counts; patients with IMNM and nIMNM are visualized as open and closed squares, respectively; sarcolemmal MHC class I expression and endothelial complement deposition (A), and regeneration of muscle fibers (B), are elevated in IMNM compared with nIMNM. Significantly more CD68⁺ macrophages and CD4⁺ and CD8⁺ lymphocytes (C) can be quantified in IMNM. A definition of the scale is given in Materials and Methods (0, minimum; 4, maximum). For cell count, the average number of cells per 10 high-power fields is shown. Data are given as mean with SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Figure 3. Trans-sarcolemmal MHC class I expression (**A** and **B**) is more prominent in an IMNM biopsy specimen compared with a representative nIMNM specimen. **C** and **D**: Double immunofluorescence illustrates important complement (MAC; C5b9) deposition (red) on endomysial capillaries stained by factor VIII (green) in IMNM, whereas nIMNM capillaries do not show complement deposition; a necrotic fiber (red) is stained in the center of the photomicrograph (**D**). In addition, sarcolemmal decoration of muscle fibers with complement C5b9 (**E**) in an IMNM case coincides with sarcolemmal α B-crystallin staining, indicating X fibers (**F**). **Arrows**, immunoreactivity; **asterisks**, necrotic muscle fiber.

intense trans-sarcolemmal immunoreactivity of MHC class I molecules on many nonnecrotic muscle fibers (Figures 2A and 3A). MHC I expression was diffusely distributed and particularly strong in biopsy specimens derived from patients with anti-SRP antibodies, antisynthetase syndrome with respective autoantibodies, and paraneoplastic necrotizing myopathies. A perifascicular pattern of atrophic fibers, such as in DM, was not a feature of IMNM, nor was a gradient of increasing capillary density toward the center of the fascicles. An electron microscopic investigation excluded the presence of tubuloreticular formations, which are characteristic of DM, in endothelial cells of IMNM muscle. In contrast, muscle biopsy specimens from patients with nIMNM showed MHC class I expression confined only to the sarcoplasm of necrotic fibers, and a scant sarcolemmal staining pattern on a few adjacent fibers. None of the nIMNM biopsy specimens showed a significant or diffuse up-regulation of MHC class I molecules on nonnecrotic fibers (Figures 2A and 3B).

Furthermore, complement deposition of MAC (C5b9) was particularly prominent in IMNM biopsy specimens, especially in patients with anti-SRP autoantibodies and antisynthetase syndrome, and in biopsy specimens derived from patients with a paraneoplastic cause with severe general systemic signs of disease (Figures 2A and 3C). In contrast, complement deposition on capillaries in the nIMNM group was insignificant or completely absent

(Figures 2A and 3D). In addition to the previously mentioned complement deposition on capillaries that was also previously described,¹ an interesting and discriminating feature of severe IMNM cases was the decoration of the sarcolemma of nonnecrotic fibers by MAC (Figure 3E). This feature was consistently absent in biopsy specimens derived from patients with nIMNM (data not shown). In addition, in IMNM muscle samples, we identified some of these fibers as so-called X fibers,³⁹ characterized by their labeling with α B-crystallin in the sarcoplasm (Figure 3F); they appeared otherwise normal by trichrome stain (Figure 1, A and B). This might indicate that these X fibers were committed to be attacked by the immune system.³⁹ X fibers were consistently absent in nIMNM specimens (data not shown).

IMNM Skeletal Muscle Biopsy Specimens Are Characterized by a CD4⁺ and CD8⁺ T-Cell Infiltrate

Many researchers believed that IMNM, especially SRP-associated NM, was characterized by a poor cellular infiltrate. Therefore, we thoroughly investigated the presence and specific distribution of interstitial immune cells on serial sections in our samples using cell counts.

Quantitatively, CD68⁺ macrophages clearly constituted the dominant mononuclear cellular infiltrate. Biopsy specimens derived from patients with IMNM displayed many CD68⁺ macrophages in the endomysium and perimysium, independent of myophagocytosis, whereas in nIMNM, CD68⁺ macrophages were exclusively confined to necrotic fibers that underwent myophagocytosis (Figures 1, C and D, and 2C). In IMNM, a perimysial and endomysial inflammatory infiltrate consisting of CD4⁺ (Figures 1, E and F, and 2C) and CD8⁺ (Figures 1, G and H, and 2C) T cells was identified. In some cases, T cells were also identified in a perivascular location, but necrotizing vasculitis was not a feature in IMNM biopsy specimens. In contrast, nIMNM biopsy specimens displayed only scant T cells in the endomysium and in proximity to fiber necrosis and myophagocytosis (Figures 1, D, F, and H, and 2C).

We hypothesized that regenerating processes would differ in the muscle biopsy specimens derived from patients of either subgroup. Antibodies against neural cell adhesion molecule (CD56) and myosin heavy chain neonatal were used to identify these fibers. We found significantly more pronounced regeneration in IMNM, as indicated by the presence of numerous myosin heavy chain neonatal-positive fibers and neural cell adhesion molecule-positive (CD56) muscle fibers, whereas in biopsy specimens from patients with nIMNM, these antibodies were less frequently detected (Figure 2B; see also Supplemental Figure S3 at <http://ajp.amjpathol.org>).

B Cells and Prominent B-Cell Chemoattraction Are Features of a Subgroup of IMNM Biopsy Specimens

As a trial, B-cell depletion therapy with rituximab was used in a few severely ill patients with SRP-autoantibody–

Figure 4. Illustration of CD79a⁺ B cells in IMNM (A) versus nIMNM (B). Quantification reveals more B cells (CD79a) and plasma cells (CD138) in immune-mediated cases (C). The expression of the B-cell chemoattractant, CXCL13, by qPCR is illustrated (D). Some patients with conspicuous B-cell infiltration also show highly increased expression levels of CXCL13 (marked by colored squares: blue and orange, Jo1 patients; green and purple, SRP patients). Statistics are performed with the *U*-test (C) and the Kruskal-Wallis test (D). **P* < 0.05, ***P* < 0.01. Arrows, immunoreactivity for CD79a; asterisk, necrotic muscle fiber. ns, not significant.

mediated IMNM, which did not respond to corticosteroid therapy.^{4,40–42} In the present study, we aimed to define molecular markers of B-cell invasion of skeletal muscle during the immune process in IMNM. Of the 16 muscle biopsy specimens, three showed few, but conspicuous, amounts of CD19⁺, CD20⁺, or CD79a⁺ B cells and one showed single CD138⁺ plasma cells in the endomysium, whereas the nIMNM biopsy specimens did not display any striking numbers of B or plasma cells (Figure 4, A–C). Interestingly, molecular analysis of the B-cell chemoattractant, CXCL13,^{43–45} in the muscle biopsy specimens revealed significantly higher expression of this chemokine in IMNM compared with those in nIMNM. Furthermore, most of the histologically proven biopsy specimens with B-cell invasion showed highly increased expression of CXCL13. One of these patients had Jo1 syndrome and two had SRP-associated myopathy (Figure 4, C and D).

Immune Response in IMNM Is Essentially Characterized by Th1-M1 Mediators

Based on our quantitative analysis of inflammatory mononuclear infiltrates in the IMNM biopsy specimens, we hypothesized that the immune response was essentially T-cell mediated and that the interaction between T cells and macrophages played an important role in disease pathogenesis and progression. To specify the immune response, we analyzed key Th1 and Th2 markers by quantitative real-time PCR, and mediators of macrophage polarization, for classic or alternative activation status phenotypes.^{46–49} In contrast to nIMNM, IMNM was characterized by a significant up-regulation of the Th1 master cytokine, IFN- γ . Consequently, and in keeping

with this finding, the cytokines TNF- α and IL-12 and the transcription factor STAT1^{46,48–50} were significantly up-regulated compared with healthy controls. In nIMNM samples, the respective Th1 cytokines were expressed at significantly lower levels (Figure 5, A and B).

However, the effector molecules, iNOS and cyclooxygenase 2, were not differentially regulated in either group, which may mirror comparable cytotoxic activities of macrophages in both groups (see Supplemental Figure S4 at <http://ajp.amjpathol.org>). A significant Th2 response, as measured by the expression of the master cytokines IL-4 and IL-13, was not detected (Figure 5C). Furthermore, expression of the common IL-4 receptor α (CD124), which mediated IL-4 and IL-13 signaling, and expression of the macrophage mannose receptor (CD206) were expressed at similar levels in both groups (Figure 5D).

Discussion

In addition to the classic entities, PM, DM, and sporadic IBM, IMNM is a further and important subgroup of immune-mediated myopathies. In this study, we defined molecular markers that help to delineate IMNM as a distinct entity, which can potentially be targeted by immunosuppressive/immunomodulatory therapy. In addition, we provide evidence that IMNM is essentially a Th1-M1-mediated disease in which high levels of IFN- γ , TNF- α , IL-12, and STAT1 functionally regulate the immune re-

Figure 5. Illustration of quantitative real-time measurements (qPCR) in IMNM and nIMNM: cytokines driven by the Th1 pathway (A) and markers of classically activated macrophages (B) are significantly up-regulated in IMNM compared with healthy controls, and compared with nIMNM in case of IFN- γ and STAT1. The respective cytokines measured in nIMNM specimens compared with healthy controls do not differ. The Th2 cytokines IL-4 and IL-13 are, if at all detectable, expressed at low levels in all groups (C); expression of the M2 markers CD206 and IL-4 receptor (IL-4R) α (CD124) is on the same level in immune- and non-immune-mediated patients, but is elevated compared with healthy controls (D). It is not possible to perform statistics with the Kruskal-Wallis test for the expression of IL-12, IL-4, and IL-13, because the respective cytokines are not expressed in healthy controls. **P* < 0.05, ***P* < 0.01.

sponse, whereas Th2-mediated processes seem to be of less importance.

Both IMNM and nIMNM have in common the presence of a dominant necrotizing component with myophagocytosis, which is by far less prominent in PM and DM. Furthermore, specific ultrastructural alterations, such as tubuloreticular inclusions, which may be found in DM and rarely in other inflammatory myopathies (eg, HIV- or lupus erythematoses-associated myositis), are not a characteristic feature of IMNM or nIMNM. The use of MHC class I and MAC and staining for T and B lymphocytes are of further discriminating value. These markers, in concert with staining of diverse membrane proteins, may also help to differentiate these entities from, for example, limb-girdle muscular dystrophies and dystrophinopathies, because the latter entities may also reveal inflammatory cells.^{2,51} In IMNM, the identification of a significant amount of CD4⁺ and CD8⁺ T cells, grouped in the endomysial and perimysial compartment, and a strong and ubiquitous up-regulation of MHC class I molecules on the sarcolemma and MAC deposition on endomysial capillaries in most biopsy specimens support the diagnosis of an immune-mediated process. In contrast, nIMNMs with a toxic cause do not show a significant T- or B-cellular infiltrate, and sarcolemmal MHC class I expression is generally low, as is MAC deposition on capillaries. Some specimens of either subgroup did not differ significantly in these parameters and, therefore, clinical and laboratory results, especially measurements of myositis-specific and myositis-associated antibodies, are helpful; additional molecular analysis by qPCR of IFN- γ , TNF- α , IL-12, and STAT1 may further increase diagnostic accuracy in individual patients.

The expression of MHC class I molecules in IMNM has been assessed, with divergent results in different studies. Although some researchers found at least some sarcolemmal MHC class I expression,^{19,38} others have not.²⁷ More important, the absence of perifascicular atrophy, evidenced in MHC class I preparations in IMNM, is in line with previous studies.^{27,52} In statin-induced NMs with HMG-CoA autoantibodies, up-regulation of sarcolemmal MHC class I is described as a valuable diagnostic tool.^{26,29,53} Furthermore, Karpati et al⁵⁴ have described MHC class I expression on regenerating fibers in patients with muscular dystrophy and also studied several groups of IIMs, showing a specific pattern of sarcolemmal and partial sarcoplasmic expression of the protein. Similar findings were reported at approximately the same time by McDouall et al.⁵⁵

Although some authors note complement (MAC) deposition on a few capillaries, others describe the absence of any MAC immunoreactivity in their IMNM biopsy specimens.^{6,28,38} Interestingly, the previously described pronounced MAC deposition on significantly thickened pipestem capillaries associated with necrotizing myopathy³² is rarely seen among IMNM biopsy specimens in general, and the character of MAC deposition in these cases and the one in DM is clearly different.^{1,4} The relevance of MAC staining in paraneoplastic myopathies has been underlined on several occasions.^{33,34,56} Strong capillary MAC deposition in our three specimens with a paraneo-

plastic cause follows the reported characteristics. In summary, MAC deposition and expression of sarcolemmal MHC class I in IMNM clearly reveal a distinct pattern compared with DM; in addition, middle-sized vessels are targeted by the immune response,¹ and undulating tubules are regularly found by ultrastructural investigation.

A hallmark of IMNM, described in nearly all reports, is the relative paucity of inflammatory infiltrates; however, there is no specification about which cells are relatively rare.^{3,19,20,27,28,38,56,57} In our series of IMNM biopsy specimens, the lymphocytic infiltrate was less pronounced than in highly active DM, IBM, or PM; however, also in these entities, considerable variation concerning the density of lymphocytes exists and is well-known in daily routine. At variance with many reports, we have identified a significant endomysial infiltrate consisting of CD8⁺ T cells, CD4⁺ cells, and few B cells in our IMNM biopsy series, which were pronounced, for instance, in biopsy specimens from patients with antisynthetase syndrome. In IMNM, CD8⁺ T cells are frequently found in association with myophagocytosis, but their focal accumulation, as seen in PM or IBM, is not present. The endomysial distribution of the infiltrate, sometimes combined with a perivascular accumulation in conjunction with large areas of MHC class I up-regulation, strongly argues for a CD8-MHC class I-restricted immune component. CD8⁺ T cells, CD4⁺ plasmacytoid dendritic cells, and T-helper cells have been studied extensively in PM, IBM, and DM,^{11,44,58–62} demonstrating their pathogenic relevance in IIMs. Thus, the presence of a conspicuous lymphocytic infiltrate should always prompt for consideration of an immune-mediated cause of NM.

Because cytokines and chemokines orchestrate many aspects of the immune response, we have analyzed a comprehensive set of mediators involved in both the Th1-M1- and the Th2-M2-mediated immune responses. We found a significant up-regulation of TNF- α in patients with IMNM compared with healthy control biopsy specimens in which this cytokine is expressed at low levels, as previously described.^{63–65} Given the overwhelming dominance of macrophages, we hypothesize that TNF- α expression can most likely be attributed to them. The important role of TNF- α in IIMs has been elegantly demonstrated,^{66,67} but has not been addressed in detail in necrotizing myopathies. In addition to TNF- α , we have also found elevated levels of the proinflammatory cytokine IL-12, indicating an M1-biased immune response. Furthermore, we have observed a synergistic up-regulation of IFN- γ and TNF- α in our specimens, underlining an active Th1-M1 response by the well-defined stimulatory effect of IFN- γ by CD4⁺ cells, leading to TNF- α production by effector cells, such as macrophages. High expression of the Th1 master cytokine, IFN- γ , as shown in IMNM specimens, strongly supports our results of the often ubiquitous trans-sarcolemmal up-regulation of MHC class I molecules in our series, because IFN- γ and TNF- α are major inducers of the major histocompatibility complexes.⁶⁸ Interestingly, in earlier reports, not much IFN- γ has been described in IIMs.^{40,65,69} However, type I interferons play important parts in PM and DM.^{70–73} The expression of master cytokines of the Th2 response, such

as IL-4 and IL-13, is low or barely detectable in both IMNM and nIMNM, which is in line with studies in PM, DM, and IBM.^{65,69} Markers of an alternative activation status of macrophages (M2), such as the mannose receptor (CD206), and the IL-4 receptor α (CD124) are not expressed at different levels in both groups.

We have identified three patients in the IMNM group displaying antisynthetase and anti-SRP autoantibodies, with few B cells accompanied by high levels of CXCL13 expression, a chemoattractant of B cells, which has also been recently observed in patients with juvenile DM.⁷⁴ B cells and B-cell chemokines have been identified by others, mainly in DM, and a potential for the development of new therapeutic targets has been noted. Thus, it is worth considering the possibility of CD20⁺ B-cell targeting agents, such as rituximab, B-cell activating factor belonging to the TNF family, or others.^{12,44,75} Rituximab has been administered in refractory IIMs and in IMNM with anti-SRP autoantibodies and antisynthetase syndrome, with some encouraging and some disappointing results.^{22,76–80} The rationale for use of this treatment, however, is theoretically well defined, but has not yet been supported by molecular data, as indicated herein. Given the fact that TNF- α was expressed at high levels in many specimens, TNF blockade may also be an alternative treatment approach in IMNM. Although in principle successful, TNF blockade has not consistently produced promising results in IIMs.^{41,81–83}

Few data on targeted therapy as an alternative to standard corticosteroid treatment^{42,75} are available for IIM and for IMNM specifically, which have a presumed autoimmune etiopathogenesis in common. Yet, we believe that individual assessment of key cytokines and chemokines in IMNM muscle biopsy specimens, and in perspective also in the patients' blood, may provide useful biomarkers that can open new avenues for developments of a meaningful treatment.

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