Solo la ricerca dell'impossibile può
condurre a ciò che è realizzabile.
Anonimo

Table of contents

Chapter 1

Introduction

The idiopathic inflammatory myopathies

The idiopathic inflammatory myopathies (IIMs) constitute a heterogeneous group of subacute, chronic or acute acquired disorders affecting skeletal muscle, sharing the predominating clinical symptom of muscle weakness and histopathological signs of inflammation in muscle tissue.[1,2] On the basis of clinical, demographic, histological and immunopathological criteria, IIMs are divided in three major and discrete subgroups: dermatomyositis (DM), polymyositis (PM), and sporadic inclusion body myositis (IBM) (*box1*). There are differences in the age and gender distribution of the three major subtypes of inflammatory myopathies. Dermatomyositis may occur in childhood or adult life, whereas polymyositis arises mainly after the second decade of life. Both conditions are more common in females, whereas IBM prevails in males and occurs mainly over the age of 50 years. Few data on the relative incidence of the different forms of IIMs are available and few studies have distinguished between PM and IBM.[3]

Box 1.

Classification of idiopathic inflammatory myopathies

I Dermatomyositis
Juvenile
Adult

II Polymyositis
T-cell mediated (α/β, γ/δ;)
Eosinophilic
Granulomatous

III Overlap syndromes
With polymyositis
With dermatomyositis
With inclusion body myositis

IV Cancer-associated myositis
V Inclusion body myositis
VI Other forms:
Focal: orbital myositis; localized nodular myositis; inflammatory pseudotumor
Diffuse: macrophagic myofasciitis; necrotizing myopathy with pipestem capillaries; infantile myositis

General clinical features

For all the three forms of IIMs, the onset is typically subacute (over several weeks), but in DM may be acute (days) or insidious (several months) with patients complaining of increasing fatigue for example in rising from a chair, lifting their arms, climbing steps.[4] In IBM muscle weakness develops insidiously and the diagnosis may not be made for several years. The most common clinical signs are reduction

of the muscular strength in the proximal muscles, contractures and, late in the course of the disease, muscular atrophy (40% of cases). In DM and PM the pattern of muscle weakness is nonselective; it is usually more severe in the shoulder and pelvic girdle muscles. In IBM weakness and atrophy are first observed in the quadriceps femoris muscles in the lower limbs and in the forearm flexor muscles in the upper limbs (*Fig. 1*). The early involvement of the flexor digitorum profundus muscles and subsequently also of the flexor digitorum sublimes and finger extensors lead to a characteristic inability to maintain flexion of the distal phalanges, with impairment of fine motor tasks, such as holding and manipulating objects.

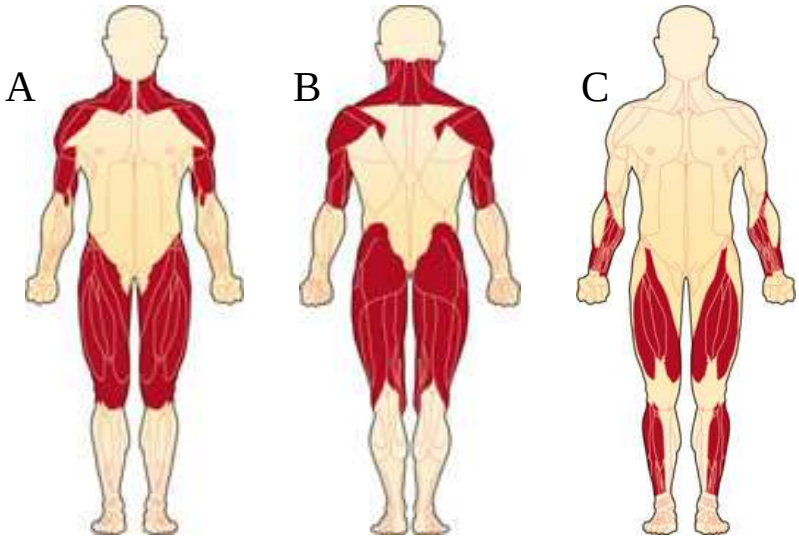


Fig. 1. (A, B) Polymyositis and Dermatomyositis mostly affect muscles of the hips and thighs, the upper arms, the top part of the back, the shoulder area and the neck.

(C) The first muscles affected in inclusion-body myositis are usually those of the wrists and fingers, and those at the front of the thigh. Peroneus longus and brevis, tibialis anterior and the extensor digitorum longus may also be affected.

Extramuscular manifestations

Characteristic of DM is a skin rash very useful tool for the diagnosis of this condition; it may accompany or precede of several months or years the onset of muscle weakness. The classic skin manifestations include a purplish discoloration of the eyelids (heliotrope rash), often associated with periorbital edema, and papular, erythematous, scaly lesions over the knuckles (Gottren's sign).[5] In addition, a flat, erythematous, sun-sensitive rash may appear on the face, neck and anterior chest (V-sign), on the shoulders and upper back (shawl sign), and on the elbows, knees and malleoli. In some patients, the rash is the dominant feature and weakness may be minor or absent (*amyopathic dermatomyositis*). Subcutaneous calcifications occur particularly in the juvenile form of DM, but occasionally also in adult cases, and take the form of nodules or plaques over the elbows, forearms, knuckles, axillae or buttocks which may be painful and may ulcerate through the skin and become infected.

Specific clinical features

Dermatomyositis

Dermatomyositis is identified by characteristic skin manifestations accompanying or, more commonly, preceding muscle weakness. The typical and possibly pathognomonic cutaneous features of DM are the heliotrope rash and Gottron's papules. The heliotrope rash consists of a violaceous to dusky erythematous rash with or without edema in a symmetrical distribution involving periorbital skin.[6] Colour changes are often accompanied by scaling and desquamation. Sometimes this sign is quite subtle and may involve only a mild discoloration along the eyelid margin, while at other times there may be massive edema that develops. Gottron's papules are found over bony prominences, particularly the metacarpophalangeal joints, the proximal interphalangeal joints, and/or the distal interphalangeal joints. They may also be found overlying the elbows, knees and/or feet. The lesions consist of slightly elevated, violaceous papules and plaques. Telangiectasia is often found within the lesions. Several other cutaneous features are characteristic of the disease despite not being pathognomonic. The skin lesions of DM are probably photoaggravated despite the lack of symptoms suggestive of photosensitivity reported by patients. Clinical observations suggest that not only is the skin disease exacerbated by light, but muscle disease may be worsened after sun exposure. In small case series, it

has been suggested that some of the cutaneous manifestations may be more common in patients with an associated malignancy.

Juvenile Dermatomyositis

Juvenile dermatomyostitis (JDM) is the most common form of the idiopathic inflammatory myopathies in children, accounting for 85% of the pediatric inflammatory myopathy group.[7] It is an occlusive small-vessel vasculopathy, involving arterioles and capillaries. Its most obvious effects are seen in skeletal muscle and in the skin, although other organ systems can be involved, including the gastrointestinal tract, heart, and lungs. As the adult form, it is classically characterized by symmetric, frequently progressive, proximal muscle weakness, and inflammatory cutaneous lesions, including but not limited to, erythematous scaly lesions over the metacarpophalangeal and/or interphalangeal joints (Gottron's papules), a violaceous hue over the eyelids (heliotrope), with or without periorbital edema, malar erythema, periungual telangiectasia, and erythematous scaly rashes over the neck, upper back, and extensor surfaces of the extremities (*Table 1*).

Table 1. [*Rheumatic disease clinics of North America,* 2002; 28: 579-602]

Clinical characteristics at diagnosis of untreated children with JDM (n = 79)

Symptom	N (%)
Rash	79 (100)
Weakness	79 (100)
Muscle Pain	58 (73)
Fever	51 (65)
Dysphagia	35 (44)
Hoarseness	34 (43)
Abdominal Pain	29 (37)
Arthritis	28 (35)
Calcifications	18 (23)
Melena	10 (13)

From Pachman LM, Hayford JR, Chung A, Daugherty CA, Pallansch MA, Fink CW, Gewanter HL, Jerath R, Lang BA, Sinacore J, Szer IS, Dyer AR, Hochberg MC. Juvenile dermatomyositis at diagnosis: clinical characteristics of 79 children. *J Rheumatol*, 1998; 25: 1198-1204.

Among children with JDM, 3 to 5% have the amyopathic subtype with no clinical evidence of muscle weakness, but with the pathognomonic cutaneous manifestations of the disease. It has been estimated that approximately 34 to 40% of affected children have an acute course that resolves within a 2-year period and remains in remission indefinitely; these children have a monocyclic disease course. The remaining 60-66% have chronic disease, requiring immunosuppressive therapy for more than two years and having either disease that remains continuously active or that is characterized by remissions and exacerbations (polycyclic course).

Polymyositis[8]

Polymyositis generally presents in patients over the age of 20 and is more prevalent in women. There are rare cases of myositis beginning in infancy, although it is suspected that most of these cases are in fact congenital muscular dystrophies with secondary inflammation. Diagnosis is often delayed, unlike DM, because there is no associated rash to serve as a "red flag" to patients and their physicians. Patients present with neck flezor and symmetrical proximal arm and leg weakness that typically develops over several weeks or months. Distal muscles also may become involved, but are not as weak as the more proximal muscles. Muscle pain and tenderness frequently are noted. Dysphagia occurs in approximately one-third of patients secondary to oropharyngeal and esophageal involvement. Mild facial weakness occasionally may be demonstrated. Sensation is normal and muscle strech reflexes usually are preserved.

Most patients with PM respond favorably to immunosuppressive therapies but usually require life-long treatment. Some retrospective studies suggest that PM does not respond to immunosuppressive agents as well as DM. Poor prognostic features are older age, interstitial lung disease (ILD), cardiac disease, the presence of anti-Jo-1 or anti-SRP antibodies, and a delay in treatment or previous inadequate treatment.

Inclusion Body Myositis[8]

Inclusion body myositis is characterized clinically by the insidious onset of slowly progressive proximal and distal weakness, which generally develops in patients over 50. The slow evolution of the disease process probably accounts in part for the delay in diagnosis, which averages approximately 6 years from the onset of symptoms. Men are much more commonly affected than women. The clinical hallmark of IBM is early weakness and atrophy of the quadriceps, volar forearm muscles (i.e., wrist and finger flexors), and the ankle dorsiflexors. Muscle involvement in IBM is often asymmetric, in contrast to the symmetric involvement in DM and PM. The presence of slowly progressive, asymmetric quadriceps and wrist/finger flexor weakness in an elderly patient strongly suggests the diagnosis of IBM. Dysphagia occurs in up to 40% of patients and can be debilitating, requiring cricopharyngeal myotomy. Likewise, mild facial weakness can be detected on examination in at least 33% of IBM patients, however, this weakness is clinically insignificant. Extraocular muscles are spared. Although most patients have no sensory symptoms, evidence for a generalized peripheral neuropathy can be detected in up to 30% of patients on clinical examination and electrophysiologic testing. Muscle stretch reflexes are normal or slightly decreased. In particular, the patellar reflex is lost early.

Most of the IBM patients are older, and life expectancy does not appear to be significantly altered. IBM is slowly progressive and does

not respond well to immunosuppressive medications. Most patients remain ambulatory, although they frequently require or at least benefit from a cane or a wheelchair for long distances. However, some patients become severely incapacitated and require a wheelchair or become bed-ridden within 10 to 15 years. Many patients with so called steroid-resistant or “refractory” PM are eventually diagnosed with IBM. Importantly, these are patients whose illness clinically resembles IBM but in whom a definitive diagnosis cannot be confirmed on muscle biopsy.

Diagnostic assessment

The diagnosis of inflammatory myopathies is suspected on the basis of clinical features and confirmed by laboratory tests, serum muscle enzyme concentration, presence of autoantibodies, electromyography and muscle biopsy. Moreover, muscle magnetic resonance imaging and ultrasound have been recently introduced in the diagnostic work up of patients with inflammatory myopathies.[4]

A number of attempts has been made to develop sets of diagnostic criteria for inflammatory myopathies, taking into account clinical and pathological criteria as well the presence of myositis-specific autoantibodies and immunogenetic markers. The best known of these criteria are those of Bohan and Peter, which were developed for PM and DM, but did not include IBM. More specific diagnostic criteria

for DM, PM and IBM have recently been proposed by neurologists and rheumatologists.[3]

Autoantibodies

Antibodies to nuclear or cytoplasmic constituents have been reported in patients with inflammatory myopathies. These are classified as myositis-specific autoantibodies (MSA), occurring only in patients with inflammatory myopathies, and myositis-associated autoantibodies (MAA), found also in other autoimmune diseases without myositis (*Table 2*).[3,4] MSA are present in 30-40% of patients with PM-DM and are directed against cytoplasmic aminoacyl tRNA synthetases, a heterogeneous family of enzymes that catalyze the ATP-dependent binding between an amino acid and its cognate tRNA during protein synthesis. Antibodies directed against six of the 20 aminoacyl tRNA synthetases have been described and among them, anti-hystidil-tRNA synthetase (anti-Jo-1) antibodies seem to be the most frequent. Anti-Jo-1 antibody positivity is strongly associated with the DR3, DRw52, and DQA1*0501 HLA haplotype and it presents high specificity for the *antisynthetase syndrome* in which myositis is associated with interstitial lung disease, joint pain or arthritis, Raynaud's phenomenon, and skin lesions of the hands ("mechanic's hands"). Autoantibodies against other synthetases specific for alanine (anti-PL-12), treonine (anti-PL-7), glicine (anti-EJ), isoleucine (anti-OJ) and asparagine (anti-KS) are less frequent (3-

5%). Anti-SRP antibodies are directed against a ribonucleoprotein of a six proteins complex bound to a RNA molecule of about 300 nucleotides. The presence of these antibodies in the serum identifies a group of patients (generally affected by PM), with acute necrotizing myositis who do not respond well to therapy. Anti-Mi-2 antibodies are directed against a nuclear protein of seven subunits, which is part of a protein complex involved in gene transcription. These antibodies are present in 10-15% of DM and PM cases and seem to be associated with acute onset of disease, good prognosis and good response to the therapy.

The MAA may be found also in other connective tissue diseases. Antibodies to the PM-Scl nucleolar antigen complex (originally referred to as anti-PM-1) have been reported in a high proportion of white patients with the scleroderma-myositis overlap. Almost 50% of patients with the antibody have this overlap syndrome but some have PM or scleroderma alone. The anti-Ku antibody has a high specificity, but low sensitivity, for the scleroderma-myositis overlap syndrome in Japanese patients. High titers of antibodies to nuclear ribonucleoprotein (nRNP) are a diagnostic marker for mixed connective tissue disease (MCTD) in which there is an overlap between features of scleroderma, polymyositis, SLE, and rheumatoid arthritis. Antiproteasomal antibodies are found in a high proportion of patients with autoimmune myositis, SLE, and Sjögren's syndrome, and their titer has been found to correlate with disease activity. Antiendothelial antibodies are found in DM and in other types of

inflammatory myopathy, and in one study they were found to be markers for the presence of interstitial lung disease.

Other non-disease-specific antibodies have been reported in patients with IBM, but myositis-specific antibodies such as anti-Jo-1 are only rarely found in this condition. A high incidence of monoclonal gammopathies has been found in some series of IBM cases.

Table 2. Characteristics of myositis autoantigens. [from *Nature Clinical Practice – Rheumatology*, 2008; 4(4): 201-209]

Autoantigen	Structural and functional features	Nucleic acid binding	Caspase substrate	Granzyme B substrate	Clinical associations	References
Myositis-specific antigens						
HRS	N-terminal regulatory domain most often targeted by autoantibodies	tRNA	No	Yes	Antisynthetase syndrome: interstitial lung disease, myositis, arthritis, Raynaud's phenomenon	2,3
Mi-2	Component of NuRD complex, required for hair follicle development	Chromatin	Yes	Yes	Dermatomyositis; esp. with skin rash	33–35
SRP	Required for cotranslational translocation of proteins into ER	7SL-RNA	Yes	Yes	Severe disease with rapid onset; necrotic muscle fibers with little inflammation	36,37
Myositis-associated antigens						
PM/Scl	Complex of 11 proteins; binds exosomal complex	RNA	Yes—PM/Scl-75	Yes	Polymyositis, dermatomyositis, or polymyositis–scleroderma overlap	38–41
PMS1/2	DNA mismatch repair proteins	DNA	No	Yes	Dermatomyositis and polymyositis	42,43
Ro52	E3 ligase activity; polyubiquitinated *in vitro*; autoantibodies associated with Jo-1	RNA binding—probably through binding partners	No	No	Antisynthetase syndrome	44,45
DNAPK	Required for DNA repair by NHEJ along with XRCC4, Artemis, Ku70/80, and DNA ligase IV	DNA	Yes	Yes	Dermatomyositis and polymyositis	46,47
Ku70/80	NHEJ protein	DNA	Yes	Yes	Dermatomyositis and polymyositis	48

Abbreviations: DNAPK, DNA-dependent protein kinase; ER, endoplasmic reticulum; HRS, histidyl-tRNA synthetase; NHEJ, nonhomologous end joining; NuRD, nucleosome remodeling and deacetylase complex; PMS1/2, postmeiotic segregation increased 1/2; PM/Scl, polymyositis/scleroderma autoantigen; SRP, signal recognition particle; tRNA, transfer RNA.

Treatment

The treatment of the immune-mediated inflammatory myopathies is still largely empirical as there is limited data from controlled clinical trials to allow an evidence-based approach (*Table 3*).[3]

The goals of therapy are to improve the ability to carry out activities of daily living by increasing muscle strength and to ameliorate extramuscular manifestations (rash, dysphagia, dyspnoea, arthralgia, fever).[4]

Treatment has traditionally relied upon the use of corticosteroids and immunosuppressive agents, whose actions on the immune system are largely nonselective, and more recently on other forms of immunomodulatory therapy such as intravenous immunoglobulin. Recent experimental approaches which hold promise for the treatment of resistant cases include cytokine-directed therapies and the use of monoclonal antibodies targeting B and T cells involved in the immune response. More specific immunotherapy awaits a better understanding of the effector mechanisms and the identification of the target antigens against which the immune response is directed.[3]

Table 3. Immunosuppressive therapy for inflammatory myopathies [from *Current Opinion in Neurology*, 2003; 16: 569-575].

Therapy	Route	Dose	Side effects	Monitor
Prednisone	p.o.	100 mg/day for 2–4 weeks, then 100 mg every other day; single a.m. dose	Hypertension, fluid and weight gain, hyperglycemia, hypokalemia, cataracts, gastric irritation, osteoporosis, infection, aseptic femoral necrosis	Weight, blood pressure, serum glucose/potassium, cataract formation
Methylprednisone	i.v.	1 g in 100 ml/normal saline over 1–2 h, daily or every other day for 3–6 doses	Arrhythmia, flushing, dysgeusia, anxiety, insomnia, fluid and weight gain, hyperglycemia, hypokalemia, infection	Heart rate, blood pressure, serum glucose/potassium
Azathioprine	p.o.	2–3 mg/kg/day; single a.m. dose	Flu-like illness, hepatotoxicity, pancreatitis, leukopenia, macrocytosis, neoplasia, infection, teratogenicity	Monthly blood count, liver enzymes while adjusting the dose then every 3 months
Methotrexate	p.o.	7.5–20 mg weekly, single or divided doses; 1 day a week dosing	Hepatotoxicity, pulmonary fibrosis, infection, neoplasia, infertility, leukopenia, alopecia, gastric irritation, stomatitis, teratogenicity	Weekly liver enzymes, blood count while adjusting the dose and then monthly
	i.v./i.m.	20–50 mg weekly; 1 day a week dosing	Same as p.o.	Same as p.o.
Mycophenolate mofetil	p.o	Adults: 1–1.5 g twice daily; no more than 1 g per day in adults with renal failure. Children: 600 mg/m^2/dose twice daily	Bone marrow suppression, hypertension, diarrhea, tremor, nausea, vomiting, headache, sinusitis, cough, amblyopia, confusion, infections, neoplasia, teratogenicity	Blood counts are performed weekly for 1 month, twice monthly for the 2nd and 3rd month, and then once a month for 1 year
Cyclophosphamide	p.o.	1.5–2 mg/kg/day; single a.m. dose	Bone marrow suppression, infertility, hemorrhagic cystitis, alopecia, infections, neoplasia, teratogenicity	Monthly blood count, urinalysis
	i.v.	1 g/m^2	Same as p.o. (although more severe), and nausea/vomiting, alopecia	Daily to weekly blood count, urinalysis
Chlorambucil	p.o.	4–6 mg/day; single a.m. dose	Bone marrow suppression, hepatotoxicity, neoplasia, infertility, teratogenicity, infection	Monthly blood count, liver enzymes
Cyclosporine	p.o.	4–6 mg/kg/day, split into two daily doses	Nephrotoxicity, hypertension, infection, hepatotoxicity, hirsuitism, tremor, gum hyperplasia, teratogenicity	Blood pressure, monthly cyclosporine level, creatinine/ blood urea nitrogen (BUN), liver enzymes
Tacrolimus	p.o.	Adults: 0.1–0.15 mg/kg/day divided into 2 doses, 12 h apart. Children: 0.15–0.20 mg/kg/day divided into 2 doses, 12 h apart	Nephrotoxicity, hypertension, infection, hepatotoxicity, hirsuitism, tremor, gum hyperplasia, teratogenicity	Blood pressure, monthly tacrolimus level, creatinine/BUN, liver enzymes
Intravenous immunoglobulin	i.v.	2 g/kg over 2–5 days; then every 4–8 weeks as needed	Hypotension, arrhythmia, diaphoresis, flushing, nephrotoxicity, headache, aseptic meningitis, anaphylaxis, stroke	Heart rate, blood pressure, creatinine/BUN

p.o., orally; i.v., intravenously; i.m., intramuscularly. Modified from Amato and Barohn [1] with permission.

Prognosis

The prognosis of the disease significantly improved after the introduction of steroids in the fifties, at least a third of patients are left with mild to severe disability. Early diagnosis and treatment are crucial for avoiding atrophic changes of the muscle with consequent loss of function. Factors predictive of a bad prognosis are old age at

onset, bulbar, cardiac and pulmonary involvement and poor response to therapy. CK levels and the extent of muscular involvement at onset do not seem to be determinant for the prognosis.[4] In a small cohort of patients, the 5-year survival was 95% and the 10-year survival 84%.[1]

Immunopathogenesis

The pathogenesis and etiology of idiopatic inflammatory myopathies is not yet fully understood and numerous pathogenic concepts have been proposed.[9] The most characteristic common histopathologic finding is presence of inflammatory cell infiltrates mainly composed of a mixed population of macrophages and T lymphocytes within the affected skeletal muscle together with degenerating and regenerating muscle fibers. Presence of circulating specific autoantibodies (i.e., anti-Jo-1, anti-Mi-2, and anti-signal recognition particle), particularly in PM and DM, other than of T lymphocytes in muscle tissue suggest that IIM are autoimmune disorders but the specificity of the immune reactions is unknown, and why muscles are the major targets of the immune system in these disorders is unclear. Several studies suggest that the degree of inflammatory cell infiltrates varies substantially between patients and does not consistently correlate with the severity of muscle weakness or with the extent of structural changes in the muscle fibers. Hence, non-immune mechanisms might also play a pathogenic role in these disorders. One such non-immune-mediated muscle dysfunction could be caused by a disturbed microcirculation

due to capillary loss or to phenotypically changed endothelial cells in the capillaries. These aberrations may affect the microenvironment of muscle tissue and lead to local tissue hypoxia with development of a secondary metabolic myopathy. Another possible non-immune-mediated mechanism leading to muscle dysfunction is the newly identified endoplasmic reticulum (ER) stress response in myositis. The ER stress response is thought to be a consequence of the up-regulation of major histocompatibility complex class I in muscle fibers.[10]

Immunopathology of dermatomyositis

The primary antigenic target in DM is the endothelium of the endomysial capillaries (*Fig.2*).[1]

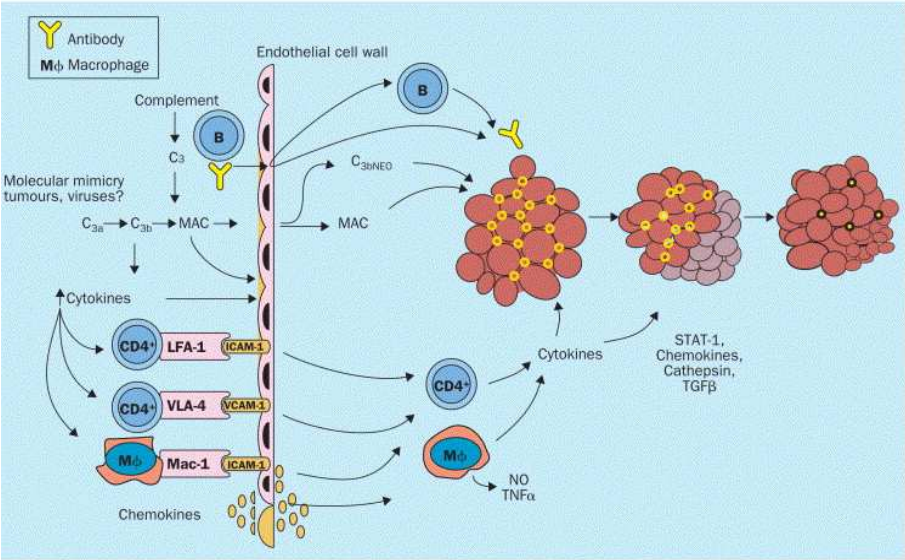


Fig. 2. Proposed sequence of immunopathological changes in DM [from *The Lancet* 2003; 362: 971-982].

The disease begins when putative antibodies directed against endothelial cells activate complement C3. Activated C3 leads to formation of C3b, C3bNEO, and C4b fragments and C5b-9 membranolytic attack complex (MAC), the lytic component of the complement pathway. MAC, C3b, and C4b are detected early in patients serum and are deposited on capillaries before inflammatory or structural changes are seen in the muscle. Sequentially, the complement deposits enduce swollen endothelial cells, vacuolization, capillary necrosis, perivascular inflammation, ischaemia, and destruction of muscle fibers. The current model for DM proposes that the characteristic perifascicular atrophy may reflect endofascicular hypoperfusion, which is prominent distally. However, perifascicular

muscle fibers have not been shown to be preferentially vulnerable to ischaemia (experimental models suggest in fact that perifascicular myofibers are less vulnerable than central muscle fibers to ischaemia) nor has any evidence been found that such fibers are indeed damaged by ischaemia in DM.[11]

Cytokines and chemokines related to complement activation are released; they upregulate vascular-cell adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM-1) on the endothelial cells and facilitate the egress of activated T cells to the perimysial and endomysial spaces. T cells and macrophages through their integrins (very late activation antigen 4, VLA-4, and leucocyte-function-associated antigen 1, LFA-1) bind to the adhesion molecules and pass into the muscle through the endothelial cell wall. The perivascular/perimysial infiltration of mononuclear cells consists predominantly of CD4+ T lymphocytes, occasionally together with B lymphocytes and macrophages,[12] consisting with a humorally mediated process. In peripheral blood of active DM, a decreased percentage of CD3+ and CD8+ T lymphocytes and decreased IFN-γ expression by CD4+ and CD8+ T lymphocytes and an increase in B lymphocyte and IL-4 producing CD4+ T cell frequencies were found.

In addition to an adaptive immune response, an innate immune response characterized by infiltration of dendritic cells (DCs) may be important in the pathogenesis of DM. Immature and mature DCs (CD1a+ and DC-LAMP/CD83+, respectively) have been detected in skeletal muscle of DM patients; in particular, a higher number of

plasmacytoid DCs (pDCs) was found in comparison with the amount of myeloid DCs. pDCs are natural interferon-producing cells because of their capacity to produce over 1,000 times the amount of IFNα than any other known cell type.[11] They also act as antigen presenting cells for T helper and T regulatory cells, and stimulate B cell development into plasma cells. These cells express CD4 and may account for some of the relatively large numbers of endomysial CD4+ cells that have previously been seen in DM muscle and interpreted as T helper cells. pDCs are typically found in perimysial regions and this peculiar localization may represent the explanation of the high local concentration of interferon-α/β observed in these areas and it may contribute, through unidentified mechanisms, to the typical perifascicular atrophy. pDC interferon-α/β production occurs through engagement of Toll-like receptors 9 (TLR-9) and TLR-7, that ligate sequence specific viral DNA or RNA; therefore, it seems possible to suppose that a chronic viral infection is present in DM muscle, although viral genetic material has not yet been detected.

Immunopathology of polymyositis and inclusion body myositis

The immunologic processes occurring within muscle in IBM and PM have been modeled identically, yet these diseases have different responsiveness to several immunotherapies.[11] In IBM compared with PM, less frequent myofiber necrosis and more frequent invasion of non-necrotic myofibers by mononuclear cells have been observed.

There is a degenerative process in IBM of an uncertain nature and models for it. The nature of the relationship between the autoimmune process and the degenerative process present in IBM is currently unknown. In view of this consideration, in this study we decided to focus our attention exclusively on JDM, adult DM and PM, in which the autoimmune response is thought to play a primary role in determining the pathological phenotype of the myopathy.

A close interaction between $CD8^+$ T-cells and non-necrotic fibers has been consistently demonstrated in PM and IBM, combined with the intracellular polarization of perforin toward affected muscle fibers in the invading T-cells.[3,13] No specific T-cell-reactive autoantigen has been defined in the diseases and there is a paucity of evidence to support a role for the antigens recognized by the autoantibodies characteristic of PM. In PM, restricted T-cell receptor V gene usage by invading T cells suggests an antigen-specific reaction. In IBM, there is some debate as to whether the T cell infiltrate represents a true antigen-driven autoimmune reaction, although some recent data suggest limited T cell receptor heterogeneity among muscle-invading T cells (*Fig. 3*).[14]

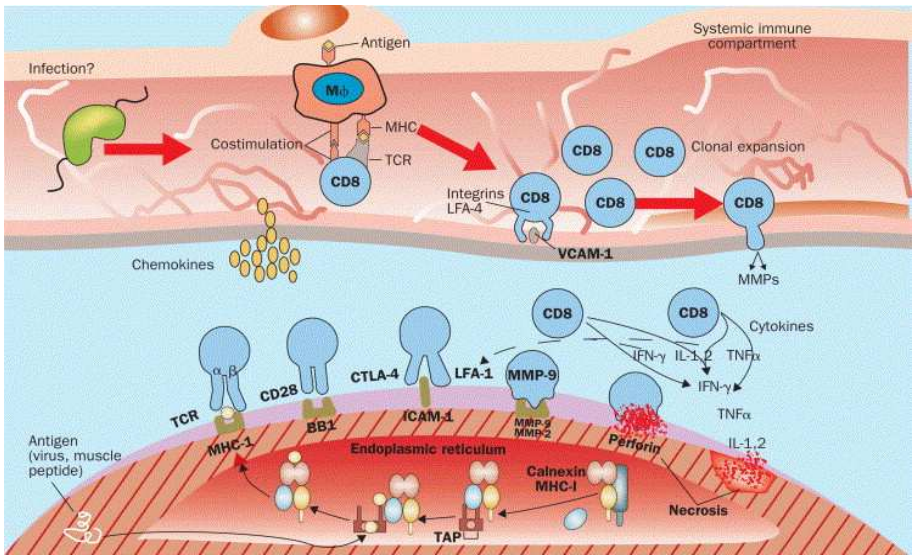


Fig. 3. Molecules, receptors and ligands involved in transgression of T cells through endothelial cell wall and recognition of antigens on muscle fibers in PM [from *The Lancet* 2003; 362: 971-982].

In PM and IBM, there is a great deal of evidence to suggest that T cell products are involved in the pathogenic process. Cytotoxic $CD8^+$ T lymphocytes can release three different cytotoxic proteins: perforin, granzyme and granulysin.[12] It is known from earlier studies that in PM and IBM, $CD8^+$ T lymphocytes and macrophages surrounding the non-necrotic muscle fibers expressing MHC class I antigen do express perforin. Perforin may cause a leak in the sarcolemmal surface through which granzymes could invade the sarcoplasm to initiate muscle fiber necrosis. Recently, granulysin has also been demonstrated in both PM and IBM.[15]

Expression of cytokines such as interleukin (IL)-1 (α and β), IL-6, interferon-γ, lymphotoxin, and tumor necrosis factor-α (TNF-α) has been demonstrated in mononuclear cells in affected muscles in each disease group.[3] Collectively, these cytokines have a variety of functions that can contribute to local inflammatory responses and the generation or perpetuation of autoimmune response, and may act synergistically to damage muscle cells. These functions include T cell activation, recruitment of lymphocytes and mononuclear phagocytes, stimulation of expression of MHC molecules on a variety of cell types, and direct damage to target cells. Furthermore, several chemotactic and inflammatory chemokines, such as IL-8, monocyte chemoattractant protein-1 (MCP-1), regulated-upon activation normal T cell expressed and secreted (RANTES), macrophage inflammatory protein-1α (MIP-1α), other than anti-inflammatory cytokines (transforming growth factor-β, IL-10, IL-4), has been shown in affected muscle in IIMs. The expression of these cytokines in muscle induces the upregulation of cell surface antigens that contribute to T cell emigration into muscle, their adherence and subsequent activation. If the autoinvasive $CD8^+$ T lymphocytes are driven by specific antigens, as the clonally expanded TCR gene rearrangements indicate, the MHC-I molecule on the muscle fibers should be able to present antigenic peptides to the TCR.[1] For primary T cell antigenic stimulation a second signal is required and provided by the B7 family of costimulatory molecules. Muscle fibers do not express the classic costimulatory molecules B7-1 (CD80) or B7-2 (CD86); instead, they

express a functional B7 related molecule defined by the monoclonal antibody BB-1. Indeed, the MHC-I/BB1-positive muscle fibers make direct cell-to-cell contact with their CD28 or CTLA-4 ligands on the autoreactive $CD8^+$ cells. The inducible co-stimulator ligand (ICOSL), member of the B7 family of co-stimulatory ligands, and the costimulatory molecule CD40 are also upregulated on muscle fibers.[16-17] Another MHC molecule, the non-polymorphic non-classic HLA G, is upregulated *in vitro* by interferon-γ and is expressed on muscle fibers of patients with PM and IBM. Because HLA G protects human muscle cells from immune-cell-mediated lysis *in vitro*, it could also partially protect muscle fibers *in vivo*.

In sporadic IBM, the nature of the T cell response remains unclear. Intimate interaction between CD8, DR+ (i.e. activated) T cells which express perforin and granzyme, has been shown, but evidence of antigen-specific autoimmunity is scarce. Deposition of the Aβ fragment of the amyloid precursor protein is a feature of affected muscle in IBM and it has been shown that muscle cells can secrete Aβ. Interactions of Aβ with muscle cells in turn can stimulate IL-6 production by these cells, and its interaction with macrophages can induce TNF-α and IL-1 secretion. Thus, Aβ may contribute to a positive feedback loop that exacerbates inflammation in affected muscles. The stimulus for excessive Aβ production in IBM is unknown, and whether this precedes inflammation, or vice versa, remains to be determined.

For IBM and PM, the abundance of T cells and sparseness of B cells have contributed to a model of these as cytotoxic T cell mediated autoimmune diseases. Nevertheless, circulating autoantibodies have been recognized as present in some patients with IBM and PM.[11] Microarray studies have found that for IBM and PM muscle compared to normal muscle, the most abundant transcripts were derived from immunoglobulin genes. As these genes are only transcribed in B cells and their progeny, this finding stood in contrast to the previously performed immunohistochemical studies suggesting no significant role for B cells in muscle pathology in these diseases. The identification of differentiated B lymphocytes in the form of $CD138^{+}$ plasma cells in the endomysium of IBM and PM muscle might clarified this discrepancy. These plasma cells have undergone affinity maturation, in which they have mutated their immunoglobulin genes, switching their isotype from IgM to IgG and IgA. Considering that generally plasma cells are viewed as acting from a distance, residing in bone marrow, and secreting their antibodies into the circulation, the presence of clonally related but distinct B or plasma cells within muscle in IBM and PM suggests that B cells can mature into plasma cells locally within muscle.

The presence of T lymphocytes in all subsets of IIMs indicates a permanent immune response that requires the presence of antigen-presenting cells (APCs). Myeloid DCs have only recently been recognized in PM and IBM muscle, mainly surrounding and sometimes invading non-necrotic muscle fibers. They are also present

within dense collections of inflammatory infiltrates displacing myofibers in their differentiated stellate morphology suggesting that they may be activating T cells locally within muscle, a process generally believed to occur in lymph node.

Inflammatory myopathies and infections

Several clinical, epidemiologic and experimental evidences support the hypothesis that autoimmune diseases are triggered by infections and many autoimmune disorders such as systemic lupus erythematosus (SLE), anti-phospholipid syndrome (APS), Sjögren's syndrome, reactive arthritis and multiple sclerosis have been associated with a variety of infectious agents representing the initiating event of autoimmunity.[4]

For some autoimmune diseases, established associations have been described, for example: Group A streptococcus and rheumatic fever; hepatitis B and C viruses and mixed cryoglobulinemia; and for others the causative infectious factor is suspected, but not definitely demonstrated, for instance: mycobacteria and mycoplasmas and rheumatoid arthritis, cytomegalovirus and type I diabetes mellitus.

In patients affected by PM and DM, several data have been published regarding the role of infections in the induction of autoimmunity (*Table 4*). Many case reports evidenced a medical history compatible with respiratory infection prior to symptom onset in a substantial number of patients affected by juvenile dermatomyositis (JDM).[18]

Aspiration pneumonia, produced by Gram-positive and anaerobic bacteria, is the most common infection in IMs, occurring in 15% to 20% of PM/DM patients.[19] *S. aureus* infections involving the soft tissue and skin around calcinotic lesions are frequently described in patients with JDM and calcinosis cutis. Other bacterial infections in DM constitute only isolated reported cases. For example, *Streptococcus pyogenes* myositis has been described in patients with juvenile and adult DM with or without calcinosis; in these patients the myopathy probably favored the bacterial colonization of muscle from bacteremia originating in dermal lesions. Among the opportunistic infections, disseminated *Nocardia brasiliensis* involving the skin and the lung was reported in a pediatric patient receiving immunosuppressive therapy with methotrexate and prednisone for severe DM, and extra pulmonary infections produced by *Mycobacterium tuberculosis* and generalized *M. avium- intracellulare* have been described in patients with PM/DM. Although opportunistic infections in these patients are uncommon, early diagnosis and adequate treatment are essential to improve survival.

Table 4. Evidences of infection-induced PM–DM [from *Autoimmunity*, 2006; 39: 191-196].

	Reference
Induction of anti-tRNA synthetase autoantibodies	[13,14]
During virus replication, viral and host proteins are presented to the immune system breaking self tolerance	
Molecular mimicry between host and microbial proteins	[17,18]
1. Histidyl-tRNA synthetase matches with ECRF4 protein from Epstein Barr virus	
2. Alanyl-tRNA synthetase matches with:	
- Exon associated protein (IIIA) from adenovirus 2	
- BPLF1 protein from Epstein Barr virus	
- Haemoagglutin in molecules from two strains of influenza viruses	
3. Human skeletal myosine matches with group A streptococcal type 5 M protein	
Seasonal pattern of disease which may correspond to the seasonal pattern of the inciting infection	[19,21–23]
Anti-Jo-1 positive patients tend to develop disease in April	
Anti-SRP positive patients tend to develop disease in November	

Some picornaviruses, especially enteroviruses and cardioviruses, have been supposed to play a pathogenic role in human chronic PM and DM,[20-22] although viral antigens have rarely detected in muscle cells after the acute phase of the disease. *Herpes zoster* is a common viral infection in patients with PM/DM and usually occurs during periods of disease inactivity. Cytomegalovirus is an uncommon opportunistic viral infection in patients with PM/DM; however, some cases of severe and fatal infection, such as interstitial pneumonia, have been noted in patients receiving corticosteroid or immunosuppressive therapy. Some studies have demonstrated a temporal relationship between coxsackie virus, parvovirus B19, hepatitis C, and other

enterovirus infections and the onset of PM/DM; however, the role of these pathogens in the etiology of IMs remains speculative.

Among fungal infections, *Pneumocistis carinii* pneumonia (PCP) is frequently fatal in patients with IM. This infection has a rapid and severe course, and most patients require critical care support. Known risk factors for the development of PCP are interstitial pulmonary disease, lymphopenia, and the use of corticosteroids and immunosuppressive drugs. Candidiasis is another fungal infection occurring in patients with DM.[23] In a very large study of more than 40,000 patients with various skin disorders, the frequency of mucocutaneous candidiasis was three times higher in patients with DM, bullous pemphigous, tinea inguinalis, or condylomata acuminata than in the general population, suggesting that pre-existing dermal involvement may increase the susceptibility of patients with DM to this infection. Finally, disseminated histoplasmasis involving the muscles and fascia has been reported in a patient with DM being treated with corticosteroids and methotrexate, which were initially thought to have a disease flare.

Type I and type II interferons and the host defense against viral and bacterial infections

The multiple subtypes of α-interferons and the single β-interferon belong to the type I family of interferons (IFNs) and, together with the related but distinct single-member type II IFN (IFN-γ), constitute one

of the most important classes of cytokines, named for their potent ability to "interfere" with viral replication.[24,25] The IFN system represents the first line of defense designed to block the spread of virus infection in the body, sometimes at the expense of accelerating the death of the infected cells. In addition to their role of mediators of cellular homeostatic responses to virus infection, IFNs have pleiotropic effects on many aspects of cell physiology, including cell growth, cell motility and cell functions.

The type I IFN family consists of multiple IFN-α members, single IFN-β, ε, κ, and ω subtypes, as well as δ and τ subtypes found in pig and ovine, respectively.[25] Recently, it was identified a novel class of human type I-like IFNs, termed IFN-λ1, -λ2, and –λ3, or IL-28A, IL-28B, and IL-29,[26] functionally similar to type I IFNs but with distinct gene sequence and chromosomal locations.

Signaling of type I IFNs starts with the binding of these cytokines to a common receptor designated IFNAR (*Fig. 4*). These molecules, unique among cytokine receptors for their ability in mediating responses to more than 15 different ligands, are composed of heterodimers of two types of ectoproteins with single transmembrane domains, IFNAR1 and IFNAR2, that lack intrinsic enzymatic activity. Their cytoplasmic domains recruit specific protein kinases that get activated when IFNs bind to the extracellular domains of the receptors.[27] The activation process involves receptor dimerization and tyrosine phosphorylation of the receptor proteins by specific tyrosine kinases of the Janus kinase (Jaks) family, TYK2 and JAK1. Activated

Jaks phosphorylate themselves, the receptor subunits, and some members of the family of proteins called Signal Transducers and Activators of Transcription (STAT), recruited to the receptor-bound Jaks either directly or by SH2 domain-containing adapter proteins with high affinity for phosphorylated tyrosine residues. Once phosphorylated, STATs dissociate from the receptors, dimerise and translocate to the nucleus to regulate gene transcription. The classical transcription factor complex identified in the type I IFN signal transduction pathway is the Interferon Stimulated Gene Factor (ISGF) 3 complex, composed of STAT1, STAT2 and IRF9, which binds Interferon Stimulated Response Elements (ISREs) in the promoters of certain Interferon Stimulated Genes (ISGs), thereby initiating their transcription.

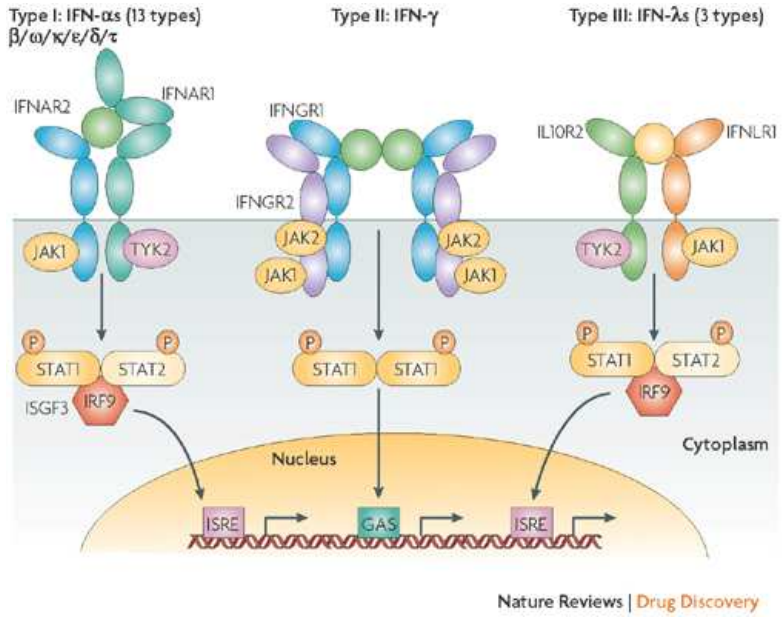


Fig. 4. Receptor activation or ligand-receptor complex assembled by type I, type II or type III interferons [*Nature Review drug discovery*, 2007; 6: 975-990].

Other homodimeric or heterodimeric IFN-induced STAT complexes bind another type of element – known as an IFN-γ-activated site (GAS) element – that is present in the promoter of ISGs. Of the hundreds of known ISGs, some have only ISREs or only GAS elements in their promoters, whereas others have both elements; therefore, combinations of different STAT-containing complexes might be required for the optimal transcriptional activation of a particular gene.[28]

The transcription of type II IFN (IFN-γ)-dependent genes is regulated by GAS elements, and STAT1 is the most important IFN-γ-activated transcription factor for the regulation of these transcriptional responses. After engagement of the type II IFN receptor by IFN-γ, JAK1 and JAK2 are activated and regulate downstream phosphorylation of STAT1. Such phosphorylation results in the formation of STAT1-STAT1 homodimers, which translocate to the nucleus and bind GAS elements to initiate transcription. In contrast to type I IFNs, IFN-γ does not induce the formation of ISGF3 complexes and thereby cannot induce the transcription of genes that have only ISREs in their promoter.

The primary activity of type I IFNs is their ability to render cells resistant to viral infection.[29] Mechanisms of IFN-induced inhibition of viral replication are viruses- and cell type-dependent; multiple stages

in the viral life cycle are targeted by ISGs including viral entry, transcription, initiation of translation, maturation, assembly and release. The 2'-5' oligoadenylate synthetase is an IFN-induced, double-stranded RNA-dependent enzyme that catalyses the formation, from ATP, of 2'- to 5'-linked oligoadenylates that activate a latent ribonuclease, called RNase L, that degrades viral RNA. Protein kinase R (PKR) is another IFN-induced, dsRNA-dependent enzyme that phosphorylates various substrates including the protein synthesis initiation factor eIF2α and blocks the translation of viral RNA. The Mx proteins are IFN-induced GTPases that form complexes with dynamin disrupting trafficking or activity of viral polymerases, thereby interfering with viral replication. In particular, Mx proteins have been shown to have a protective effect mainly against two RNA viruses, influenza virus and vesicular stomatitis virus (VSV).[30]

IFNs have been shown to affect many molecules involved in the control of cell cycle including the rapid shutdown of c-myc expression, and the production of growth factors and growth factor receptors. Type I IFNs regulate the expression of several molecules involved in cell survival/apoptosis. These include p21/waf1, IRF1, STAT1, PKR, RNase L, PML, Daxx, BCL2 family members, caspase 4 and 8, TNF family receptors and ligands (TRAIL and CD95L), phospholipids scramblase and death-associated protein kinases. All the effector cells of both the innate and adaptive immune systems are regulated by type I IFNs. They promote NK cell-mediated proliferation, differentiation and cytotoxicity *in vitro* and *in vivo*, the

survival of memory T cells and the differentiation of T cells. The generation and activation of immature myeloid DCs are also targets of IFN action. Type I IFNs regulate IL-7-stimulated B-cell differentiation and promote maturation and immunoglobulin class switching. They also regulate the recruitment of immune cells to the site of inflammation via the induction of chemokines and chemokine receptors, including CXCL10 and CCL12, which modulate the recruitment of NK cells, macrophages and T lymphocytes. Not only the effector arm of immune response is stimulated by IFNs, but also antigen presenting machinery and cell surface molecules on target cells. Type I IFNs are involved in the induction of major histocompatibility complex, in the production of co-stimulatory molecules such as CD40 and CD86 and also in the increase of the expression of several tumour antigens.

The aforementioned properties of the type I IFNs, particularly antiviral, antiproliferative and immunoregulatory, have led to their development as therapeutics. Currently, IFNα is used in the treatment of hepatitis B and C, certain haematological cancers and multiple sclerosis. However, while IFNs have properties that are clearly beneficial to the host, they also induce side effects including leucopenia, nausea, malaise and occasionally neurotoxicity, autoimmunity and death. The regulation of IFN effects occurs at several levels. Firstly, the induction of IFN genes is subjected to positive (IRFs 1, 3, 5 and 7) and negative (IRFs 2, 4 and 8) regulation; and the selection of the subtypes of IFN produced will determine the

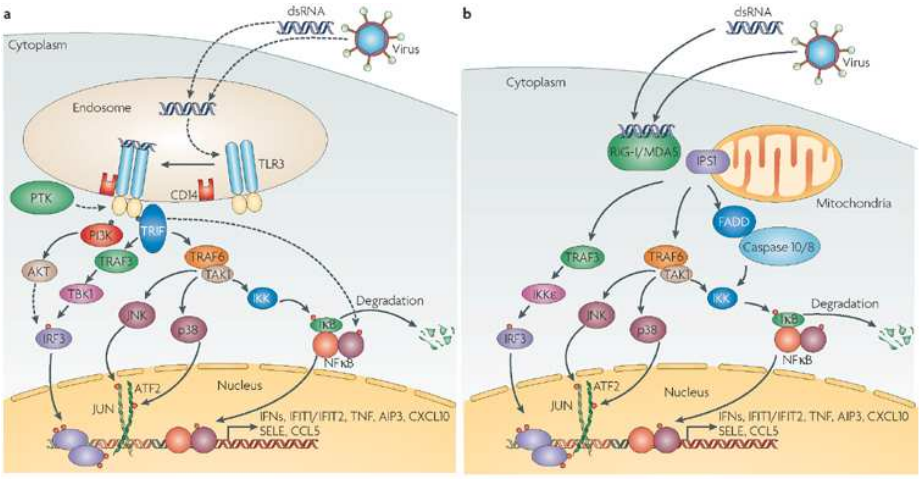
potency and perhaps the nature of the effects. The distribution of cell surface and soluble receptors can modulate the strength and duration of IFN signal. In addition, there are several mechanisms of negative regulation of cytoplasmic signaling including the suppressors of cytokine signaling (SOCS) 1 and 3, UBP43 and protein inhibitors of activated STAT proteins.[29]

The induction of type I IFNs: the role of Toll-like receptors and cytosolic RNA helicases [30]

Two complementary receptor systems account for most virus detection; they include a class of receptors (exemplified by RIG-I and MDA5 proteins) expressed ubiquitously and localized within the cytosol where it detects viral nucleic acids produced upon infection, and a group of receptors (members of the Toll-like receptor [TLR] family) able to detect viral nucleic acids in endosomes and only in specialized cell types.[30] This latter mode of recognition does not require that the IFN-producing cells are infected themselves and hence need not be ubiquitous.

Several of the mammalian TLRs recognize different type of nucleic acids, and together they provide enough coverage to detect most types of viruses. TLR3 recognizes double-stranded RNA, a common feature of both DNA and RNA viruses that until recently was thought to be the principal (or even only) form of viral nucleic acid recognized by the immune system (*Fig. 5*). TLR7 and TLR8 detect single-stranded

RNA (ssRNA) and viruses that contain ssRNA genomes; TLR9 detects unmethylated CpG motifs in DNA that are common in DNA viruses and also bacteria. The only known exception to the rule that all receptors that activate type I IFNs detect nucleic acids is TLR4, which detects lipopolysaccharides (LPS) derived from Gram-negative bacteria and activates type I IFNs in conventional dendritic cells (DCs) and macrophages through a signaling pathway tha utilizes the adaptor protein TRIF. TLR2 also recognizes cell wall components (peptidoglycans) but does not activate IFNs. It is likely that the TLR4-TRIF axis arose to harness some of the functions of IFNs in enhancing T cell differentiation and NK cell function. TLRs are primarily expressed on key sentinel cells of the innate immune system: macrophages and DCs.



Nature Reviews | Drug Discovery

***Fig. 5**. Different interferon (IFN) signaling pathways activated by dsRNA and viruses* [*Nature Reviews drug discovery,* 2007; 6: 975-990].

TLR3 is expressed by conventional DCs, whereas TLRs 7 and 9 are expressed by both conventional and plasmacytoid DCs (pDCs). pDCs have a specialized signaling pathway that links activation of TLRs 7 and 9 to the production of copious amounts of type I IFNs, and they are primarily responsible for the systemic concentrations of IFNs detected during many experimental viral infections. One feature shared by the four TLRs that sense nucleic acids is their intracellular localization: unlike all other TLRs, which are located at the cell surface, TLRs 3, 7, and 9 signal from within an acidified endosomal compartment. This intracellular localization is important for the ability of these TLRs to discriminate between self and non-self nucleic acids. A key feature of TLR-dependent nucleic acid recognition is directly tied to their intracellular location: TLRs sample material entering cells from the outside and thus do not detect the presence of infection from within. This material often consists of viral particles that become uncoated or degraded upon endosomal acidification, thus exposing their nucleic acids for recognition. TLR3 also samples apoptotic cells for viral dsRNA as they are engulfed and degraded in the phagosome. Considering the strong link between viral infection and apoptosis in vertebrates and the role of TLRs in antigen presentation, this mechanism would provide an efficient means for viral antigens within dead cells derived from any tissue to be presented to T cells by

uninfected DCs, a process known as crosspresentation. The separation of viral infection from viral recognition in DCs has another important consequence: most viruses encode proteins that potently antagonize type I IFN expression and signaling within infected cells, but uninfected DCs would be refractory to such inhibition.

Although some TLRs can activate IFNs in a subset of specialized cells, almost all nucleated cells respond to viral infection by producing type I IFNs. This alternative broadly expressed virus-detection system comprises the caspase recruitment domain (CARD)-containing RNA helicases retinoic acid inducible gene-I (RIG-I) and melanoma differentiation antigen 5 (MDA5) as sensors that link detection of dsRNA to activation of type I IFNs. These molecules use the CARD-containing adaptor protein MAVS (also known as IPS-1, VISA, or CARDIF) to activate the IFN-β gene. Although the two receptor systems can independently activate the type I IFN response, they are not functionally redundant. Moreover, this also implies that type I IFNs are not sufficient for antiviral defense and that these cytosolic receptors may also control essential antiviral functions in an IFN-independent manner.

In addition to intracellular recognition of viral dsRNA, foreign DNA is also recognized by an intracellular sensor that triggers type I IFN production. This DNA recognition system presumably evolved to detect DNA derived from DNA viruses during infection. DNA recognition is a cell-autonomous and, presumably ubiquitous virus-recognition system. The signaling pathway induced by this still

unknown sensor is distinct from RIG-I and MDA5 signaling in that it does not result in activation of NF-κB transcription factor or MAP kinases, but does require IRF3 for the induction of interferon genes and it is MAVS-independent.

A potent type I IFN response is also induced by several intracellular bacterial infections (*Fig. 6*). Gram-negative bacteria, including *Salmonella typhimurium* and *Escherichia coli* or LPS derived from these bacteria, induce type I IFN production through TLR4 signaling pathway.[25] Other bacterial components that induce type I IFN include bacterial DNA and Gram-negative flagelling. Contrary to what observed with Gram-negative bacteria, the production of type I IFNs during infections of Gram-positive bacterial components are usually detrimental to the host, with the induction of several proapoptotic gene programs including PKR, the death-receptor ligand TRAIL, and Daxx.

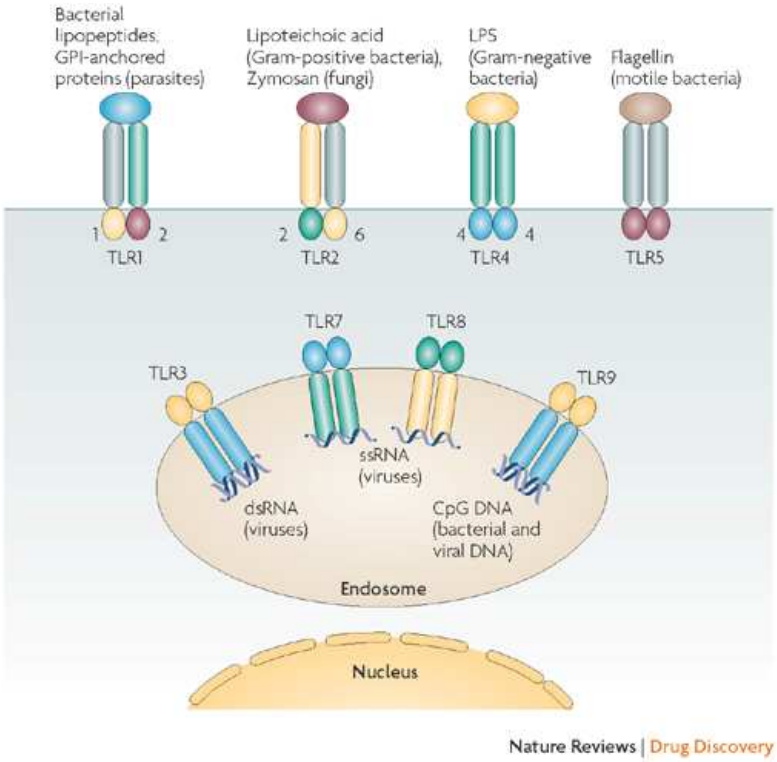
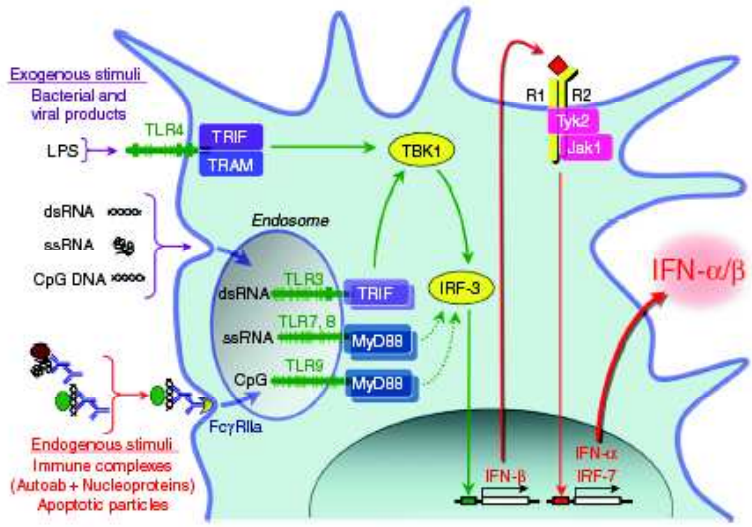


Fig. 6. *Mammalian TLRs and their bacterial and viral ligands* [*Nature Reviews drug discovery*, 2007; 6: 975-990].

Nucleic-acid sensing TLRs: compartmentalization as an effective ploy against autoimmunity

Although TLRs appear to have evolved as a warning system to detect infections, in some cases they can be triggered accidentally by self molecules.[31] This is best established for the TLRs that detect nucleic acids, including TLR3 (activated by double-stranded RNA), TLR7

and TLR8 (activated by single-stranded RNA), and TLR9 (activated by unmethylated CpG motifs within ssDNA) (*Fig.7*).



Immunol Rev 2005

Fig. 7. *Interferon-α/β (IFN-α/β) production through Toll-like receptor (TLR) engagement.*

Nucleic acid components of certain pathogens have the ability to induce not only a powerful innate immune reaction but also an inflammatory response that is directed to "self" structures.[32] In addition, endogenous ligands such as RNA and DNA can activate TLRs under certain conditions and promote or sustain autoimmune diseases such as systemic lupus erythematosus (SLE). To some

degree, specificity in the detection of foreign nucleic acids by these TLRs may be provided by their localization in an endosomal compartment, from which self nucleic acids are normally excluded but through which pathogens commonly transit. An additional level of specificity seems to be provided by the fact that vertebrate nucleic acids have several modifications that functionally reduce the probability of activating TLRs. Modified nucleosides that are commonly present in vertebrate rRNA and tRNA reduce the activation of TLR3, TLR7, and TLR8, and the methylation and suppression of CpG dinucleotides in vertebrate DNA prevents it from having the same immune stimulatory activity as bacterial DNA.

Another safety mechanism limiting the improper activation of TLRs is the control of their intracellular trafficking. Nucleotide-sensing TLRs are sequestered in the ER in steady state and, upon ligand stimulation, traffic from the ER to endolysosomes, where they detect internalized viral RNA and DNA.[33] Interestingly, signals triggering nucleotide-sensing TLR translocation from the ER to endolysosomes are not limited to nucleotides but include other stimulants such as lipopolysaccharide (LPS). Unnecessary enhanced translocation of TLRs to endosomal compartments in inflammatory situations may predispose to autoimmune diseases. Among the control mechanisms to avoid detrimental responses to self-products, the need of the acidification of endolysosomes for the proper activation of TLRs by their ligands is the most important.

The precise molecular mechanisms underlying the unusual intracellular trafficking of endosomal TLRs are not known. Recent studies[34] have been shown that a poorly characterized multi-transmembrane-domain-containing protein, UNC93B1, seems to play an essential role in signaling by the nucleotide-sensing TLRs, specifically binding to them via their transmembrane domains, and also in TLR7 and TLR9 translocation from the ER to lysosomes. It is not known if this protein may be involved also in the TLR3 trafficking to endosomal compartment or to plasma membrane. Considering that the subcellular locations of early and late endosomes and lysosomes, and their movements, result from their interactions with the actin and microtubule networks and the forces generated by actin- and microtubule-dependent motors,[35] it might be supposed that cytoskeletal components and adaptor proteins could have a role in the TLR trafficking.

The cytoskeleton

The cytoskeleton is a cellular “scaffolding” or “skeleton” contained within the cytoplasm. The cytoskeleton is present in all cells; it was once thought this structure was unique to eukaryotes, but recent research has identified the prokaryotic cytoskeleton. It is a dynamic structure that maintains cell shape, protects the cell, enables cellular motion (using structures such as flagella, cilia and lamellipodia), and

plays important roles in both intracellular transport (e.g., the movement of vesicles and organelles) and cellular division.

The eukaryotic cytoskeleton

Eukaryotic cells contain three main kinds of cytoskeletal filaments: microfilaments, intermediate filaments, and microtubules. The cytoskeleton provides the cell with structure and shape, and by excluding macromolecules from some of the cytosol it adds to the level of macromolecular crowding in this compartment. Cytoskeletal elements interact extensively and intimately with cellular membranes.

- **Actin filaments/Microfilaments**

Around 6 nm in diameter, this filament type is composed of two intertwined actin chains. Microfilaments are most concentrated just beneath the cell membrane, and are responsible for resisting tension and maintaining cellular shape, forming cytoplasmatic protuberances (like pseudopodia and microvilli- although these by different mechanisms), and partecipation in some cell-to-cell or cell-to-matrix junctions. In association with these latter roles, microfilaments are essential to transduction. They are also important for cytokinesis (specifically, formation of the cleavage furrow) and, along with myosin, muscular contraction. Actin/myosin interactions also help produce cytoplasmic streaming in most cells.

- **Intermediate filaments**

These filaments, around 10 nm in diameters, are more stable (strongly bound) than actin filaments, and heterogeneous constituents of the cytoskeleton. Like actin filaments, they function in the maintenance of cell-shape by bearing tension. Intermediate filaments organize the internal tridimensional structure of the cell, anchoring organelles and serving as structural components of the nuclear lamina and sarcomeres. They also participate in some cell-cell and cell-matrix junctions.

Different intermediate filaments are:

- made of vimentins, being the common structural support of many cells;
- made of keratin, found in skin cells, hair and nails;
- neurofilaments of neural cells;
- made of lamin, giving structural support to the nuclear envelope.

- **Microtubules**

Microtubules are hollow cylinders about 23 nm in diameter (lumen = approximately 15 nm in diameter), most commonly comprised of 13 protofilaments that, in turn, are polymers of alpha and beta tubulin. They have a very dynamic behaviour, binding GTP for polymerization. They are commonly organized by the centrosome. In 9 triplet sets (star-shaped), they form the centrioles, and in 9 doublets

oriented about two additional microtubules (wheel-shaped) they form cilia and flagella. The latter formation is commonly referred to as a "9+2" arrangement, wherein each doublet is connected to another by the protein dynein. As both flagella and cilia are structural components of the cell, and are maintained by microtubules, they can be considered part of the cytoskeleton.

They play key roles in:

- intracellular transport (associated with dyneins and kinesins, they transport organelles like mitochondria or vesicles);
- the axoneme of cilia and flagella;
- the mitotic spindle;
- synthesis of the cell wall in plants.

Microtubule associated motor proteins

The majority of active transport in the cell is driven by three classes of molecular motors: the kinesin and dynein families that move toward the plus-end and minus-end of microtubules, respectively, and the unconventional myosin motors that move along actin filaments. Each class of motor protein has different properties, but in the cell they often function together.[36] Motor proteins are able to recognize the microtubule polarity, and so the organization of the rails combined with the specific motor employed determines the direction of transport. Kinesin-mediated transport is usually used to bring cargoes toward the cell periphery, while dynein moves in the opposite

direction, toward the microtubule minus-end, and is tipically used to move cargoes toward the cell center (and nucleus).

Actin filaments are more randomly oriented, and can be used by unconventional myosin motors, such as myosin-V, to ferry cargoes. Actin filaments are significantly shorter than microtubules, and have been suggested to bridge the gap between microtubules. In this way, local transport can occur on actin filaments in region where there are few microtubules, as at the axon terminal. As with microtubules, the organization and density of each actin filaments is cell-type specific. In some cases, actin filaments have an ordered structure close to the cell surface, with barbed (plus) ends pointed outwards, which could allow myosin-V – which moves toward the actin filament plus-end – to transport cargoes to the very edge of the cell.

In some systems, the same cargo can move on both microtubule and actin filaments, switching between motors in the course of motion. Cargoes moving this way include pigment granules, axonal vesicles, mitochondria, and endosomes. A functional collaboration can exist between microtubule and actin filament networks, and there have been suggestions that motors associated with each network coordinate to achieve the requisite subcellular distribution of cargo. Motors also often appear to work together locally: intracellular transport often employs multiple motors of different classes on the same organelle.

Scope of the Thesis

The aim of this work was to investigate the role of type I interferons (IFN-α/β) and of endosomal Toll-like receptors 3, 7 and 9 in the pathological alterations of idiopathic inflammatory myopathies (IIMs), and, in a second part of the study, to evaluate the role of cytoskeletal motor proteins in Toll-like receptor trafficking, in order to identify new possible therapeutic targets for the treatment of IIMs.

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Chapter 2

"Type I interferon molecules and Toll-like receptors in inflammatory myopathies"

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Abstract

Objective: The triggering factor of inflammation in idiopathic inflammatory myopathies (IIMs) is still unknown and an involvement of viruses or bacteria has been put forward. We sought to investigate the expression of type I interferons (IFNα/β) and of endosomal Toll-like receptors (TLRs) in IIM muscles.

Methods: Ten IIM and 5 control muscle biopsies were assessed by microarray analysis for the expression of approximately 16,000 genes; 37 additional samples from IIM and controls were studied for IFNα/β-dependent genes and intracellular TLR expression using immunohistochemistry, confocal microscopy, real-time quantitative and qualitative PCR.

Results: IFNα/β-dependent gene transcripts were up-regulated in all IIM muscle specimens compared to controls. In juvenile dermatomyositis (JDM) ISG15 (408-fold), IFIT3 (261-fold), MX1 (99-fold), IRF7 (37-fold) were those most expressed. TLR3, TLR7

and TLR9 were differentially expressed in IIM muscles: TLR3 was highly up-regulated in JDM, localized on vascular endothelial cells, muscle infiltrating cells and regenerating myofibers; TLR7 and TLR9 were frequently detected in polymyositis (PM), mainly on cell infiltrates (particularly plasma cells), and on some injured myofibers.
Conclusion: Transcriptome analysis indicated that IFNα/β-induced molecules play a key role in the pathogenesis of IIMs, particularly in JDM. Endosomal TLRs represent important effector molecules that link innate and adaptive immune responses in affected muscles, showing their potential as new therapeutic target for the IIM treatment.

Introduction

The idiopathic inflammatory myopathies (IIMs) constitute a heterogeneous group of chronic disorders characterized by muscle weakness and inflammation within muscle tissue.[1] They include polymyositis (PM), dermatomyositis (DM) and inclusion-body myositis (IBM). PM and DM have probably an autoimmune origin in muscle injury, although the target antigens remain still unknown.[1] Clinical, epidemiological and experimental evidences suggest a contribution of pathogens to autoimmunity in IIMs. Among infectious agents, Coxsackie viruses, parvoviruses, enteroviruses, retroviruses (i.e. human T-lymphotropic virus and human immunodeficiency

virus), have been proposed as initiating factors, but results are still controversial.[2,3]

Antiviral immune responses are regulated by type I interferons (type I IFNs), particularly IFN-α and IFN-β.[4] These cytokines play a key role in innate response to a wide variety of infectious agents[5] and also provide potent regulatory interaction with the adaptive immune system, by activating the microbiocidal functions of macrophages, up-regulating antigen processing by professional antigen presenting cells (APCs), and inducing cytokine expression that modulates lymphocyte and myeloid cell function.[5] Induction of type I IFN genes is mediated by two main classes of receptors defined as pattern recognition receptors (PRRs): Toll-like receptors (TLRs) and cytoplasmic RNA helicases RIG-I and Mda-5.[6] Among TLRs, the most potent promoters of type I IFN production are TLR3, TLR7 and TLR9, all localized in the endolysosomal compartments.[7] TLR3 is expressed by conventional dendritic cells (DCs) and macrophages as well as non-immune cells including fibroblasts, epithelial and skeletal muscle cells;[8] TLR7 and TLR9 are expressed by myeloid (mDCs) and plasmacytoid DCs (pDCs), these last considered the main IFN-α source.[9]

Microarray studies assessing the pattern of muscle gene expression in IIM patients showed that most of the highest differentially expressed gene (DEG) transcripts belong to a common pathway of type I and II IFN-inducible genes, especially in DM.[10-12] We, therefore, investigated the expression of TLRs associated with IFN-response in

IIM muscle biopsies, to better characterize the relationship between innate immune system and IIM pathological alterations. Our results indicate that type I IFN-mediated molecules are key players in IIM muscles, particularly in the juvenile form of DM (JDM). Furthermore, the differential expression of endosomal TLRs in DM and PM muscle tissues may be linked to their different aetiopathological mechanisms in these two disorders.

Materials and methods

Inflammatory myopathy muscle biopsies

Gene expression profiling was performed on muscle biopsies from 3 patients with JDM (mean age 9.3 ± 3.2), 3 with DM (44 ± 23.1), 4 with PM (49.5 ± 20.4) and 5 subjects (43.2 ± 10.5) who had undergone muscle biopsy for diagnosis, but whose clinical, electromyographic, and histological findings showed them to be free of muscle disease (referred as controls). All IIM patients met the diagnostic criteria for either idiopathic DM or PM.[13] No other neurological or immunological diseases were diagnosed in these patients. Muscle histopathology and inflammatory infiltrates were characterized according to a routine diagnostic protocol.[14] All patients and controls received no immunosuppressive drugs before muscle biopsy. For additional molecular and immunohistochemical experiments muscle samples from 37 IIM patients (6 JDM: mean age 8.6 ± 6.3; 13 DM: 57.4 ± 10.6; 18 PM: 54.2 ± 16.1) and 14 controls

(29.7 ± 20.8) were included. Muscle biopsies were obtained by needle biopsy, frozen and stored in liquid nitrogen pending assay. Written informed consent for muscle biopsy for diagnostic purposes and for tissue storage for research purposes was obtained from patients or their parents/guardians, as required by the Ethical Committee of the Foundation Neurological Institute.

Microarrays: target preparation, hybridization, and signal detection

Total RNA from 10 mg of DM, PM and control muscle tissue, was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA yield and integrity were assessed using an Agilent Lab-on-a-Chip Bioanalyzer (Agilent Technologies, Palo Alto, CA). cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen) and a T7-(dT)24 primer (Affymetrix, Wooburn Green, UK). Biotin-lebeled cRNA was produced through *in vitro* transcription of 6 μg of cDNA using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Life Sciences, Farmingdale, NY). The cRNA was fragmented and hybridized together with control targets (according to Affymetrix recommendations) on HG-U133A GeneChips (Affymetrix). The GeneChips were washed and stained using streptavidin phycoerythrin (Molecular Probes, Eugene, OR), and scanned by the Affymetrix Scanner.

Microarrays: data processing, analysis and visualization

Data handling was mainly done using Bioconductor.[15,16] The GC Robust Multi-array Analysis (GCRMA)[17] was employed to calculate probe set intensity. Normalization was performed by a quantile method. The identification of differentially expressed genes was addressed using linear modeling approach (Limma) and empirical Bayes methods[18] together with false discovery rate correction of the *p*-value (Benjamini Hochberg ed) and a *p*-value threshold of 0.01. Functional classifications were based on the Gene Ontology (GO) classification using the R annotation package.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from 10-20 mg of muscle biopsy using TRIzol reagent (Invitrogen) and subjected to DNase I treatment (Ambion, Austin, TX). Random-primed cDNA was prepared using the AffinityScript Multiple Temperature cDNA Synthesis Kit (Stratagene, La Jolla, CA) and stored at -20°C pending PCR amplification.

Semi-quantitative PCR analysis

cDNA (corresponding to 150 ng of total RNA) was amplified in a PCR reaction containing 1X PCR buffer (Finnzymes, Expoo, Finland), 0.1 mM each dNTPs (Applied Biosystems, Foster City, CA), 1 U DynaZyme DNA polymerase (Finnzymes) and 1 μM each of specific primer. Primer sequences for the target mRNA (STAT-1) were designed with Primer3 Software and purchased from NBS

Biotech (Milan, Italy). The PCR primer sequences were as follows: STAT-1 forward 5'-TTCAGAGCTCGTTTGTGGTG-3'; STA-1 reverse 5'-GGTGCCAGCATTTTTCTGTT-3' (fragment length 314 bp). PCR products were run on 2% ethidium bromide-stained agarose gels. β-actin was used as internal control to carry out the normalization for densitometric analysis by ImageJ software (a public domain software from National Institutes of Health).

Real-time quantitative PCR

TLR3 primer/probe sequences were designed using the Primer Express software package (Applied Biosystems): TLR3 forward 5'-CCTGGTTTGTTAATTGGATTAACGA-3'; TLR3 reverse 5'-TGAGGTGGAGTGTTGCAAAGG-3'; TLR3 probe 5'FAM-ACCCATACCAACATCCCTGAGCTGTCAA-3'. Optimal concentrations for TLR3 primers and probe, efficiency and validation experiments were performed following the procedures already described.[19] For MX1, TLR7, TLR9 and IFN-γ, pre-designed functionally tested assays (all from Applied Biosystems) were used: assay ID MX1: Hs00182073_m1; TLR7: Hs00152971_m1; TLR9: Hs00152973_m1; IFN-γ: Hs00174143_m1. Each cDNA sample (corresponding to 50 ng total RNA) was amplified in triplicate using 7500 Fast Real-Time PCR System (Applied Biosystems). For IRF7, MyD88, IFN-α1, quantitative PCR assays were done at least in triplicates using the Platinum TaqDNA Polymerase (Invitrogen) and the SYBR Green I (BioWhittaker Molecular Applications, Rockland,

ME) on a LightCycler Instrument (Roche Diagnostics, Basel, Switzerland). The sequences of the primer pairs used for the quantification of GAPDH, IRF-7, IFN-α1 and IFN-β have been previously described.[20,21] The primers used for mRNA quantification of the following genes were: MyD88_for: 5'-TGGGACCCAGCATTGAGGA-3'; Myd88_rev: 5'-CGCTGGGGCAATAGCAGA-3'. Relative and fold difference expression were calculated using the Ct and $2^{-\Delta Ct}$ method after normalization with human GAPDH.

Immunohistochemistry

For the diagnosis of IIM, cellular infiltrates were characterized by immunohistochemistry on acetone-fixed 6-μm-thick cryosections of muscle biopsy. The following mouse anti-human monoclonal antibodies were used: anti-CD3, anti-CD4, anti-CD8, anti-CD22, anti-CD163, anti- MHC class I and II (all from Dako, Golstrup, Denmark). The Abs were applied for 2 hours at RT in a humid chamber, followed by a 60 min incubation with the DakoCytomation EnVision + System Labelled Polymer-HRP Anti-Mouse or Anti-Rabbit (Dako). Sections were mounted with Bio Mount medium (Bio-Optica, Milan, Italy) and observed under a Zeiss microscope.

Immunohistochemical analysis for the identification of pDCs, TLR3, TLR7 and TLR9 was performed on 2 PM, 2 JDM, 2 DM and 2 control muscle biopsies, using the following primary antibodies: mouse monoclonal anti-CD303 (BDCA-2) (clone AC144, Miltenyi Biotec,

Bergisch Gladbach, Germany); rabbit polyclonal anti-TLR3 (clone H-125), rabbit polyclonal anti-TLR7 (clone H-114) and rabbit polyclonal anti-TLR9 (clone H-100) (all from Santa Cruz Biotechnology, Santa Cruz, CA). The protocol utilized was the same reported above.

Confocal microscopy

For confocal microscopy six μm-thick cryostat cut sections from 2 PM, 2 JDM, 2 DM and 2 control muscle biopsies were prepared and stained with antibodies against TLR3, TLR7, TLR9 (Santa Cruz), CD34, CD68, CD3, CD4, CD8, CD138 (all from Dako), CD1c, BDCA-2 (both from Miltenyi), integrin αVβ3 (Millipore) and MHCdev (Novocastra Laboratories, Newcastle, UK), followed by incubation with Cy2-conjugated goat anti-mouse IgG and Cy3-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Fluorescence images were captured from confocal laser scanning microscope Nikon Eclipse TE2000-E and analyzed with the EZ-C1 3.70 imaging software (Nikon, Tokyo, Japan).

Quantitative analysis

On double immunofluorescence sections stained to reveal the angiogenic marker αVβ3 or regenerating myofibers (MHCdev-positive) and TLR3, single and double positive cells were counted on 3 or 8 adjacent field areas per section from the three different subtypes

of IIM at X40 magnification under the confocal laser-scanning microscope.

Statistical analysis

Data are presented as means ± standard deviation (SD). Analysis of variance (ANOVA) with appropriate post-hoc test (Bonferroni/Dunn's Multiple Comparison) was performed to assess statistical significance of results. Differences were considered significant when $P < 0.05$. Statview 5 for Macintosh (Abacus Concepts, Berkeley, CA) and GraphPad Prism version 4.0 for Macintosh (GraphPad Software, San Diego, CA) programs were used for data elaboration.

Results

Microarray analysis of muscle gene profile distinguished juvenile dermatomyositis from adult dermatomyositis and polymyositis

The hierarchical clustering method applied to our samples yieled a tree structure, which provides a clear difference in gene expression between a group of DM patients (DM1-3) and the other IIM (DM and PM) patients (Fig 1). The group of DM patients showing a gene profile distinct from the other IIM patients was constituted by patients affected by JDM. The most representative GO terms were those related to immune response (GO0006955) and response to viruses (GO0009615): genes, whose expression is induced by IFN and linked to an IFN-mediated antiviral response, were highly transcribed in DM muscles; in our hands this was true mainly for JDM since adult DM patients showed levels similar to those calculated in PM patients (see Table 1 for details). The most up-regulated gene was the interferon-stimulated protein of 15 kDa (ISG15), an ubiquitin-like modifier: 408.27 ± 273.69 fold in JDM compared to 51.37 ± 64.64 in adult DM and 22.1 ± 30.44 fold in PM (Bonferroni's Multiple Comparison test, $P < 0.001$). Interestingly, the second most up-regulated gene is that coding for the interferon-induced protein with tetratricopaptide repeats 3 (IFIT3), a protein up-regulated after IKKε activation in interferon-mediated antiviral immunity.[22] Further robust differences between JDM and DM or PM were observed for the following genes: myxovirus resistance 1 (MX1), interferon-induced protein with

tetratricopeptide repeats 1 (IFIT1), interferon-stimulated transcription factor 3 gamma (ISGF3G), DEAD box polypeptide 58 (DDX58), and interferon-regulatory factor 7 (IRF7). Among the chemokine family regulated by type I and II IFNs, CXCL10 showed the highest expression levels in JDM than in the other forms of IIM; CXCL12, together with the chemokine receptor CXCR4, and PECAM1, were also up-regulated in PM muscle biopsies compared to juvenile and adult DM.

Quantitative RT-PCR validation of differentially expressed genes confirms the importance of IFN-inducible genes and innate immunity in inflammatory myopathy muscles.

We validate some of the DEGs reported in Table 1 by quantitative and qualitative PCR. We focused on the transcripts for IFN-α1, IFN-β, IFN-γ, MX1, IRF7, MyD88 and STAT-1 (Fig 2). IFN-α1 was undetectable in the majority of IIM and control muscle samples, except for three DM patients, one PM and one control in whom IFN-α1 levels ranged from 4.8E-05 to 2.2E-04 (data not shown). IFN-β was expressed in almost all myopathic muscles, especially in JDM, and in one control at a very low level. IFN-γ showed high expression levels in all IIM muscles, particularly in PM samples. We observed high levels of MX1 expression in all IIM muscles (DM, adult and juvenile: 9.2 ± 11.9; PM: 3.9 ± 5.8; control 0.2 ± 0.2); in particular, MX1 resulted strongly up-regulated in the muscles of patients affected by JDM (JDM: 14.3 ± 17.7 versus adult DM: 6.1 ± 5.9). Furthermore,

JDM muscles showed higher expression levels of IRF7 (one important interferon regulatory factor) [23] in respect to PM and control samples.

MyD88, the universal adapter protein used by all TLRs (except TLR3) to activate the transcription factor NF-κB, was over-expressed in all myopathic samples compared to controls, with no remarkable differences among the subtypes of IIM. Finally, by semiquantitative PCR we investigated the expression levels of the signal transducer and activator of transcription 1 (STAT-1), the main transcription factor involved in the cell response to IFN-α and IFN-γ. Densitometric analysis showed an up-regulation in the expression of STAT-1 in all IIM samples compared to controls, although without no statistically significant difference (Figs 2B and C).

Upon up-regulation of IFN-inducible genes was confirmed, we focused on the analysis of those genes known to trigger a type I IFN response: TLR3, TLR7 and TLR9. These molecules were up-regulated in all IIM muscles compared to controls (Fig 2A). In particular, TLR3 and TLR7 transcript levels showed a significant increase in JDM and adult DM than in PM and controls, while the expression levels of TLR9 were comparable among all IIM subtypes, with statistically higher values in PM muscles ($p < 0.05$).

TLR3 is expressed by the endothelial cells of blood vessels, by mononuclear infiltrating cells and by regenerating myofibers both in DM and PM muscles.

The immunohistochemical analysis showed a strong positive staining for TLR3 in all the IIM muscle biopsies (Fig 3A-I) compared to control samples (Fig 3J-L). In JDM and adult DM muscle biopsies, TLR3 was mainly localized on the $CD34^+$ vascular endothelial cells (both on large vessels and capillaries) and on some myofibers (Fig 4). In JDM muscles, a high proportion of $TLR3^+$ fibers in the perifascicular atrophy areas were $MHCdev^+$ (83.2%) (Table 2 and Fig 5), indicating that they are regenerating fibers. Among muscle infiltrating cells, $CD3^+$ T cells, $CD68^+$ macrophages and $CD1c^+$ mDCs were all positive for TLR3 (data not shown). Although $BDCA-2^+$ pDCs did not express TLR3 on their surface, they were interspersed in muscle infiltrating cells positive to TLR3, suggesting a spatial correlation between pDCs and $TLR3^+$ cells (Fig 6).

In PM biopsies, TLR3 staining was particularly intense among infiltrating mononuclear cells (Fig 3) ($CD3^+$ T cells, both on $CD4^+$ and $CD8^+$ cells, $CD68^+$, $CD1c^+$) and on some $MHCdev^+$ myofibers (Fig 5 and Table 2).

TLR3 might be involved in the angiogenic process occurring in DM muscle biopsies

CD34 represents a classical marker for endothelial cells of blood vessels, whose expression is also associated with hematopoietic stem

cells (HSCs). As previously reported,[24,25] there is a significant up-regulation of several pathways that initiate angiogenesis in myositis, especially in DM. To investigate a possible correlation between TLR3 expression and the new blood vessel formation, we stained 6 frozen muscle sections (2 DM, 2 PM and 2 control samples) with the antibody specific for αVβ3, a neoangiogenesis marker. By confocal microscopy, we observed numerous capillaries and large blood vessels strongly stained for αVβ3 in the diseased muscles: DM 59.3 ± 0.59 versus JDM 56.7 ± 8.62 (P = 0.6213), and versus PM 12 ± 7.81 (P = 0.0005) (Fig 7). In all IIM muscle biopsies a high percentage of the $\alpha V\beta 3^+$ blood vessels co-expressed TLR3 antigen (Table 2). Control tissues showed occasionally $\alpha V\beta 3^+$ large vessels but on the whole they were negative for the angiogenic marker.

TLR9 and TLR7 staining in inflammatory myopathies

Among IIM subtypes, TLR9 showed a strong positive signal mainly in PM biopsies whereas it was weak or absent in control muscle specimens (Fig 8). In PM muscles, TLR9 localized within muscle infiltrating mononuclear cells, in particular on DCs (both $CD1c^+$ mDCs and $CD303^+$ pDCs), macrophages ($CD68^+$) and some T cells ($CD3^+$) (Figs 8 and 9). Numerous myofibers, both small and medium in diameter, were $TLR9^+$; they were surrounded by inflammatory and endothelial cells expressing both HLA-ABC and HLA-DR antigens (Fig 10). Co-localization between TLR9 and HLA-ABC/HLA-DR

was observed at the level of sarcolemma. Among the regenerating myofibers, 19% were positive for TLR9 (Fig 10 A-C).

Since previous studies demonstrated the presence of plasma cells within muscle in PM patients,[26] and due to the importance of these cells for the secretion of immunoglobulins, activation of a humoral immune response and their ability to express TLR9, we investigated the presenceof $CD138^+$ cells in PM muscle specimens and their relationship with TLR9 expression. By double immunofluorescence, we observed several plasma cells, mainly localized in the endomysial space or close to microvessels, 64% of which were $TLR9^+$ (Fig 9 M-O).

As for TLR9, positivity for TLR7 was observed mainly in PM muscle specimens, particularly at the level of the endomysial space in correspondence of infiltrates and occasionally on myofibers, in the areas where immune cells infiltrate muscle tissue; control muscle biopsies showed weak or no positivity for TLR7 (Fig 11). Among infiltrating cells, mDCs ($CD1c^+$), macrophages ($CD68^+$) and rarely T lymphocytes ($CD3^+$) were positive to TLR7 staining (Fig 12); double immunofluorescence for TLR7 and CD138 plasma cells showed that 28.5% of cells were double positive (Fig 12 J-L).

Discussion

The transcriptome analyses of DM and PM muscle biopsies confirmed a role for innate immunity in IIM pathogenesis, in particular for

interferon-α/β-mediated immune response.[10-12,27,28] The most expressed gene was ISG15, encoding an ubiquitin-like modifier inducible by type I IFNs[29] followed by IFIT3, MX1 and IFIT1, genes encoding three proteins known to be associated with ISG15.[30] All these molecules reached the highest levels of expression in JDM muscles and to minor extent in adult DM. Recently Salajegheh et al.[12] observed in DM muscles a cluster of highly up-regulated type I-IFN-inducible transcripts, including ISG15 and two members of the ISG15 conjugation pathway. Our study have partially confirmed these data since ISG15 was found over-expressed in juvenile and adult DM muscles but was detectable also in PM muscles. Salajegheh et al.[12] speculated that in DM muscle fiber abnormalities might result by an altered ISG15 conjugation pathway and that type I IFN might be responsible for the ISG15 overproduction, although no increase in IFN-α or -β transcripts was detected. Our data support a contribution of type I IFNs in IIM, particularly in DM; we were unable to detect IFN-α in IIM muscles, thu7s confirming Salajegheh et al. Observations,[12] but IFN-β transcripts were found up-regulated in IIMmuscles, particularly in DM patients; however, as a difference with Salajegheh et al.,[12] we were able to detect a 3.5-fold increase of IFN-β in JDM versus DM and also differential expression between DM and PM. These results fit well with an involvement of these molecules in the generation of the perifascicular atrophy and the different expression profiles, observed in different populations, might result by different genetic background or environmental pressure.

STAT-1 transcript levels were increased in IIM, but no statistically significant differences were found between patients and controls. Among IIM subtypes, PM showed a 1.1-fold and 1.2-fold increase of STAT-1 expression versus JDM and adult DM, respectively; this was probably due to the local IFN-γ secretion (confirmed also by real-time PCR) by the muscle infiltrating $CD8^+$ T lymphocytes and macrophages, as previously reported.[31]

Our results are relevant for the comprehension of the pathogenetic mechanisms underlying DM and PM and highlight the differences between juvenile and adult DM and between DM and PM. Type I IFNs and proinflammatory cytokines are produced by specialized immune cells in response to pathogens through the engagement of TLRs, RIG-I-like helicases (RLHs) and nucleotide-oligomerization domain (NOD)-like receptors (NLRs).[8] Analysis of our gene profilingt data revealed that molecules involved in TLR (see Table 1) and RLH pathways (data not shown) were differentially expressed in IIM muscles. Among TLRs, TLR3, TLR7 and TLR9 are the most potent promoters of type I IFN production. We therefore analyzed their expression in IIM and control muscles both on muscle fibers and immune infiltrates. TLR3 expression was increased in all IIM muscles, with respect to control muscles (Fig 3). Schreiner et al.[8] reported TLR3 expression only in muscle fibers of patients with idiopathic IBM or IBM/PM associated with HIV infection. Our data showed a differential TLR3 positive staining in relation to the different diagnosis: in PM TLR3 was mainly expressed on muscle

infiltrating cells and on some myofibers, whereas in juvenile and adult DM a strong immunostaining was observed on muscle fibers and on vascular endothelial cells of small and large blood vessels (Fig 4). Based on these findings several hypothesis on the role of TLR3 in DM was formulated: *i)* TLR3 expression on endothelial cells may be directly induced by a viral pathogen and trigger an immune response4; *ii)* TLR3 expression may be secondary to the activation of the type I IFN pathway and induce an antiviral response at the level of the endothelium; *iii)* RNA from damaged cells may be recognized by TLR3 and trigger an immune response without the presence of viral antigens; *iv)* TLR3 expression may be secondary to oxidative stress, generated by ischemia/reperfusion; *v)* TLR3 may participate in an angiogenic process within muscle tissue. This last hypothesis was very controversial: several studies sustain an anti-angiogenic activity of TLR3, through the production of IL-12, IFN-γ and the CXCR3 ligands (i.e. CXCL9, CXCL10 and CXCL11),[39,40] other studies indicate that TLR3 activation may result in a repair process by enhancing the production of pro-angiogenic, anti-inflammatory, anti-fibrotic and chemotactic mediators (such as IL-9, IL-10, IL-11 and Viperin/cig5) that support regenerative responses.[32] Recently, Greenberg[35] revisited the theory on DM perifascicular atrophy caused by ischemia following capillary injury, suggesting that capillary and muscle fiber damages can be caused by a chronic intracellular overproduction of IFN-α/β-induced proteins. Our findings reinforce this hypothesis and the distribution of TLR3 throughout the muscle

might explain why capillaries are affected along the entire fascicle and not only at the perifascicular areas (as questioned by Hohlfeld et al.)[36]. It remains to understand if TLR3 activation precedes or follows new vessel formation in myopathic tissues and what is its exact role in the angiogenic context.As previously demonstrated,[24] neoangiogenesis is highly activated in DM muscles, likely not only by traditional pro- and anti-angiogenic genes, but also by genes involved in leukocyte trafficking and complement activation. Accordingly, our microarrays data showed that many genes implicated in angiogenesis and dendritic cell migration, (i.e. CXCL10, CXCL12, CXCR4, PECAM), were up-regulated in IIM muscles compared to controls. Interestingly, PM samples showed the highest expression levels for the pro-angiogenic genes (i.e. CXCL12, PECAM and CXCR4), while in DM muscles, particularly in those affected by the juvenile form of disease, we observed the overexpression of some important anti-angiogenic genes, including CXCL10.

Interestingly, we found that TLR3 was expressed on most regenerating muscle fibers in all the myopathic samples analyzed (Fig 5). In JDM a proportion of $TLR3^{+}$ $MHCdev^{+}$ muscle fibers were located in the perifascicular atrophic areas, supporting the idea of a regulatory role of TLR3 in the regeneration or differentiation mechanisms in injured muscle tissue. However, contrasting results about this matter have been reported and the question remains open.[37-39]

Immunohistochemistry performed on PM muscle biopsies showed TLR3 positivity either on some myofibers and on mononuclear infiltrating cells. Among these immune cells, not only $CD4^+$ T helper cells but also activated $CD8^+$ T cells expressed TLR3. Tabiasco et al.[40] demonstrated that TLR3 is a functional co-receptor of human effector $CD8^+$ T lymphocytes. Triggering of TLR3 on $CD8^+$ T cells might constitute, in IIM muscle tissue, an efficient amplification system for the overall antiviral effect mediated by Ag-specific CTL responses, enhancing the IFN-γ production (as observed by real-time PCR, see Fig 2A) and activating cytotoxic immune response.

Besides TLR3, the TLRs involved in the production of IFN-α/β are TLR7 and 9, strongly expressed by plasmacytoid and conventional DCs, and macrophages.[9] Numerous evidences indicate a potential role of TLR7 and TLR9 in the pathogenesis of a wide range of immune-mediated inflammatory disorders, such as systemic lupus erythematosus, and psoriasis.[9] Greenberg et al.[11,41] showed high concentration of pDCs in perifascicular areas of DM muscle tissue and of mDCs around or inside nonnecrotic myofibers in PM/IBM muscles. In view of this, we investigated TLR7 and TLR9 expression in the different subtypes of IIM. We found that TLR7 and 9 transcripts were up-regulated in all the IIM muscles compared to controls, in particular in adult DM (Fig 2A), results confirmed by immunohistochemistry. Among IIM subtypes, we observed a preponderance of TLR7 and TLR9 positive staining in PM muscle biopsies, probably due to the highest number of infiltrating immune

cells in PM than in DM, including pDCs, mDCs, macrophages and plasma cells. As for TLR3, we found that TLR9 was expressed on some regenerating myofibers (Fig 10 A-C), suggesting its possible involvement in muscle regeneration.

According to Greenberg et al.,[10] our microarray data showed upregulation of transcripts for immunoglobulin genes in all PM muscle biopsies compared to other IIM subtypes and control samples (data not shown). As suggested by our immunohistochemical analysis, production of autoantibodies in PM might be due to the abundance of plasma cells among immune infiltrateswithin muscle tissue. As previously demonstrated,[42] maturation of naive B cells into memory cells or in plasma cells might be sustained by stimulation of TLR7 and TLR9 through a MyD88-dependent signaling pathway. Additionally, neither BCR activation nor CD4 T cell-derived costimulation via CD40 ligand nor T-cell deived cytokines was required for Ig production by naive B cells.[42] Moreover, activation of TLR9 on pDCs, whose presence, although in a minor extent than in DM, was shown in PM by double immunofluorescence, further amplifies the susceptibility to autoimmunity, increasing IFN-α/β and subsequent IgM production. A role for TLR9 in antigen presentation might also be suggested by its relationship with HLA I^+ and II^+ infiltrates, as observed in our immunohistochemical stainings (Fig 10 D-I).

In conclusion, type I IFNs are mainly associated with JDM although they can be detected also in adult DM and PM; this indicates that also in IIMs a link between innate and adaptive immune responses is

necessary to induce pathological alterations. Furthermore, elements of the innate immunity, such as TLRs, may have a pleiotropic and diversified functional activity in the context of the different pathological alterations characteristic of the different forms of IIM. Finally, our results indicate type I IFNs, type I IFN-induced genes and TLRs as new possible therapeutic targets for the treatment of IIMs.

TABLES

Table 1. Interferon-α/β-inducible genes differentially expressed in muscle tissue of inflammatory myopathy patients compared to controls

Affy ID	Symbol	JDM	DM	PM	Gene description
205483_s_at	ISG15	408.3±273.7	51.4±64.6	22.1±30.4	ISG15 ubiquitin-like modifier
204747_at	IFIT3	261.5±191.6	23.2±19.8	9.9±8.5	Interferon-induced protein with tetratricopeptide repeats 3
204533_at	CXCL10	127.8±78.1	34.2±26.2	62.9±69.6	Chemokine (C-X-C motif) ligand 10
202086_at	MX1	99.1±110.9	14.1±16	4.2±3.1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)
203153_at	IFIT1	62.4±41.6	7.9±5.4	3.3±1.4	Interferon-induced protein with tetratricopeptide repeats 1
203882_at	ISGF3G	55.2±24.6	17.5±4.4	13.8±7.5	Interferon-stimulated transcript factor 3, gamma 4
202269_x_at	GBP1	52.5±37.4	18.8±15.3	19.8±19.5	Guanylate binding protein 1, interferon-inducible, 67
218943_s_at	DDX58	49.8±33.7	5.7±4.1	2.7±2.2	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58
202953_at	C1QB	45.4±55.9	16.5±12.7	25.6±7.8	Complement component 1, q subcomponent, B chain
208436_s_at	IRF7	37.8±25	3.2±2.6	3±2.6	Interferon regulatory factor 7

AFFX-HUMISG	STAT1	34.5±9.8	18.4±14.8	20.6±15.4	Signal transducer and activator of transcription 1
214022_s_at	IFITM1	33.4±18.2	8.5±6.8	6.1±2.3	Interferon induced transmembrane protein 1
209417_s_at	IFI35	33.3±17.4	4.5±0.8	5.2±4.8	Interferon induced protein 35
215049_x_at	CD163	29.9±44.8	4.5±3.1	21.1±16.2	CD163 molecule
202411_at	IFI27	27.8±12.5	6.9±1.6	7.9±5.8	Interferon, alpha-inducible protein 27
218232_at	C1QA	25.3±32.6	8.8±9.0	19.3±8.8	Complement component 1, q subcomponent, A chain
202307_s_at	TAP1	25.0±3.9	17.4±8.9	10.8 ± 6.2	Transporter 1, ATP-binding cassette, subfamily B (MDR/TAP)
208451_s_at	C4B	24.6±11.8	4.4±1.5	8.8±5.5	Complement component 4B (Child blood group)
201601_x_at	IFITM1	21.8±11.7	4.8±2.9	2.2±0.8	Interferon induced transmembrane protein 1
201743_at	CD14	17.7±21.9	3.6±0.2	13.6±5.6	CD14 molecule
206584_at	LY96	17.3±10	5.1±4.3	17.9±7.6	Lymphocyte antigen 96
212203_x_at	IFITM3	16.3±8.7	4.6±2.2	4±1.1	Interferon induced transmembrane protein 3
203595_s_at	IFIT5	15.6±8.5	2.3±0.0	2.6±0.8	Interferon-induced protein with tetratricopeptide repeats 5
201422_at	IFI30	14.9±8.5	5.1±2.6	10.6±4	Interferon, gamma-inducible protein 30

202531_at	IRF1	14.0±10.4	6.8±5	11.6±11.6	Interferon regulatory factor 1
201315_x_at	IFITM2	9.6±2.7	3.3±1.9	2.9±0.9	Interferon induced transmembrane protein 2
206332_s_at	IFI16	8.0±2.4	2.8±0.3	6.1±3.1	Interferon, gamma-inducible protein 16
202748_at	GBP2	7.2±4.7	4.9±2.9	9.1±6.0	Guanylate binding protein 2, interferon-inducible
215051_x_at	AIF1	6.4±4.7	3.3±1.5	8.5±2.9	Allograft inflammatory factor 1
208747_s_at	C1S	5.9±1.5	3.3±0.0	6.2±2.2	Complement component 1, s subcomponent
209732_at	CLEC2B	5.6±3	2.3±0.8	5.5±1.1	C-type lectin domain family 2, member B
214329_x_at	TNFSF10	4.9±1.7	1.8±0.2	1.9±1	Tumor necrosis factor (ligand) superfamily, member 10
209124_at	MYD88	4.1±0.7	1.7±0.2	2.6±0.5	Myeloid differentiation primary response gene (88)
200986_at	SERPING1	3.8±1.2	2.0±0.3	2.2±0.8	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1,(angioedema, hereditary)
208829_at	TAPBP	3.1±1.2	2.5±0.6	2.5±0.9	TAP binding protein (tapasin)
209687_at	CXCL12	3.1±1.4	2.3±0.7	4.7±1.9	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
217871_s_at	MIF	2.9±0.7	1.9±0.3	3.6±1.7	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)
202948_at	IL1R1	2.7±1.6	1.6±0.1	3.0±1.3	Interleukin 1 receptor, type 1

201587_s_at	IRAK1	2.4±0.7	1.4±0.1	2.5±0.4	Interleukin-1 receptor-associated kinase 1
208982_at	PECAM1	2.4±0.5	1.7±0.4	2.5±1	Platelet/endothelial cell adhesion molecule (CD31 antigen)
213699_s_at	YWHAQ	2.3±0.6	2.0±0.4	3.6±1.5	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta
200052_s_at	ILF2	2.3±0.6	1.9±0.4	2.1±0.5	Interleukin enhancer binding factor 2
202491_s_at	IKBKAP	1.9±0.3	2±0.0	1.5±6	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein
212271_at	MAPK1	1.9±0.3	1.5±0.1	2.5±0.8	Mitogen-activated protein kinase 1
217028_at	CXCR4	1.2±0.6	1±0.1	3.5±2	Chemokine (C-X-C motif) receptor 4

Mean value ± standard deviation of the fold-change of differentially expressed genes in juvenile dermatomyositis (n = 3), adult dermatomyositis (n = 3) and polymositis (n = 4) patients compared with controls (n = 5).

JDM = juvenile dermatomyositis; DM = adult dermatomyositis; PM = polymyositis.

Table 2. Results of double-immunofluorescence analysis for MHCdev and integrin αVβ3, and TLR3 in IIM muscle specimens

	JDM	**DM**	**PM**
REGENERATION			
MHCdev+ cells[a]	12.4±7.1	4.9±2.8	3.8±2.1
TLR3+ cells[a]	12.2±6.1	1.1±1.3	1.9±1.6
Co-localization (%)[b]	83.2±36.1	26.3±31	41±36.1
ANGIOGENESIS			
αVβ3+ cells[a]	56.7±8.6	59.3±0.6	12±7.8
TLR3+ cells[a]	53.3±11.9	39.3±5.1	31.7±3.2
Co-localization (%)[b]	40.1±5.4	46.6±5.6	92.7±6.5

Single (MHCdev-, αVβ3- or TLR3-positive) and double (MHCdev-, αVβ3- and TLR3-positive) cells were counted on 8 adjacent field areas per section from each muscle biopsy as described in Material and Methods.

[a] The mean number of MHCdev+ or αVβ3 or TLR3+ cells per field area of the muscle section with the relative standard deviation is reported.

[b] The mean percentage of MHCdev+ myofibers or αVβ3+ blood vessels expressing TLR3 per field area of the muscle section with the relative standard deviation is reported.

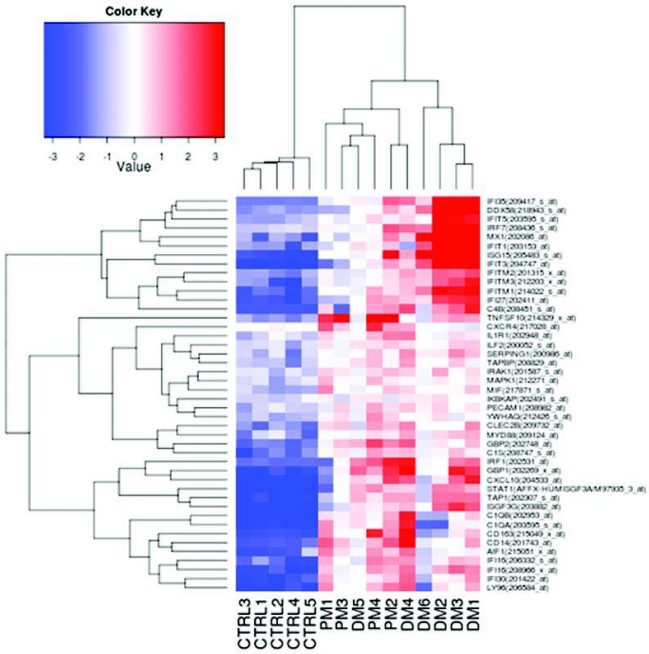

Fig. 1

Figure 1. Heat map. The ratio of the log2 expression value and the median value of each probe is reported. Red color indicates up-regulated and blue color down-regulated probes. Probe sets (rows) and samples (columns) are clusterized based on their similarity by hierarchical clustering using complete linkage (Euclidean distance).

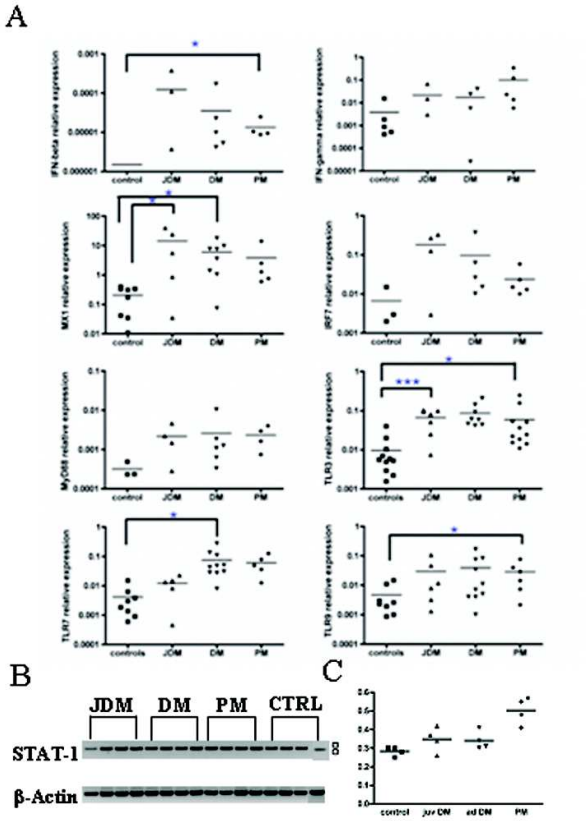

Fig. 2

Figure 2. Quantitative real-time PCR and semiquantitative PCR of some of the most expressed genes obtained with microarray technique and of endosomal TLRs. (A) Type I (IFN-β) and II (IFN-γ) interferon expression levels are higher in IIM muscle biopsies than in control samples; a statistically significant difference is observed for PM muscles (versus controls p = 0.0182, *). Other genes related to type I IFNs, such as IRF7 and MX1, show high levels of expression

mainly in the juvenile form of DM (JDM); in particular, MX1 transcript levels are statistically different compared to control samples ($p = 0.0407$, *). Among endosomal TLRs, TLR3 and TLR7 are strongly up-regulated in DM muscle biopsies (juvenile and adult) compared to PM or control muscles. TLR3 transcript levels are increased in a statistically significant manner in JDM ($p = 0.0003$, ***) and adult DM ($p = 0.0139$, *) versus controls, while TLR7 shows higher expression levels in adult DM ($p = 0.0249$, *) than in controls. An increase in TLR9 expression level was observed in all IIM muscles with a statistically significant difference in PM muscle biopsies versus controls ($p = 0.0337$, *). No significant differences were observed in the expression of the adapter molecule MyD88 between IIM and control muscles. (B) Gel electrophoresis of 314-bp amplification products of STAT-1, the main transcription factor of the type I and II interferon-mediated pathway, from 4 JDM, 4 DM, 4 PM and 4 control muscle biopsies. β-actin (234 bp) was amplified for RNA integrity and retro-transcriptiion efficiency. (C) Densitometric analysis shows an up-regulation for STAT-1 in all the myopathic muscles compared to controls. In particular, PM showed the highest values of expression for the transcription factor, although without any statistically significant difference.

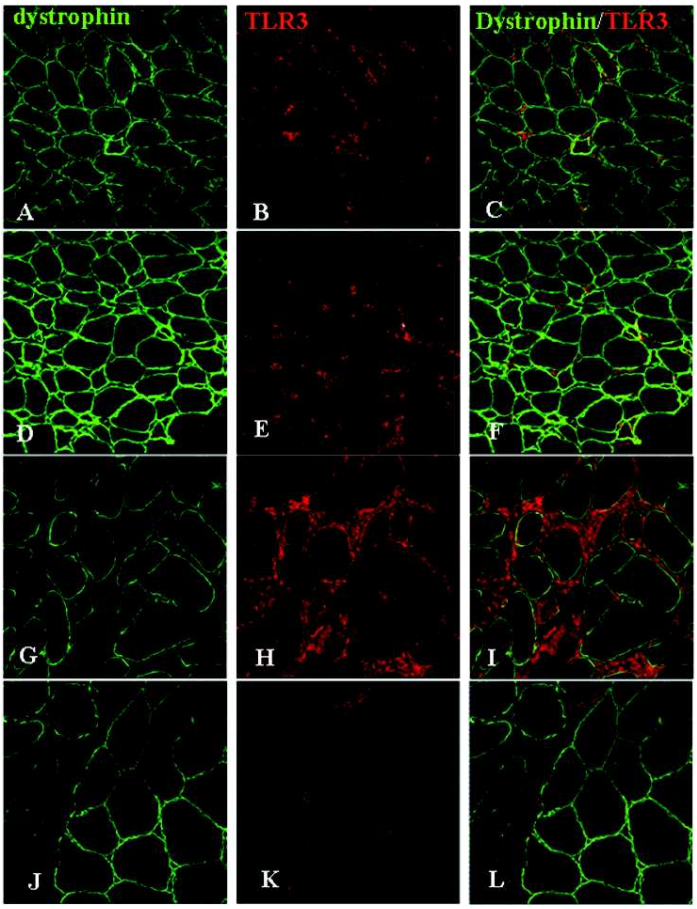

Fig. 3

Figure 3. Detection of TLR3 protein in muscle biopsy specimens from IIM patients and control subjects. (A-L) Double immunofluorescence for dystrophin (green) and TLR3 (red). TLR3 expression shows different patterns of localization among the subtypes of myopathies, with a positive staining at level of the vascular endothelium of capillaries and large vessels in juvenile (A-C) and adult (D-F) DM and of infiltrating mononuclear cells in PM (G-I).

Immunopositivity for TLR3 was also found on sarcolemma or cytoplasm of some myofibers in all muscle tissues analyzed, included the controls (J-L). Original magnification: 40X.

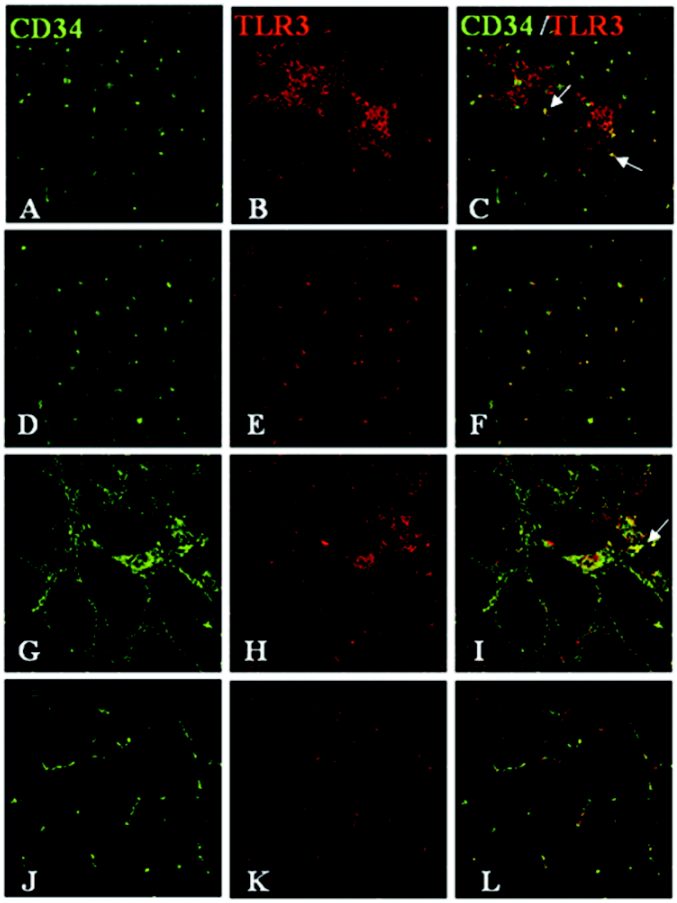

Fig. 4

Figure 4. Expression of TLR3 on vascular endothelium in IIM and control muscle biopsies. Endothelial cells of capillaries and large vessels show a strong positive staining for $CD34^{+}$ (green) in myopathic (A-I) and control muscles (J-L). Vascular endothelium of DM muscle tissues, juvenile (A-C) and adult (D-F) forms, and PM specimens (G-I) are positively stained also for TLR3 (red) (arrows in

C and I). A weak positive staining for TLR3 is observed in control sample (K). Original magnification: 40X.

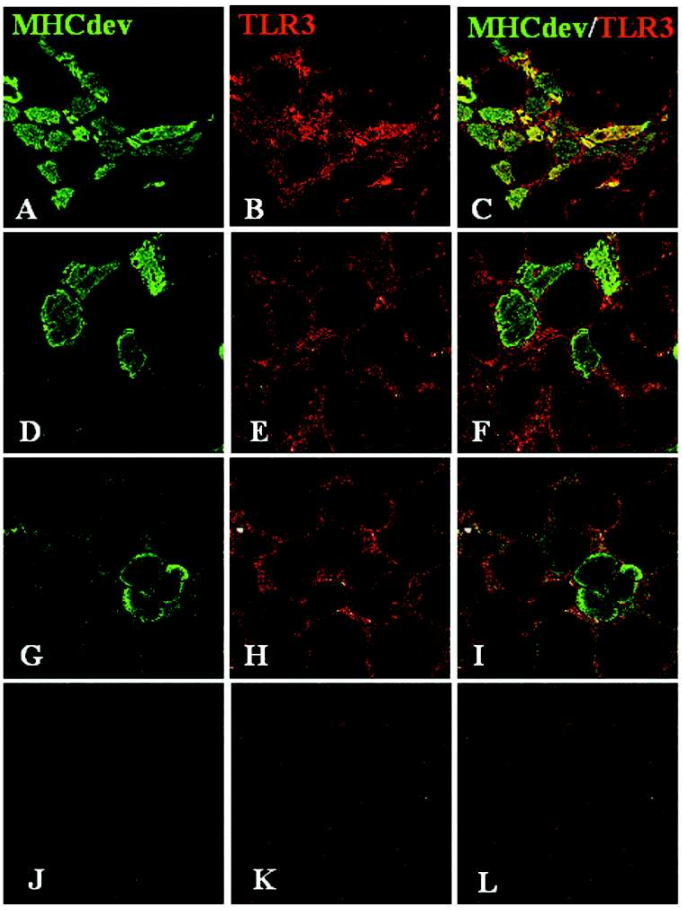

Fig. 5

Figure 5. TLR3 expression on regenerating muscle fibers. (A-L) Muscle biopsy specimens from IIM patients are stained with the anti-developmental myosin heavy chain monoclonal antibody (MHCdev, green) to detect regenerating myofibers. Juvenile DM (A-C) shows the highest number of MHCdev^{+} fibers compared to DM (D-F) and PM (G-I). Many of these muscle fibers are also positively stained for

TLR3 (red). No positive staining for MHCdev or TLR3 was observed in control (J-L). Original magnification: 40X.

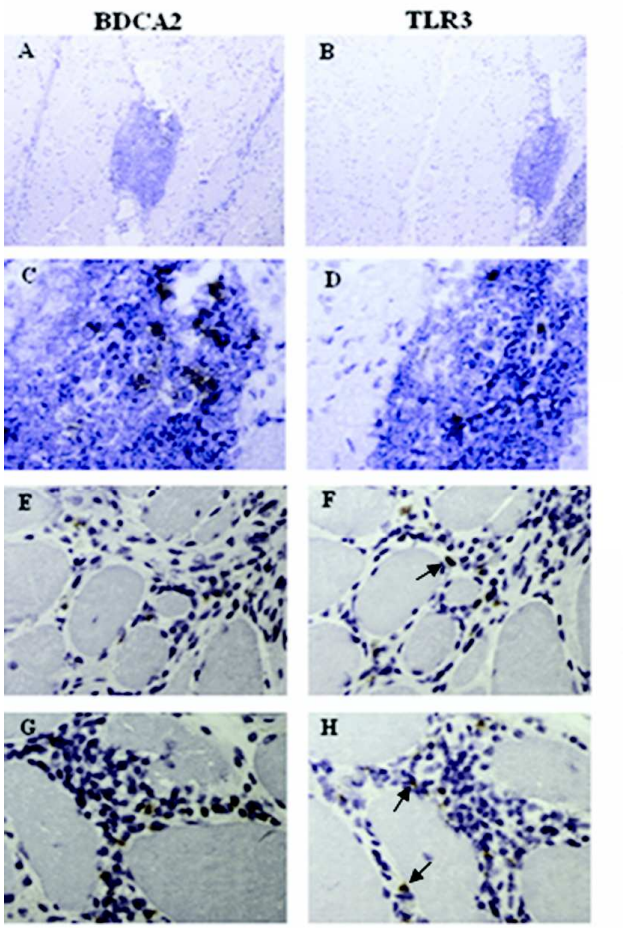

Fig. 6

Figure 6. Detection of plasmacytoid dendritic cells (pDCs) in DM and PM muscle biopsies by immunohistochemistry using BDCA-2 marker. (A, C, E, G). Several BDCA-2^+ cells with oval bodies and short cytoplasmic processes are present in a large cluster of infiltrating mononuclear cells near blood vessels in DM biopsy (A, C). In PM sample, several BDCA-2^+ cells are observed within the infiltrates

surrounding some muscle fibers (E, G). In DM and PM samples in the same area of pDCs numerous $TLR3^{+}$ cells are present (B, D, F, H), and some of them directly contact the muscle fibers (arrows in F and H). Original magnification: 40X.

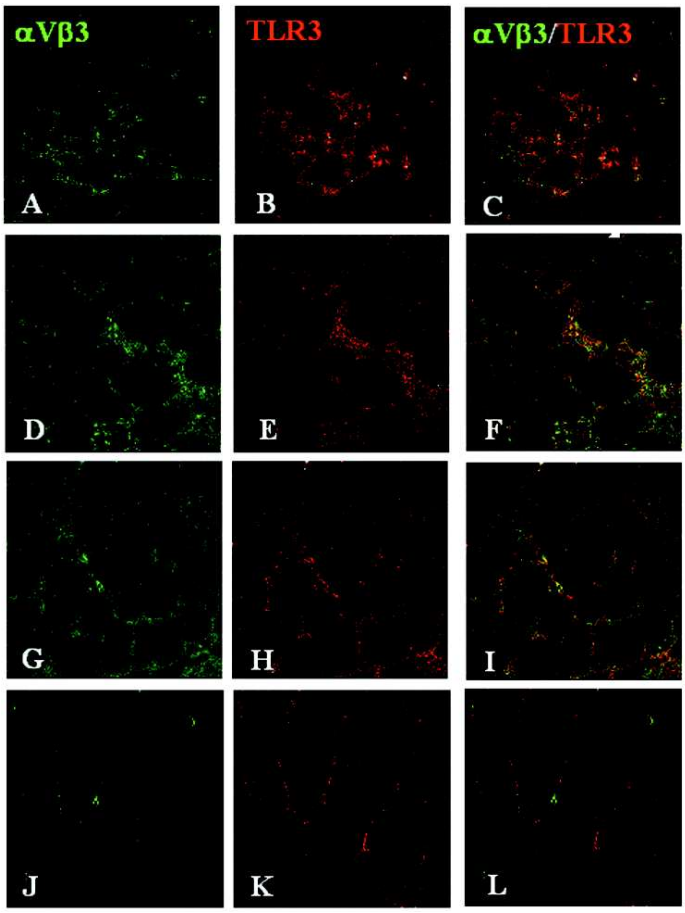

Fig. 7

Figure 7. Detection of the angiogenic marker αVβ3 and TLR3 in IIM and control muscle biopsies. In all samples affected by inflammatory myopathy numerous $\alpha V\beta 3^+$ blood vessels (green), which are positive also for the TLR3 antigen (red), are evident (A-I). αVβ3/TLR3 positivity is particularly evident in the adult form of DM (D-F). Rare $\alpha V\beta 3^+$ blood vessels are observed in control sample (J). Original magnification: 40X.

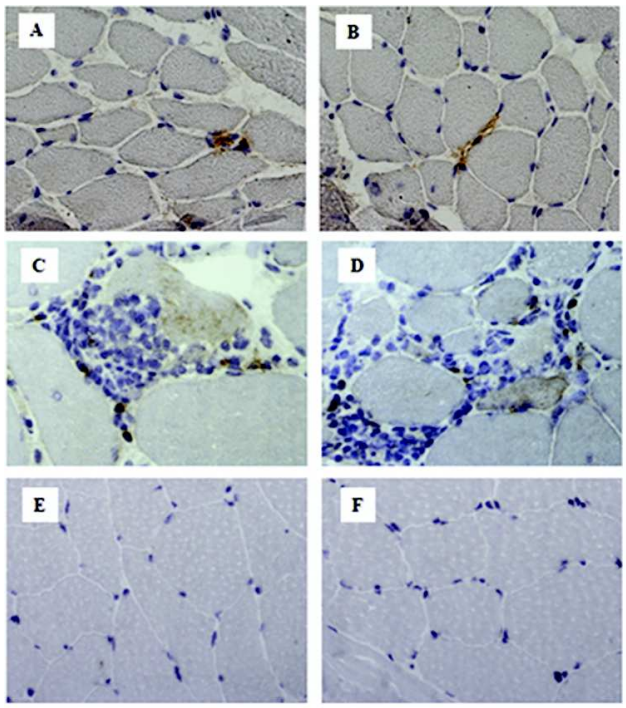

Fig. 8

Figure 8. Detection of TLR9 protein expression in muscle biopsy specimens from IIM patients and control subjects. In JDM (A) and DM (B) few $TLR9^{+}$ cells with irregular bodies are present in the endomysial areas. In PM several $TLR9^{+}$ cells, mainly with plasmacytoid morphology, are detected among immune infiltrates that surround (C) and invade (D) some muscle fibers. No positive staining for TLR9 is detected in control samples (E, F). Original magnification: 40X.

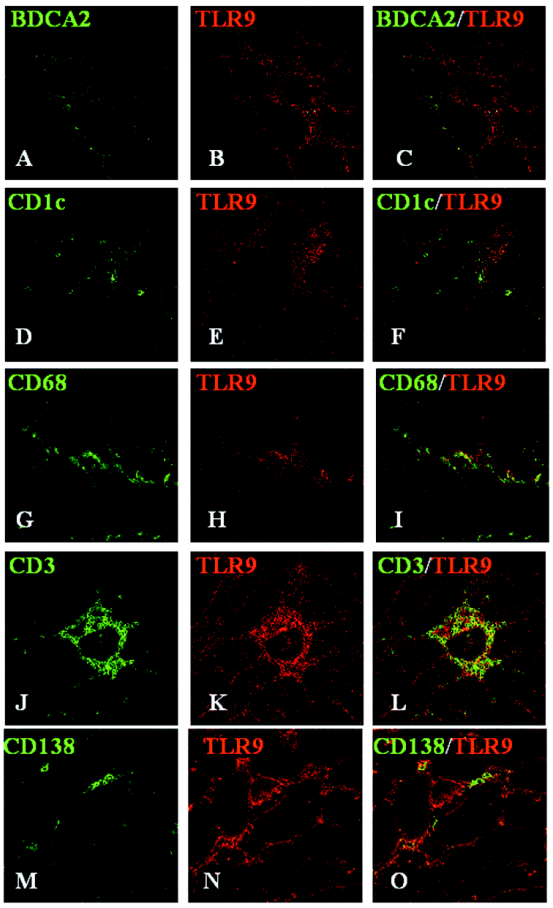

Fig. 9

Figure 9. TLR9 expression on infiltrating mononuclear cells in PM muscle tissue. Double immunofluorescence for BDCA-2 (A, green), CD1c (D, green), CD68 (G, green), CD3 (J, green) and CD138 (M, green), and TLR9 (B, E, H, K, N, red) show that TLR9 is mainly localized on macrophages ($CD68^{+}$), plasmacytoid dendritic cells ($BDCA\text{-}2^{+}$) and plasma cells ($CD138^{+}$). Original magnification: 40X.

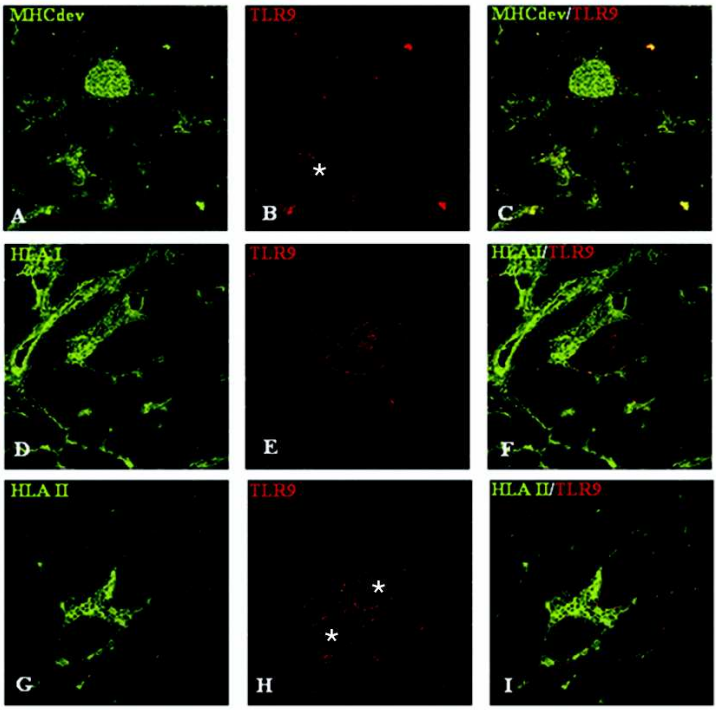

Fig. 10

Figure 10. Confocal analysis of TLR9 expression on regenerating myofibers and HLA I and II expression in PM muscles. (A) Myofibers, in particular those with small diameter, positively stained for the regeneration marker MHCdev (green) co-express the TLR9 antigen (B, red, asterisk). TLR9 is also expressed on the sarcolemma of some muscle fibers surrounded by infiltrates or on blood vessels positive for HLA class I and II antigens (F and I, respectively). In particular, within these muscle fibers, TLR9 is localized in the cytoplasmic areas in close proximity to the invading immune cells (E, H asterisks). Original magnification: 40X.

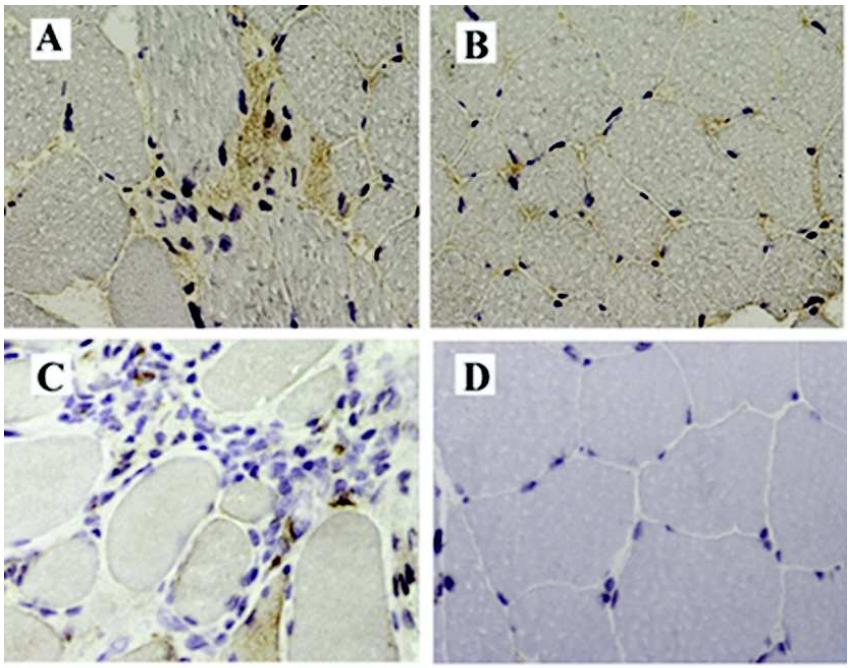

Fig. 11

Figure 11. Detection of TLR7 protein expression in muscle biopsy specimens from IIM patients and control subjects. Several $TLR7^{+}$ cells with irregular bodies are localized among infiltrates, in particular in PM muscle tissue (C). In JDM (A) and PM (C) several muscle fibers are positively stained for TLR7. Adult DM (B) show a weak immunopositivity for TLR7 mainly in the endomysial areas probably in correspondence of capillaries. No positive signal is detected in control (D). Original magnification: 40X.

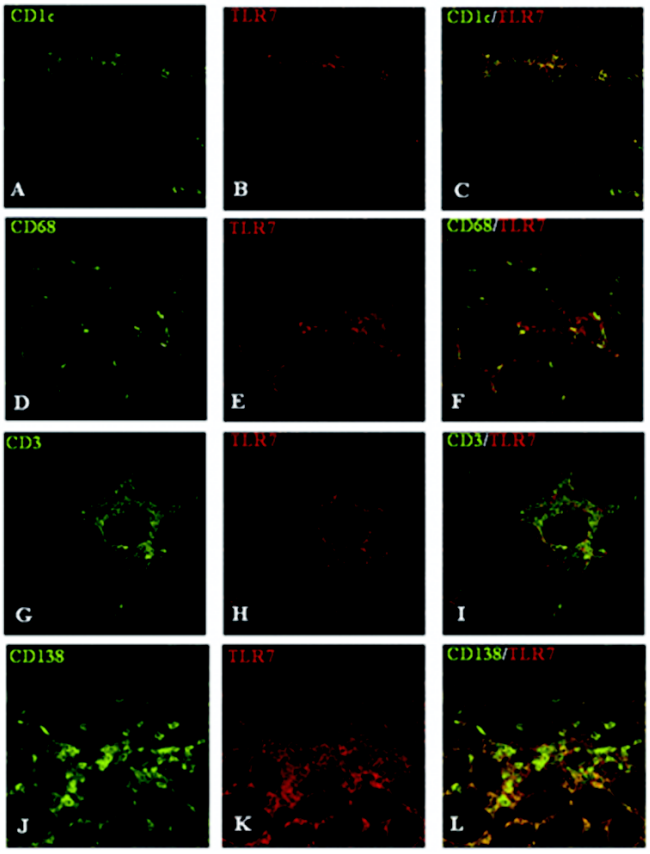

Fig. 12

Figure 12. Confocal analysis of TLR7 expression on various infiltrating mononuclear cells in PM muscle tissue. Myeloid dendritic cells (mDCs) $CD1c^+$ (A, green) and plasma cells $CD138^+$ (J, green) show a staining for TLR7 (C and L, respectively). $CD68^+$ macrophages (D, green) and few $CD3^+$ T cells (G, green) show a double immunoreactivity for TLR7 (F and I, respectively). Original magnification: 40X.

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Chapter 3

"The kinesin superfamily motor protein KIF4 is associated with immune cell activation in Idiopathic Inflammatory Myopathies"

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Abstract

The idiopathic inflammatory myopathies (IIMs) dermatomyositis, polymyositis, and inclusion body myositis are characterized by myofiber degeneration and inflammation. The triggering factors of muscle autoaggression in these disorders are unknown, but infiltrating T cells may be activated locally and proliferate in situ. T-cell polarization involving reorientation of cytoskeleton and microtubule-organizing centers mediated by motor proteins may occur within inflammatory cells in the muscle. We therefore analyzed ubiquitous and neuronal kinesin superfamily (KIF) members KIF5, dynein, and KIF4 in IIM muscle biopsies and in activated peripheral blood lymphocytes from healthy donors. Only KIF4 was altered. Transcript levels were significantly higher in IIM muscle than in controls, and $KIF4^+$ inflammatory cells were found in IIM muscles. In polymyositis and inclusion body myositis, $KIF4^+$ cells were mainly located around

individual muscle fibers, whereas in dermatomyositis, they were also near blood vessels. KIF4^{+} cells were not specific to any immune lineage, and some were Ki67^{+}. In peripheral blood lymphocytes stimulated with mitogens, inteleukin 2 or anti-CD3/CD28 antibodies, KIF4 expression was upregulated, and the protein was localized in the cytoplasm in association with lysosome-associated membrane protein 1^{+} and perforin^{+} lysosomal vesicles. These results imply that KIF4 is associated with activated T cells, irrespective of their functional phenotype, and it is likely involved in cytoskeletal modifications associated with in situ T-cell activation in IIM.

Motor proteins of the kinesin superfamily (KIF) transport organelles, protein complexes, and mRNAs in a microtubule- and adenosine triphosphate-dependent manner from the Golgi apparatus to specific destinations within the cell [1]. During mitosis and meiosis, KIFs are also involved in spindle organization and chromosome movements [2]. In neurons, KIFs are essential for the anterograde transport of various molecules from the cell body to axons and dendrites, whereas the cytoplasmic dynein are minus-end-directed microtubule motors that transport cargoes from axonal or dendritic terminals to the cell body [3]. One of the plus-end-directed microtubule motors, KIF4, was originally characterized as a mitotic motor implicated in chromosome segregation during mitosis [3]. It has been reported, however, to be highly expressed in the developing nervous system and to colocalize with membrane organelles [4,5]. It seems that KIF4 plays a role in the microtubule-based transport of vesicles containing L1, a cell adhesion

molecule, toward the tip of growth cones in developing neurons in culture [6].

Kinesin superfamily protein (KIF) 4 is absent or present at very low levels in most adult tissues: the notable exception is hematopoietic tissues in which KIF4 is abundantly transcribed and synthesized [4,5]. The *Xenopus* protein Xklp1, the homolog of human KIF4, is involved in chromosome segregation during mitosis [7], but it has recently been proposed to be involved in the regulation of microtubule dynamics [7,8]. The accumulating data on KIF4 location in adults and a possible role in microtubule dynamics suggested that it might be involved at certain stages of immune cell activation and/or proliferation. It is known that F-actin and actin-associated proteins are involved in establishing the interaction between the effector T cell and the target cell, but the detailed molecular mechanisms involved in the reorientation of the microtubule cytoskeleton and microtubule-based movement of the microtubule-organizing center to a point on or close to the cell surface, and the detachment of secretory lysosomes from the microtubules with subsequent docking to the lymphocyte membrane during maturation of the immunologic synapse, are poorly understood [9-13]. As in other systems [14,15], these processes are likely to require tight regulation of microtubule dynamics [16] and to involve the concerted action of various microtubule-stabilizing and/or microtubule-destabilizing proteins and microtubules-based molecular motors [3,16].

The idiopathic inflammatory myopathies (IIMs) are a heterogeneous group of subacute/chronic muscle disorders characterized by

inflammation-mediated muscle injury and degeneration. The major forms of IIM are dermatomyositis (DM), polymyositis (PM), and sporadic inclusion body myositis (sIBM) [17]. Evidence supporting a primary autoimmune pathogenesis in PM and DM includes the presence of muscle damage at the endomysium (infiltrating T cells in PM, complement-mediated humoral attack against endothelial cells in DM), a frequent association with other autoimmune diseases, serum positivity for autoantibodies (e.g. anti-synthetase antibodies), and positive responses to immunosuppressive treatments [17]. In sporadic IBM, it is unclear as to whether the immune response is primary or secondary [18]. The triggering factors of myositis and the processes by which the immunologic attack induces muscle weakness are still unknown. Therefore, we investigated possible KIF involvement in immune cell activation by characterizing the expression of KIF4 and other kinesin motors in muscle biopsies from patients with IIM and *in vitro* during activation of peripheral blood lymphocytes (PBLs) from healthy donors.

Material and methods

Subjects

We analyzed muscle specimens from 39 patients with IIM (16 DM, 15 PM, and 8 IBM). Eight patients who had undergone diagnostic muscle biopsy but whose clinical, electromyographic, and histologic findings showed them to be free of muscle disease (hereafter called controls)

were also included. All IIM patients met the diagnostic criteria for idiopathic DM, PM, or IBM (Table) [19,20]. No other neurologic or immunologic diseases were diagnosed. Muscle histopathology and inflammatory infiltrates were characterized by a routine diagnostic protocol [21]. Consecutive transverse muscle sections, stained to reveal infiltrating cell phenotype (CD4, CD8, macrophages, and B-lymphocytes), direct cytotoxicity, and upregulation of major histocompatibility complex (MHC) Class I, were analyzed at 10x or 20x magnification as described elsewhere [21]. Immunosuppressive drugs had been given to 2 patients only (1 DM and 1 PM) and to no controls prior to the muscle biopsy. In all cases, the biopsies had been obtained by needle biopsy, frozen in liquid nitrogen-cooled isopentane, and stored in liquid nitrogen. Written informed consent for muscle biopsy for diagnostic purposes and for tissue storage for research purposes was obtained from all patients as required by the ethical committee of the Foundation Neurological Institute.

Cells and Culture Conditions

For isolation of PBLs, heparinized venous blood from healthy volunteer donors was layered over Ficoll Hypaque Plus (Amersham Pharmacia Biotech AB, Piscataway, NJ) and centrifuged. The mononuclear cells thus obtained were washed twice with PBS (pH 7.2) and resuspended in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mmol/L of L-glutamine, 1% penicillin/streptomycin solution, 1% nonessential amino acids, 1 mmol/L of sodium pyruvate

(all from Euroclone, Pero, Italy), and 5 x 10^{-3} mmol/L of 2-mercaptoethanol (BDH Biochemical, Poole, United Kingdom). Cells adherent to plastic dishes, after incubation for 2 hours at 37°C in 5% CO_2, were eliminated. Peripheral blood lymphocytes were seeded at a concentration of 2 x 10^6 cells/ml in medium, supplemented as previously discussed, and incubated with various stimuli: concanavalin A (Con A; 1 μg/ml; Sigma, St. Louis, MO); recombinant human interleukin (IL) 2 (20 U/ml; Roche Diagnostics, Mannheim, Germany), plastic-immobilized anti-CD3 antibody (50-100 μl/well supernatant from clone TR66; American Type Culture Collection, Manassas, VA) alone or in combination with soluble anti-CD28 antibody (clone 37407; 5 mg/ml; R & D Systems, Abingdon, United Kingdom). Time courses of these experiments are reported in the Results section and figures. After incubation, the cells were harvested, centrifuged, and processed for RNA exctraction and immunocytochemistry. Peripheral blood lymphocyte proliferation was assessed by pulsing cells with 1 μCi [^{3}H]thymidine (Amersham Biosciences), and the incorporated radioactivity was determined after an additional 16 hours by liquid scintillation on a β-plate counter (MicroBeta TriLux, EG and G Wallac, Turku, Finland).

Neuroblastoma SH-SY5Y cells (ATCC CRL 2266; American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium High Glucose (Euroclone) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin solution and 1% L-glutamine (all from Euroclone) in a humidified atmosphere of 5% CO_2 at 37°C.

At confluence, the cells were harvested and processed for immunoblot.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from 20 to 30 mg of frozen muscle tissue or 5 x 10^5 PBLs using Trizol reagent (Invitrogen, Carlsbad, CA), followed by DNase I treatment (Ambion, Austin, TX). Random-primed cDNA was prepared using SuperScript II reverse transcriptase (Invitrogen) following the manufacturer's instructions and stored at -20°C pending polymerase chain reaction (PCR) amplification.

Real-time quantitative PCR

The TaqMan primer-probe combinations were designed and tested by Applied Biosystems (Foster City, CA). The assay codes were KIF4A, Hs00602211_g1; ubiquitous KIF5, Hs0018659_m1; neuronal KIF5, Hs00189672_m1; and dynein, Hs00540753_m1. Each cDNA sample (corresponding to 100 ng total RNA) was amplified in triplicate using a 7500 Fast real-time PCR system (Applied Biosystems). Amplification of human glyceraldehydes-3-phosphate dehydrogenase (Applied Biosystems) served as endogenous control for sample normalization; normalized PCR amplification from nonmyopathic muscle cDNA or untreated PBLs served as calibrators. Transcript expression was quantitated by the $\Delta\Delta C_T$ method according to the manufacturer's instructions (Applied Biosystems).

Protein extraction

For protein extraction, 1 x 10^6 PBLs were seeded and maintained in culture for 72 hours in the absence or presence of Con A (1 μg/ml). At the end of this time, PBLs were collected, washed once with PBS, and resuspended in 120 μl of ice-cold RIPA lysis buffer (1% Nonidet P-40; 6.4 mmol/L of deoxycholate; 150 mmol/L of NaCl; 50 mmol/L of Tris-HCl, pH 7.5) containing protease inhibitors (1 mmol/L of sodium orthovanadate, 1 mmol/L of EGTA, 5 μg/ml of aprotinin, 12.5 mg/L of leupeptin, 1 mmol/L of phenylmethylsulfonyl fluoride) for 30 minutes[22]. The lysates were passed through a 29-G needle and centrifuged for 10 minutes at 15,600xg (IEC Micromax microcentrifuge) to pellet the nuclear fraction. The supernatant containing the cytosol fraction was moved to fresh tubes and used for immunoblot. The SH-SY5Y cell line was processed in the same way. Total protein concentrations were determined using the Coomassie Plus-The Better Bradford assay kit (Pierce, Rockford, IL).

Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis and immunoblot

Aliquots (30 μg) of cytosol protein were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 4% stacking gel, and the gels were either stained with GelCode blue stain reagent (Pierce) or electroblotted to nitrocellulose membranes (Bio-Rad, Hercules, CA). Blots were incubated with rabbit polyclonal antibodies: anti-human ubiquitous kinesin heavy chain[23] (dilution,

1:1000), anti-human KIF4 (C-tus), and anti-*Xenopus* KIF4 (Ab03; dilutions, 1:250)[24] overnight at 4°C. After incubation with goat anti-rabbit immunoglobulin G (whole molecule)-alkaline phosphatase (Sigma), blots were washed 3 x 15 minutes in 20 mmol/L of Tris-HCl, pH 7.5, 150 mmol/L of NaCl, 0.1% Tween-20 plus 5% nonfat milk and 2 x 10 minutes in 20 mmol/L of Tris, pH 7.5, 150 mmol/L of NaCl. Immunoblot signals were developed using 1-step nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (Pierce), a 1-component sensitive precipitating alkaline phosphatase substrate. A PageRuler prestained protein ladder, a mixture of 10 recombinant, highly purified, colored proteins with apparent molecular weights of 10 to 170 kd (Fermentas, M-Medical, Florence, Italy), was loaded for monitoring protein separation, Western transfer efficiency, and protein sizes.

Immunohistochemistry

The following mouse anti-human monoclonal antibodies were used: anti-CD3, anti-CD4, anti-CD8, anti-CD22, anti-CD163, anti-MHC Class I, anti-MHC Class II (all from DAKO, Golstrup, Denmark), and anti-Ki67 (DAKO; kindly provided by Dr M. Barberis, MultiLab, Gruppo MultiMedica, Milan, Italy). For KIF4, rabbit anti-*Xenopus* polyclonal antibodies Ab03 and Ab65[24] and the recently available affinity purified goat polyclonal antibody raised against a peptide mapping within an internal region of human KIF4A (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Cryostat consecutive

transverse muscle sections (6-μm thick) were mounted on poly-lysine-coated glass slides (Bio-Optica, Milan, Italy), air-dried for 30 minutes at room temperature, and stored at -80°C until use. Frozen sections were fixed in acetone (-20°C) and rehydrated in PBS for 5 minutes. All stainings was performed using an EnVision+System-HRP (3,3'-diaminobenzidine HCl^+) kit (DAKO). After an endogenous peroxidase-blocking step and preincubation with protein block solution for 30 minutes, primary antibody at appropriate dilutions in PBS was added for 90 minutes, followed by washing in PBS and incubation (1 hour) with secondary antibody at room temperature. After 3 washes with PBS, the slides were stained by 5- to 10-minute incubation with the 3,3'-diaminobenzidine HCl^+ chromogenic substrate system, which produces brown coloration at the antigen site. After extensive washes in distilled water, slides were counterstained with Mayer hematoxylin and mounted by Bio Mount (Bio-Optica).

Confocal microscopy

Unfixed 6-μm-thick cryostat muscle sections and 4 x 10^5 PBLs fixed with paraformaldehyde and permeabilized with 0.1% Triton X-100 were incubated with 10% normal goat serum diluted in PBS for 30 minutes at room temperature to block aspecific sites. Slides were then incubated with anti-KIF4, anti-perforin (Acris Antibodies, Hiddenhausen, Germany), anti-CD3, anti-CD8, anti-CD4, anti-CD163, anti-α-tubulin (Sigma), anti-lysosome-associated membrane

protein 1 or anti-Ki67 primary antibodies and, after 3 washes with PBS, were treated with Cy2-conjugated goat anti-mouse immunoglobulin G and Cy3-conjugated goat anti-rabbit immunoglobulin G secondary antibodies (all from Jackson Immunoresearch Laboratories, West Grove, PA) for 1 hour. Fluorescence images were captured from an EclipseE600 confocal laser-scanning microscope (Nikon, Tokyo, Japan) and analyzed with LaserSharp 2000 software (Bio-Rad).

Statistical analysis

Analysis of variance with post hoc Dunn multiple comparison was used to assess the significance of differences (considered significant when $p < 0.05$). Statview 5 for Macintosh (Abacus Concepts, Berkeley, CA) and GraphPad Prism version 4.0 for Macintosh (GraphPad Software, San Diego, CA) were used for data elaboration.

Results

Expression of KIF4 transcript is upregulated in IIM muscle tissue

Kinesin superfamily protein (KIF) 4 transcript levels were assessed by real-time PCR in muscle tissue. Figure 1A shows that the expression of KIF4 mRNA was significantly higher in IIM than in control muscles ($p < 0.001$ for DM vs controls; $p < 0.01$ for IBM vs controls and PM vs controls). Kinesin superfamily protein (KIF) 4 mRNA levels in the 3 types of IIM were not significantly different. Variations

in KIF4 transcript levels among patients with the same type of IIM were high (4- to 5,500-fold increases). To investigate whether increased mRNA levels in IIM were specific for KIF4, we assessed the expression of ubiquitous KIF5 (Fig. 1B) and of neuronal KIF5 (Fig. 1C)[23,25]. The mean levels of these transcripts were 2 to 3 times higher in IIM than in controls, but the difference was significant only for ubiquitous transcripts in IBM (mean, 3.6- ± 1.9-fold; $p < 0.05$; Fig. 1B). Cytoplasmic dynein transcipts[26] were also analyzed, but expression levels did not differ between IIM and controls (data not shown).

KIF4 protein localizes in IIM muscle-infiltrating immune cells

To determine whether the increase in KIF4 mRNA in IIM muscle was accompanied by increased KIF4 protein and to identify cells expressing the protein, we used immunhistochemistry on serial sections of muscle. Kinesin superfamily protein 4 was not detected in control muscles, but there was intense KIF4 immunoreactivity associated with infiltrating mononuclear cells in IIM. The pattern of positivity in DM and PM muscles was closely similar to that of anti-CD3 (pan T cell)-, anti-CD22 (B cell)-, and anti-CD163 (macrophage)-positive cells (Fig. 2A). Similar results were obtained in IBM (not shown). Kinesin superfamily protein (KIF) 4 immunoreactivity was not associated with a specific subpopulation of mononuclear cells. In serial sections, $CD3^+$ T cells (the most prominent immune cell population) seemed to be $KIF4^+$, but $CD22^+$ B

cells and $CD163^+$ macrophages may also have expressed KIF4 (Fig. 2A).

In PM, which is characterized by cytotoxic damage to muscle fibers, $KIF4^+$ cells were mainly located close to individual muscle fibers, whereas in DM, KIF4 positivity was also found close to immune cells apparently exiting from blood vessels (Fig. 2A).

To establish whether KIF4 was indeed present on infiltrating T cells, we performed double-immunofluorescence confocal microscopy. We found that the protein colocalized with $CD3^+$ T cells. The association was greater with $CD4^+$ T cells in DM and with $CD8^+$ T cells in PM in the restricted areas of muscle in which invading mononuclear cells were present (Fig. 2B). We also found that KIF4 was present on some $CD163^+$ macrophages (data not shown).

Not all immune cells were $KIF4^+$ because there was a lack of correlation between KIF4 mRNA levels and the numbers of infiltrating cells (Fig. 3A). In contrast, there was a strong correlation between KIF4 mRNA levels and the percentage of $KIF4^+$ cells in the muscle infiltrates in all 3 IIMs (Fig. 3B).

Kinesin superfamily protein (KIF) 4 mRNA is not detected or present in low quantities in most nonproliferating adult tissues[4], but it is expressed at high levels in the thymus and spleen-the major sites of lymphocyte activation and proliferation[5]. To investigate whether $KIF4^+$ cells in IIM muscle were proliferating, serial sections of 2 DM, 2 PM, and 2 control muscle biopsies were processed by immunohistochemistry to reveal KIF4 and Ki67, a nuclear marker of

proliferating cells[27]. Although most infiltrating cells seemed to be $KIF4^+$, only a proportion of these were $Ki67^+$, indicating that a proportion was proliferating (Fig. 4). The proliferating cells were located close to muscle fibers.

Assessment of KIF4 mRNA and protein expression in in vitro-stimulated immune cells

To shed light on the factors that influence KIF4 expression by immune cells in IIM, we investigated PBLs from healthy donors incubated with various stimuli. Kinesin superfamily protein 4 mRNA and protein levels were assayed as the cells proliferated in response to stimuli. Concanavalin A (1 μg/ml) upregulated KIF4 mRNA in a time-dependent manner (mean 4-fold increase ± 0.17 at 24 hours; 48-fold ± 0.09 at 72 hours) compared with basal levels (Fig. 5A). In contrast, ubiquitous KIF5 transcript levels were unaffected by Con A throughout the stimulation period (Fig. 5A). Enhanced KIF4 expression correlated with increased [^{3}H] thymidine incorporation (R^2 = 0,993; Fig. 5B).

Peripheral blood lymphocytes were also incubated with IL-2 and anti-CD3 alone or in combination with anti-CD28. There was a mean 10-fold increase in KIF4 mRNA levels after 48 hours with IL-2, a mean 14-fold increase after 6 days with anti-CD3 alone, and a mean 42-fold increase with anti-CD3 and anti-CD28 costimulation (Fig. 5C). Increased expression of KIF4 mRNA was always associated with high cell proliferation.

Double immunofluorescence of IL-2-stimulated PBLs revealed that KIF4 was present mainly in the cytoplasm where it colocalized with lysosome-associated membrane 1 and, to a lesser extent, with perforin, indicating association with lytic granules (Fig. 6). Western Blot analysis, using 2 KIF4-specific antibodies (Ab03 and C-tus), of low-speed supernatants (nuclei removed) from PBLs cultured for 24 hours revealed no KIF4 protein in unstimulated cells but a 50-kd band after Con A stimulation (Fig. 7). These results indicate increased KIF4 protein in Con A-stimulated cells, consistent with the transcriptional data shown in Fig. 5. The same band was present in supernatants from SH-SY5Y human neuroblastoma cells. Ubiquitous KIF5 was always present in PBLs and neuroblastoma cells, but its levels did not change with Con A stimulation (Fig. 7).

Discussion

We have shown for the first time that the KIF4 motor protein (but not the other KIFs) is highly expressed in the muscle of patients with IIMs. Kinesin superfamily protein 4 transcripts were upregulated in IIM muscle compared with controls, but levels did not differ significantly between the 3 IIM forms. This indicates that upregulation is not specific for any of the IIM forms.

Serial section immunostaining indicated that the KIF4 protein was mainly associated with infiltrating mononuclear cells (T lymphocytes, B lymphocytes, and macrophages) surrounding muscle fibers.

Confocal microscopy showed that the KIF4 protein was associated with $CD4^+$ T lymphocytes in DM and $CD8^+$ T lymphocytes in PM (Fig. 2), and also with some $CD163^+$ macrophages. Not all immune cells were KIF4 positive, however, as was also indicated by the lack of correlation between KIF4 mRNA levels and numbers of infiltrating cells (Fig. 3A). This suggested to us that KIF4 might be present in the cells only at certain stages of their life cycle or function.

We first sought to determine whether KIF4 positivity correlated with proliferation and found in agreement with Lindberg et al[28] that some KIF4-positive cells in IIM muscles also expressed Ki67 (Fig. 4), a marker of proliferating cells. The association KIF4-Ki67 is consistent with the idea that KIF4 is involved in immune cell proliferation in muscle *in vivo*. This concept is further supported by our finding that many stimulated PBLs expressed both KIF4 and Ki67 proteins (Fig. 6). Furthermore, Con A-stimulated PBLs showed markedly increased expression of KIF4 transcripts that correlated with [^{3}H]thymidine incorporation (Fig. 5B), but no increase of ubiquitous or conventional KIF5 transcripts, indicating that KIF4 expression in mononuclear cells is a specifically inducible process. The results of our Western Blot analysis of PBLs with 2 different KIF4-specific antibodies demonstrated that KIF4 was present only in stimulated (Con A) cells (Fig. 7); moreover, it was present in the cellular fraction corresponding to the cytoplasm. These antibodies also recognized a band with identical electrophoretic mobility in the neuroblastoma cell

line, demonstrating that protein expressed in PBLs was the same as that expressed in neuronal cells.

In all PBL experiments, KIF4 expression continued to increase throughout the stimulation period (up to 6 days in the case of CD3/CD28), with no decline observed. By analogy with our PBL data, the KIF4-positive muscle-infiltrating cells are also likely to be activated. This might occur as a result of T-cell interaction with the peptide-MHC complex on antigen-presenting cells; dendritic cells are known to be present in IIM infiltrates[29] or on muscle fibers. This activation can also occur as a consequence of interaction of the T-cell ligands CD28/CTLA4 and CD40L with CD80 or CD40, respectively, the costimulatory molecules expressed on antigen-presenting cells[29,30]. It might also be triggered by soluble factors. Indeed, many cytokines, predominantly of the T_H1 type, have been reported in IIM muscles. Infiltrating cells, muscle fibers, and endothelial cells are potent producers of proinflammatory cytokines, including IL-1, IL-6, IL-2, and IL-18[31-33].

The fact that perforin and granzyme transcripts were present in IIM muscle[34,35] also suggests activation because these transcripts are normally present in T cells only after CD3/CD28 engagement[36,37]. We also found that perforin expression in PBLs increased after CD3/CD28 and IL-2 stimulation. All these considerations suggest that KIF4 might be associated with lytic granule transport. Indeed, we found in PBLs that KIF4 colocalized with lysosome-associated membrane protein 1, marker of lytic granules, and also, to a

considerable extent with perforin, which is normally present in the granules (Fig. 6). Thus, KIF4 may be associated with these granules. Stinchcombe et al[12] have suggested that granule delivery to the lytic synapse might be driven by reorganization of the actin and microtubule cytoskeleton, with clearing of the plus-ends of microtubules away from the area of contact between T cell and antigen-presenting cells. In light of these data, we suggest that KIF4, one of the plus-end-directed microtubule motor proteins, may contribute to the structural rearrangement of the cytoskeleton necessary for granule dissociation and release. This suggestion is consistent with recent findings that KIF4 in undifferentiated neurons (Navone et al, unpublished results), and the KIF4 homolog of *Xenopus*[7,8] affect microtubule polymerization and stability. Our finding that KIF4 is specifically upregulated in infiltrating cells in IIM muscle leads us to hypothesize that KIF4 may be a marker of cell activation, although this requires confirmation by studies on other inflammatory (immune or autoimmune) conditions.

In conclusion, we have shown for the first time that the KIF4 motor protein is present in the muscles of patients with IIM and also in activated PBLs *in vitro*. Although our experimental approaches have not enabled us to elucidate the full biologic role(s) of KIF4 in the inflammatory process, they imply that KIF4 is associated with activated T cells, irrespective of their functional phenotype. Therefore, KIF4 is likely to be involved in the cytoskeleton modifications associated with T-cell activation. Clearly, further studies are required

to elucidate the role of this motor protein in IIM and other inflammatory processes.

Table. Clinical features and immunopathologic data of inflammatory myopathy patients

	DM (n=16)	**PM (n=15)**	**s-IBM (n=8)**
Age, years (mean ± SD)	36.13 ± 22.95	57.20 ± 17.01	70.88 ± 2.42
Sex (F/M)	12/4	11/4	4/4
Therapy	1*	1*	0
CK (fold increase), mean ± SD	9.76 ± 8.17	11.11 ± 19.56	2.22 ± 1.13
Cytotoxicity‡	No	Yes	Yes
$CD8^+$ cells			
Frequency, mean ± SD✣	5.69 ± 6.98	33.41 ± 7.55	33.18 ± 19.27
Distribution	EM = PA	EM	EM
$CD4^+$ cells			
Frequency, mean ± SD✣	37.69 ± 21.69	26.01 ± 6.77	17.58 ± 15.30
Distribution	PA > PV, EM	EM > PA > PV	EM > PA
$CD22^+$ cells			
Frequency, mean ± SD✣	5.02 ± 8.68	1.25 ± 1.24	0.51 ± 1.24
$CD163^+$ cells			
Frequency, mean ± SD✣	51.59 ± 22.68	39.33 ± 8.12	48.63 ± 33.60
MHC-I	66	50	33

expression, %ℑ			

*, One DM and 1 PM patient were on dexamethasone (every day) before muscle biopsy.

‡, Direct cytotoxicity of infiltrating cells against single nonnecrotic muscle fibers.

✣, Cytotoxic ($CD8^+$) and helper ($CD4^+$) T cells, B ($CD22^+$) lymphocytes, and macrophages ($CD163^+$) were counted (see Materials and Methods section) and expressed as percentage on total number of infiltrating cells.

ℑ, Percentage of patients showing greater than 50% of muscle fibers positive for MHC-I molecules.

CK, creatine kinase; DM, dermatomyositis; EM, endomysial area; F, female; M, male; MHC-I, major histocompatibility complex class I; PA, perimysial area; PM, polymyositis; PV, perivascular area; s-IBM, sporadic inclusion body myositis.

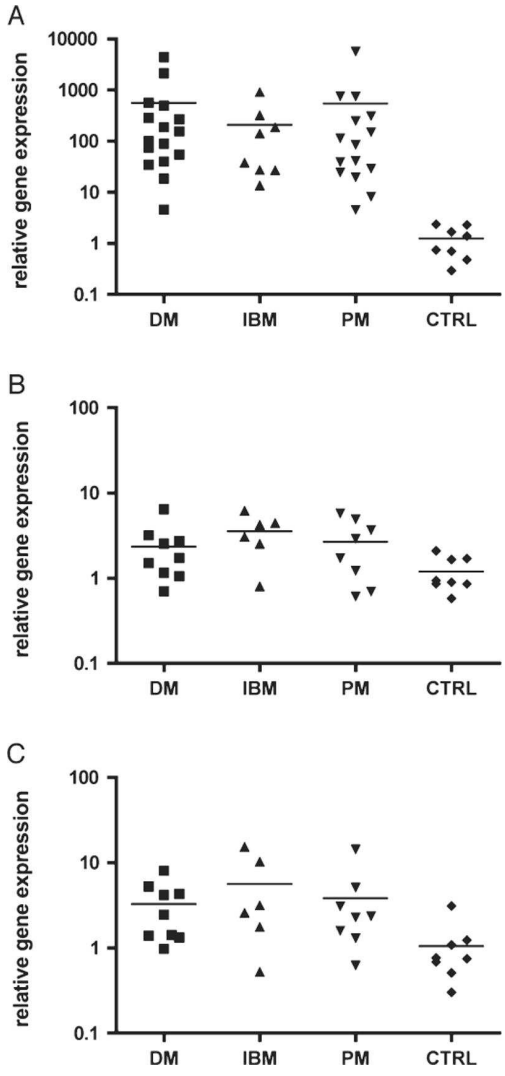

Figure 1. Kinesin superfamily protein (KIF) 4 transcript expression is increased in dermatomyositis (DM), inclusion body myositis (IBM), and polymyositis (PM) muscle compared with

control (CTRL). (A) Expression levels of KIF4 mRNA as assessed by real-time polymerase chain reaction on muscle biopsies. Data were normalized by the level of glyceraldehydes-3-phosphate dehydrogenase expression in each sample and expressed as relative values by the $2^{-\Delta\Delta CT}$ method. Values from controls were used for calibration. Horizontal bars are means. **(B)** and **(C)** show levels of ubiquitous and neuronal KIF5 mRNA, respectively. Levels are slightly increased (2- to 3-fold) in inflammatory myopathy muscles compared with control muscles.

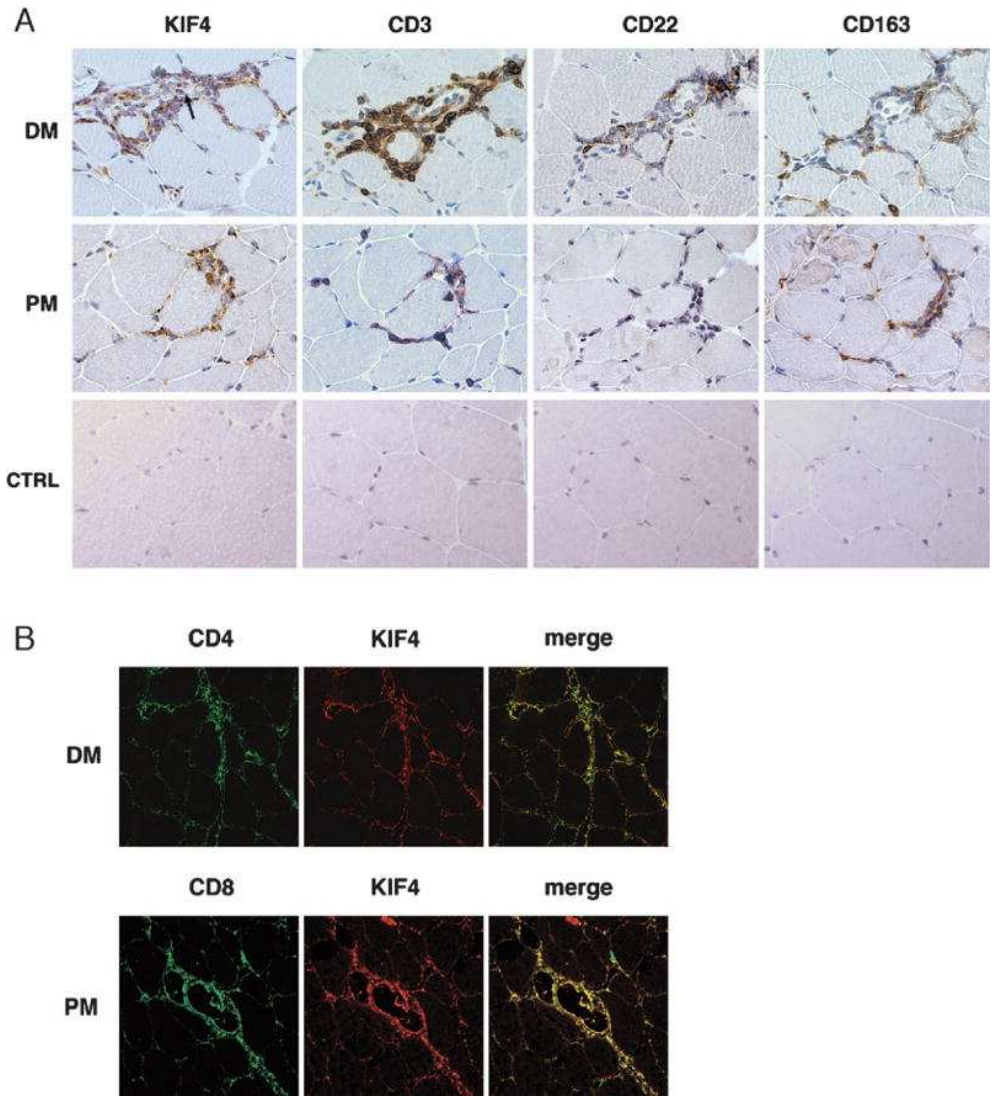

Figure 2. Kinesin superfamily protein (KIF) 4 is localized in the immune cell-infiltrating muscle in patients with inflammatory myopathies. (A) Serial sections of dermatomyositis (DM),

polymyositis (PM), and control (CTRL) muscle biopsies, immunostained to reveal KIF4, T lymphocytes (CD3), B lymphocytes (CD22), and macrophages (CD163). All are counterstained with Mayer hematoxylin. The pattern of KIF4 positivity is similar to that for CD3, CD22, and CD163 positivity, suggesting that KIF4 is expressed by infiltrating mononuclear cells but not preferentially expressed by any particular lineage. Kinesin superfamily protein 4 staining is not seen on muscle fibers. Kinesin superfamily protein (KIF) 4-positive cells surround or invade individual muscle fibers in PM or exit from blood vessels in DM (arrow). No KIF4 positivity is present in CTRL muscle. Original magnification: 40x. **(A)** Images represent 4 DM samples, 5 PM samples, and 3 CTRL samples. **(B)** Confocal microscopy of DM (upper panel) and PM (lower panel) muscle biopsies, representative of 2 DM and 2 PM samples stained. Kinesin superfamily protein (KIF) 4 is in red; CD4 (upper panel) and CD8 (lower panel) are in green. Colocalization is indicated by the merged images in yellow. Not all infiltrating mononuclear cells are $KIF4^{+}$, and kinesin positivity is mainly at the site of contact of immune cells with muscle fibers. Original magnification: 40x.

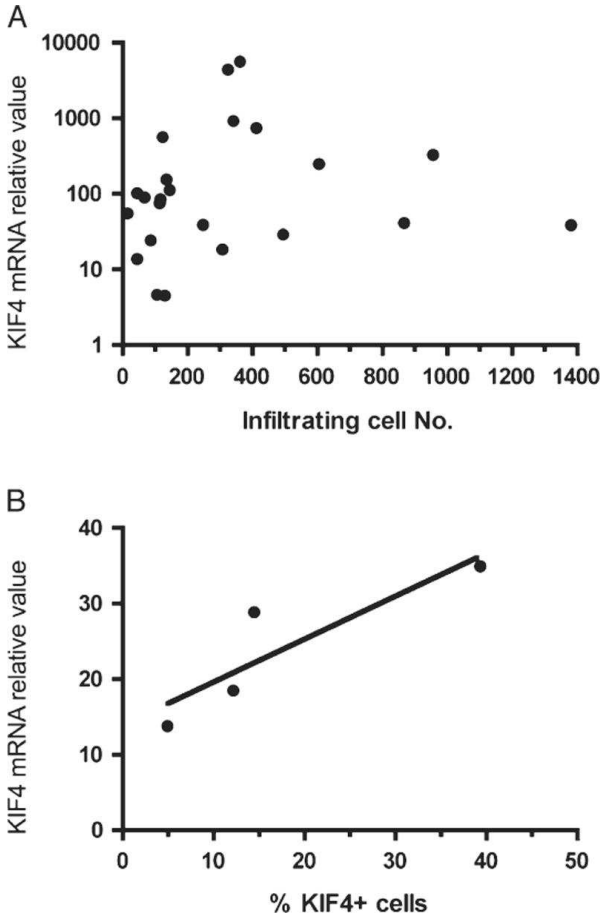

Figure 3. Kinesin superfamily protein (KIF) 4 mRNA levels correlate well with the percentage of cells positive for KIF4 protein. (A) Relative values of KIF4 mRNA, detected by real-time polymerase chain reaction (see Materials and Methods section and Fig. 1) in relation to total number of muscle-infiltrating cells, counted as described in Materials and Methods section. No correlation is

evident. **(B)** Kinesin superfamily protein (KIF) 4 mRNA levels in relation to the percentage of $KIF4^+$ cells in IIM muscle biopsies. The high levels of KIF4 transcript are associated with high proportions of cells positive for KIF4; low KIF4 transcript levels are associated with low proportions of $KIF4^+$ cells (R^2 = 0.78). The data points are representative values from biopsies of 2 DM, 1 PM, and 1 IBM patient.

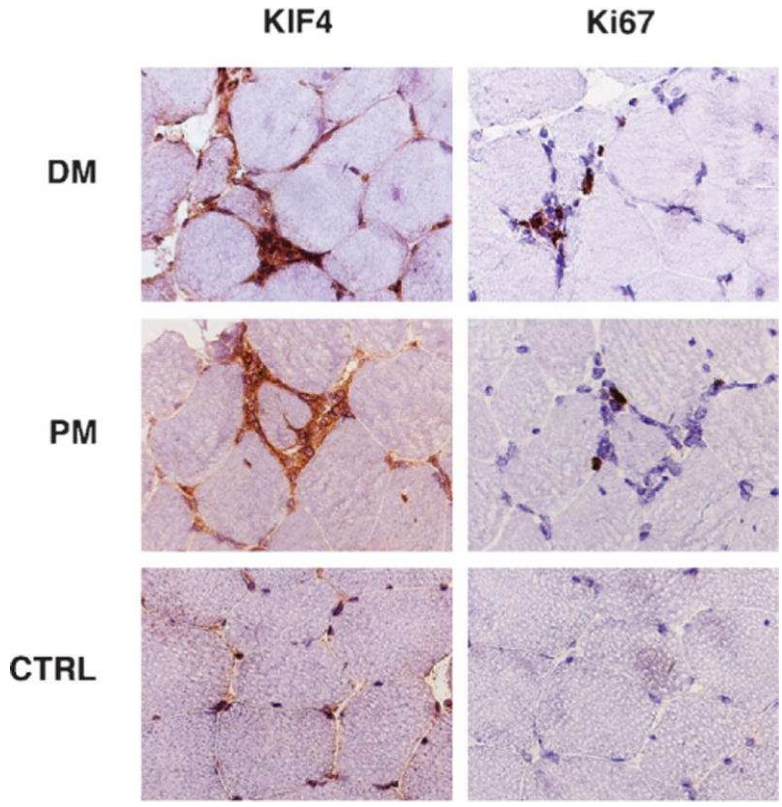

Figure 4. Kinesin superfamily protein (KIF) 4 is expressed in proliferating mononuclear cells identified by Ki67 expression on serial sections of dermatomyositis (DM), polymyositis (PM), and control (CTRL) muscle biopsies. The $KIF4^{+}$ cells surround muscle fibers both in DM and PM. However, only a few $Ki67^{+}$ cells are present in the adjacent section, indicating that a small proportion of the infiltrating mononuclear cells are proliferating. No staining for KIF4 or Ki67 is present on CTRL muscle sections. Original

magnification: 40x. Images are representative of 2 DM samples, 2 PM samples, and 2 control samples.

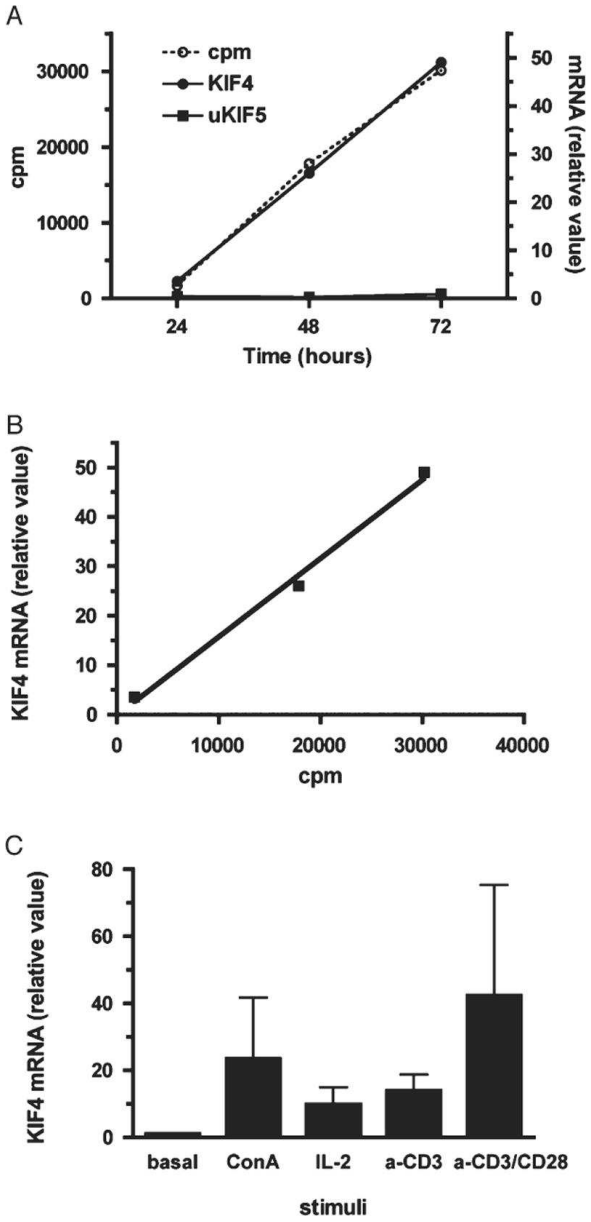
A
cpm
KIF4
uKIF5
30000
20000
10000
0
cpm
50
40
30
20
10
0
mRNA (relative value)
24
48
72
Time (hours)
B
KIF4 mRNA (relative value)
50
40
30
20
10
0
0
10000
20000
30000
40000
cpm
C
KIF4 mRNA (relative value)
80
60
40
20
0
basal
ConA
IL-2
a-CD3
a-CD3/CD28
stimuli

Figure 5. Upregulation of kinesin superfamily protein (KIF) 4 transcript correlates with cell activation and proliferation. (A) Peripheral blood lymphocytes were stimulated with mitogen concanavalin A (Con A) and harvested at 24, 48, and 72 hours and processed for real-time PCR and [^{3}H]thymidine incorporation. Transcriptional levels of KIF4 and ubiquitous KIF5 (ukif5) are expressed as relative values and cell proliferation as counts per minute (cpm). Kinesin superfamily protein (KIF) 4 mRNA levels increase in parallel with [^{3}H]thymidine incorporation during Con A stimulation, whereas uKIF5 mRNA levels remain low throughout the stimulation period. **(B)** Strong correlation between cpm and KIF4 transcript levels (R^2 = 0.993). **(C)** Peripheral blood lymphocytes were stimulated for 48 hours with Con A, 48 hours with IL-2 (which supports preferential growth and differentiation of T lymphocytes from a mixed cell population and stimulates lytic granule synthesis), and 6 days with plastic-immobilized anti-CD3 antibody alone or in combination with anti-CD28 antibody (T-cell receptor-mediated T activation). After incubation, the cells were harvested and processed for real-time PCR. The strongest activation signal (i.e. in the presence of both CD3 and CD28) is associated with greatest KIF4 expression. Data are means of 3 determinations and are representative of 3 independent experiments.

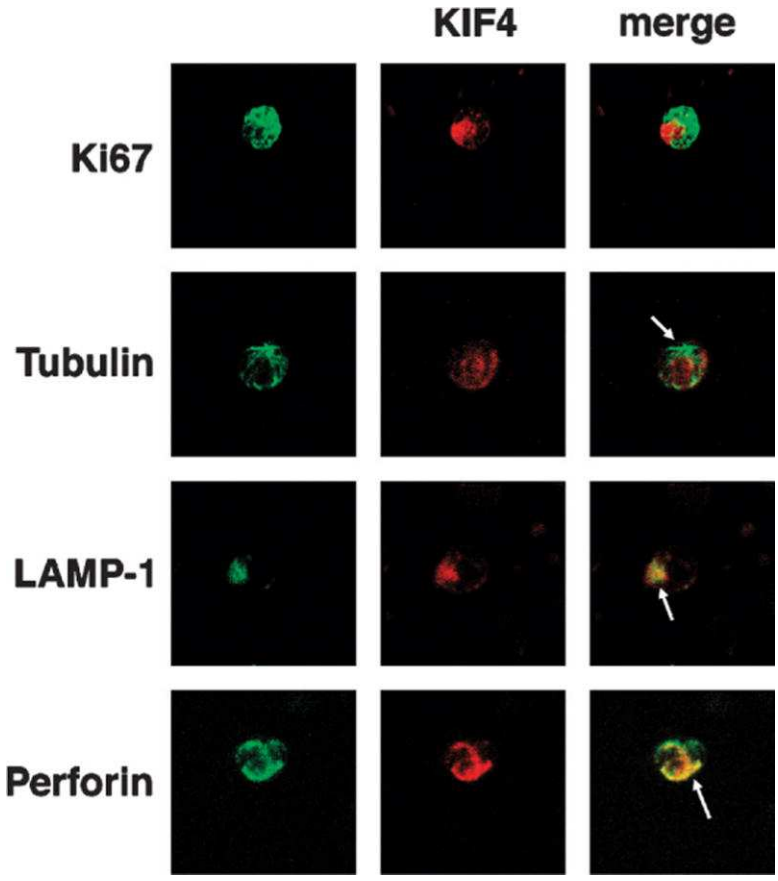

Figure 6. Intracellular distribution of kinesin superfamily protein (KIF) 4 after peripheral blood lymphocytes activation. Cells stimulated with interleukin 2 for 48 hours were processed for colocalization of KIF4 (red) with nuclear antigen Ki67, microtubule marker α-tubulin, cytolytic granule marker lysosome-associated membrane protein 1, and perforin (all in green), and analyzed by confocal microscopy. After cell activation, KIF4 localizes in the

cytoplasm (demonstrated by lack of colocalization with nuclear Ki67) near microtubules. In the tubulin panel, the white arrow indicates the microtubule-organizing center. Kinesin superfamily protein (KIF) 4 colocalizes in the cytoplasm with lytic granules, marked by lysosome-associated membrane protein 1 and containing perforin, when they were present. Original magnification: 60x.

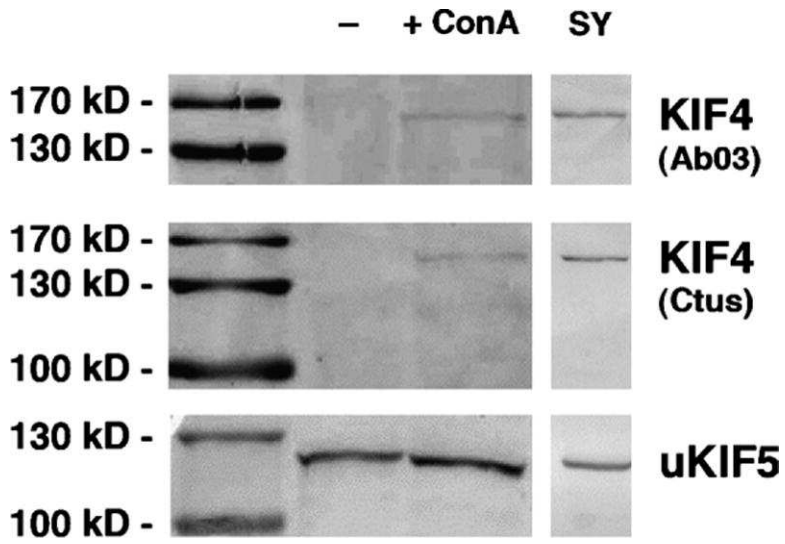

Figure 7. Kinesin superfamily protein (KIF) 4 protein levels are increased in activated peripheral blood lymphocytes (PBLs). Immunoblot of total cell extracts from PBLs unstimulated (-) or stimulated with 2 μg/ml concanavalin A (+Con A) for 72 hours. Ubiquitous KIF5 (uKIF5) was used as internal control, and undifferentiated SH-SY5Y (SY), a human-derived neurotypic cell line, was used as positive control for anti-KIF4 antibodies (Ab03 and C-tus). Left hand lane shows the prestained protein molecular weight marker. The blot is representative of 3 independent experiments.

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Chapter 4

Summary and Conclusions

Interferons (IFNs) comprise an evolutionary conserved family of secreted proteins that participate as extracellular messengers in a wide variety of responses, including antiviral, antiproliferative and immunomodulatory properties that act to maintain homeostasis and in-host defense.[1-2] These distinguishing characteristics make these cytokines, in particular type I interferons (IFN-α/β), potent therapeutic tools for the treatment of several diseases, such as chronic hepatitis C, HIV and other viral infections, other than for hematologic and non-hematologic malignancies. At the same time, as important mediators of innate and adaptive immunity, IFNs have significant disease-promoting and –inhibiting effects in several autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjögren's syndrome, and myasthenia gravis.[3] Among autoimmune neuromuscular disorders, the pathogenetic mechanisms of idiopathic inflammatory myopathies (IIMs), and in particular juvenile (JDM) and adult dermatomyositis, seem to be related to the activation and up-regulation of type I IFNs.[4-6] In the first part of the project, we confirmed these observations by a gene profile analysis of the three different subtypes of IIMs, JDM, adult DM and polymyositis

(PM). Particularly, our data highlight the existence of a distinct molecular pattern between juvenile and adult DM and between DM and PM; in fact, we observed that in DM most of the differentially expressed gene (DEG) transcripts belong to a common pathway of type I and II IFN-inducible genes, especially in the juvenile form of the disease, while in PM the most abundant transcripts are derived from immunoglobulin genes.

One of the best characterized class of mediators of type I IFN production is represented by Toll-like receptors (TLRs), a large family of pattern recognition receptors (PRRs) involved in the detection of highly conserved components of viral and microbial pathogens.[7-8] These molecules, and in particular the TLR subfamily members TLR3, TLR7 and TLR9, which are able to recognize nucleic acid components within endosomal compartments, seem to have remarkable influence on the induction and maintenance of an autoimmune response.[9-11] In this study, we describe for the first time the presence and the up-regulation of nucleic-acid specific TLRs in the muscle tissue from IIM patients. Among the different subtypes of IIMs, we show that TLR3 is preferentially expressed in DM, mainly at the level of the vascular endothelium and on some myofibers. This peculiar localization seems to support the recently proposed model for this form of myopathy, in which muscle injury and atrophy are supposed to be due to the chronic intracellular overproduction of one or more IFN-α/β inducible proteins. Moreover, our data show an overexpression of TLR3 on blood vessels of new formation,

reinforcing the hypothesis of a role of this receptor in the angiogenic process.[12-13] It remains to understand if TLR3 activation precedes or follows new vessel formation in myopathic tissues and what is its exact role in the angiogenic context. Furthermore, we describe the expression of TLR3 on some regenerating muscle fibers; we hypothesize a regulatory role of TLR3 in the regeneration or differentiation mechanisms in injured muscle tissue.[14-16]

In PM muscles we observe a preponderance of expression of TLR7 and TLR9, especially on muscle infiltrating mononuclear cells and on some myofibers. Characterization of cellular phenotype of these $TLR7^+$ or $TLR9^+$ immune cells points out the abundance of both dendritic cells (DCs) and plasma cells, the two main cell types involved in antigen presentation and antibody production, respectively. Considering the importance of TLR9 and plasma cells in the pathogenesis of some autoimmune disorders (e.g. SLE), our findings, in addition to previous observations by other groups,[17-19] suggest a possible link between IFN-mediated innate immune response and production of autoantibodies in IIM muscle, thus contributing to the development or the maintenance of the autoimmune response.[20]

Recently, we provided a first evidence of the role of microtubule-dependent motor proteins in the cytoskeletal modifications associated with T-cell activation, lytic granule dissociation and the release of cytolitic enzymes.[21] Particularly, we described the presence and the overexpression of one of the kinesin superfamily members, KIF4A, in

the muscle tissue from IIM patients, mainly at the level of immune infiltrating cells. Phenotypical characterization of these $KIF4^+$ infiltrates showed the lack of a correlation between the kinesin motor protein and a specific cell subtype (KIF4A was indifferently express on T and B cells, and on some macrophages). Furthermore, using peripheral blood leukocytes (PBLs) from healthy donors, we demonstrated that KIF4 expression in mononuclear cells is a specifically inducible process, related to the proliferation and to immune cell activation.

In view of these findings, and considering the importance of subcellular location for TLR signaling and the maintenance of self tolerance,[22-23] it is reasonable to hypothesize a possible link between TLR trafficking and cytoskeletal motor proteins.

The understanding of the mechanisms that regulate nucleic acid-sensing TLRs traffic, together with the identification of their molecular carriers, represents an important goal for the development of new therapeutic strategies directed to the regulation of TLR signaling, and to the control of the onset and propagation of the autoimmune response.

Future perspectives

We have here shown that endosomal TLRs, the main class of mediators of type I IFN pathway, are involved in several aspects of

pathophysiology of inflammatory myopathies, such as the development of muscular atrophy and the maturation of plasma cells. Future studies will be aimed to better define the regulatory mechanisms of TLR signaling; in particular, based on our previous studies[21] and on some recent observations by other groups,[24] we suggest that the employment of muscle cells derived from IIM patients, in addition to murine myoblasts, for the characterization of cytoskeletal motor proteins involved in TLR transport, will be helpful to the development of new therapeutic approaches to control TLR-induced immune responses.

Our preliminary results have already led up to the identification of two kinesin motor proteins whose expression seems to correlate with TLR3+ late and early endosomes movement during cell stimulation with synthetic pathogen-associated molecular patterns (PAMPs). Further studies to better characterize this relationship are still in progress.

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Chapter 5

Publications

- "*Human adult skeletal muscle stem cells differentiate into cardiomyocyte phenotype in vitro*".
  Invernici G, Cristini S, Madeddu P, Brock S, Spillmann F, Bernasconi P, **Cappelletti C**, Calatozzolo C, Fascio U, Bisleri G, Muneretto C, Alessandri G, Parati EA.
  Exp Cell Res, 2008; 314: 366-376.

- "*The Kinesin Superfamily motor protein KIF4 is associated with immune cell activation in idiopathic inflammatory myopathies*"
  Bernasconi P, **Cappelletti C**, Navone F, Nessi V, Baggi F, Vernos I, Romaggi S, Confalonieri P, Mora M, Morandi L, Mantegazza R.
  J Neuropathol Exp Neurol, 2008; 67: 624-632.

- "*Type I interferon molecules and Toll-like receptors in inflammatory myopathies*".
  Cappelletti C, Baggi F, Zolezzi F, Biancolini D, Beretta O, Severa M, Coccia E, Confalonieri P, Morandi L, Mantegazza R, Bernasconi P.
  Paper submitted

Chapter 6

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