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PATHOGENIC MECHANISMS IN IDIOPATHIC INFLAMMATORY MYOPATHIES

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“Vetenskapen blir egentligen intressant först ute vid den gräns där den upphör”

Justus von Liebig 1803-1873

Abstract

Idiopathic inflammatory myopathies (IIMs) are chronic inflammatory disorders characterized by muscle weakness, by low muscle endurance, and by inflammation in skeletal muscle tissue. The pathogenesis and etiology of these conditions are yet not fully understood and several different mechanisms are likely to be involved. The most characteristic histopathological finding is the presence of inflammatory cell infiltrates in muscle tissue together with degenerating and regenerating muscle fibers.

The main goal of this thesis was to increase our knowledge of the pathogenic mechanisms in IIMs, in particular how the immune reactions could cause impaired muscle performance. We characterized IIM patients and healthy subjects through muscle biopsies in different phases of disease, we performed detailed studies on the cellular level and in an animal model of IIMs, and correlated our results from *in vivo* studies with *in vitro* models.

Several new observations were made in this thesis. Firstly, we found a reduced number and morphologically changed capillaries in patients with short disease duration and without inflammatory cell infiltrates in muscle tissue. This finding correlated to an upregulated expression of the angiogenic factor vascular endothelium growth factor (VEGF) in muscle fibers. These observations may suggest that local muscle hypoxia could be a contributing factor to the impaired muscle function seen in patients. Secondly, we found that the pro-inflammatory cytokines interleukin (IL)-1 and high mobility group box chromosomal protein (HMGB)-1 were consistently expressed in muscle tissue of patients with IIMs not only in inflammatory cells but also in endothelial cells and the nuclei of muscle fibers. The expression of IL-1 and their receptors in muscle nuclei indicate that IL-1 could possess direct effects on muscle fibers and affect muscle fiber metabolism and function. In addition, HMGB-1 was found to reversibly induce major histocompatibility complex (MHC) class I expression on muscle fibers and irreversibly impair Ca^{2+} release from the sarcoplasmic reticulum during induction of fatigue, indicating a direct effect of HMGB-1 on generation of muscle force. Moreover, the expression of MHC class I in muscle fibers, which are a pathological finding in patients with IIMs, led to a specific muscle force reduction in an animal model. In this model the reduced force was associated with decreased cross-sectional area in fast-twitch muscle whereas it was due to a decrease in the intrinsic force-generating capacity in slow-twitch muscles, indicating that MHC class I upregulation affects muscle fiber contractility with differential effects depending on muscle fiber properties.

In summary, we have identified different molecular pathways that might play a pathogenic role in these disorders and how they can lead to low muscle performance. These include tissue hypoxia as a consequence of a distorted microcirculation in skeletal muscle tissue as well as direct and indirect effects of the pro-inflammatory cytokines IL-1 and HMGB-1 on muscle fiber contractility. Thus, it is likely that both immune and non-immune-mediated pathways contribute to the impaired muscle function seen in IIMs and this needs to be recognized in the development of new therapeutic modalities.

List of Publications

- I. Grundtman C, Salomonsson S, Dorph C, Bruton J, Andresson U, Lundberg IE. **Immunolocalization of IL-1 receptors in sarcolemma and nuclei of skeletal muscle in patients with idiopathic inflammatory myopathies.** *Arthritis & Rheumatism*. 2007 Feb;56(2):674-687.
- II. Dastmalchi M*, Grundtman C*, Alexanderson H, Mavragani CP, Einarsdottir H, Barbasso Helmers S, Elvin K, Crow MK, Nennesmo I, Lundberg IE. **A high incidence of disease flares in an open pilot study of infliximab in patients with refractory inflammatory myopathies.** *Ann Rheum Dis*. 2008 Feb 13; [Epub ahead of print].
- III. Grundtman C, Tham E, Ulfgren A-K, Lundberg IE. **Vascular Endothelium Growth Factor (VEGF) is highly expressed in muscle tissue of patients with polymyositis and dermatomyositis.** *Submitted*.
- IV. Salomonsson S*, Grundtman C*, Zhang S-J, Bruton J, Lanner J, Li C, Wedderburn L, Kanneboyina N, Lundberg IE, Westerblad H. **Up-regulation of MHC class I in transgenic mice results in reduced force generating capacity in slow-twitch muscle.** *Submitted*.
- V. Ulfgren A-K*, Grundtman C*, Borg K, Alexanderson H, Andersson U, Erlandsson Harris H, Lundberg IE. **Down-Regulation of the Aberrant Expression of the Inflammation Mediator High Mobility Group Box Chromosomal Protein 1 in Muscle Tissue of Patients With Polymyositis and Dermatomyositis Treated with Corticosteroids.** *Arthritis & Rheumatism*. 2004 May;50(5):1586-1594.
- VI. Grundtman C, Bruton J, Östberg T, Pisetsky DS, Erlandsson Harris H, Andersson U, Westerblad H, Lundberg IE. **High mobility group box chromosomal protein-1 (HMGB-1) induces MHC class I in muscle fibers and mediates muscle weakness -A possible key molecule in idiopathic inflammatory myopathies.** *Submitted*.

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1. Table of contents

1.	Table of contents	4-5
2.	List of abbreviations	6-7
3.	Foreword	8
4.	Idiopathic inflammatory myopathies (IIMs)	9
4.1.	Diagnostic criteria	9-11
5.	Clinical features of IIMs	11
5.1.	Polymyositis (PM)	11-12
5.2.	Dermatomyositis (DM)	12
5.3.	Inclusion body myositis (IBM)	12-13
5.4.	Brief histopathological similarities and differences in IIMs	13-17
6.	Epidemiology and etiology of IIMs	17
6.1.	Epidemiology	17
6.2.	Genetic predisposition	17-18
6.3.	UV-light	18-19
6.4.	Infections	19
7.	Autoantibodies in IIMs	19-20
7.1.	Anti-Jo-1 (histidyl tRNA synthetase)	20
7.2.	Other autoantibodies in IIMs	20-21
8.	Extra-muscular manifestations in IIMs	21
8.1.	Cutaneous manifestations	21
8.2.	Lung involvement	21-22
8.3.	Cardiac involvement	22-23
8.4.	Malignancy	23
9.	Therapy regimes in IIMs	24
9.1.	Glucocorticoid therapy	24
9.2.	Other therapies	24
9.3.	Physical exercise	25
10.	Different experimental models to study IIMs	25
10.1.	Animal models of spontaneously occurring disease	25
10.2.	Animal models of induced disease	25-26
10.3.	Transgenic animal models	26
10.4.	Muscle cultures	26-27

11.	Summary of what is known about pathogenesis of IIMs today	27-28
12.	Healthy skeletal muscle	29
12.1.	The histopathological appearance of a healthy skeletal muscle	29
12.2.	Fiber types	29
12.3.	Muscle contraction	30
12.4.	Isometric contraction	30-31
12.5.	Isotonic contraction	31-32
12.6.	Muscle fatigue	32-33
13.	Muscle weakness in IIMs	33
13.1.	Muscle weakness	33-34
13.2.	Muscle fatigue	34
14.	Patients and methods used in these studies	34
14.1.	Patients and healthy controls	34-35
14.2.	Muscle biopsy	35-36
14.3.	Imunohistochemistry and immunofluorescence techniques	36
14.4.	Whole muscle measurements	36-37
14.5.	[Ca ²⁺] _i measurements	37
14.6.	Clinical outcome measurement (used in paper II)	37
15.	Different hypotheses as to how/why muscle weakness are induced in IIMs	38
15.1.	Paper I. The pro-inflammatory cytokine hypothesis (IL-1)	38-41
15.2.	Paper II. TNF-blockade in IIMs	41-43
15.3.	Paper III. The hypoxia hypothesis	43-45
15.4.	Paper IV. The MHC class I hypothesis	45-47
15.5.	Paper V and VI. The pro-inflammatory cytokine hypothesis (HMGB-1)	47-49
16.	Concluding remarks	50-51
17.	Acknowledgements	52-53
18.	References	54-73

2. List of abbreviations

APC	Antigen presenting cell
ATP	Adenosine triphosphate
BAL	Broncho-alveolar lavage
Ca ²⁺	Calcium
[Ca ²⁺] _i	Intracellular concentration of Ca ²⁺
CFA	Complete Freund's adjuvant
CIA	Collagen induced arthritis
CK	Creatine kinase
CNS	Central nervous system
COX	Cytochrome <i>c</i> oxidase
DC	Dendritic cell
DM	Dermatomyositis
DMARD	Disease modifying anti-rheumatic drug
ECG	Electrocardiography
EDL	Extensor digitorum longus
EMG	Electromyography
ER	Endoplasmic reticulum
ESR	Erythrocyte sedimentation rate
FDB	Flexor digitorum brevis
FI	Functional index
HEV	High endothelium venules
H&E	Mayer's haematoxylin and eosin
hIBM	Hereditary inclusion body myositis
HIV	Human immunodeficiency virus
HMGB-1	High mobility group box chromosomal protein-1
HRCT	High resolution computerized tomography
HTLV-1	Human T lymphocytic virus type I
IBM	Inclusion body myositis
ICAM-1	Intracellular adhesion molecule-1
ICE	IL-1 β converting enzyme
Ig	Immunoglobulin
IFN	Interferon
IIM	Idiopathic inflammatory myopathies
IL	Interleukin
ILD	Interstitial lung disease
IL-1R	Interleukin-1 receptor
IL-1RAcP	IL-1R accessory protein
IVIG	Intravenous immunoglobulin
MAA	Myositis-associated autoantibody
MHC	Major histocompatibility complex
MSA	Myositis-specific autoantibody
MRI	Magnetic resonance imaging
MxA	IFN- α/β inducible protein
NMR	Nuclear magnetic resonance

PBMC	Peripheral blood mononuclear cell
PCr	Phosphocreatine
pDCs	plasmacytoid DCs
PM	Polymyositis
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycated end products
sIBM	Sporadic inclusion body myositis
SLE	Systemic lupus erythematosus
SR	Sarcoplasmic reticulum
SS	Sjögren's syndrome
TCR	T cell receptor
TGF	Transforming growth factor
TLR	Toll like receptors
TNF	Tumor growth factor
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelium growth factor

3. Foreword

This thesis is based on six studies investigating the pathological mechanisms in idiopathic inflammatory myopathies (IIMs). The IIMs are a heterogeneous group of autoimmune muscle disorders that are believed to be triggered by unknown environmental factors in genetically susceptible hosts. In order to be comprehensive and also for everyone who is not working in the field of Immunology and Rheumatology, I tried to write this thesis in a narrative form with a gradual integration of selected results.

The first part of this thesis comprises chapters that give a brief background on the general knowledge of these diseases. Further on, I focus on the main leitmotif of my studies, the muscle pathology and physiology of both diseased and healthy individuals. A deeper and more detailed description of the disease mechanisms follows, at least what is known today and what have been the main novel and relevant findings of my work.

The major aim of this thesis was to achieve an increased knowledge of the pathogenic mechanisms in IIMs. For that purpose my thesis work comprised studies of muscle biopsies from IIM patients with symptomatic and non-symptomatic muscles, patients in different phases of their disease, patients before and after specifically targeted therapies, and from voluntarily participating healthy controls. In addition to muscle biopsies, we also performed detailed investigations on the cellular level and in animal models of IIMs, and correlated our results from *in vitro* models that specifically induced muscle weakness with those of *in vivo* experiments.

I truly hope that you will enjoy reading my thesis.

4. Idiopathic inflammatory myopathies (IIMs)

The idiopathic inflammatory myopathies (IIMs) are a heterogeneous group of diseases characterized by proximal muscle weakness and inflammatory infiltrates of skeletal muscles. Based on clinical and histopathological differences, they are often sub-classified into three major disorders: polymyositis (PM), dermatomyositis (DM), and inclusion body myositis (IBM). However, also other more uncommon clinical conditions can be termed as subtypes of IIMs, such as juvenile myositis, malignancy-associated myositis, myositis in overlap with other connective tissue diseases, dermatomyositis sine myositis, and eosinophilic myositis (1).

The first clinical description of PM and DM was published in 1886 by Wagner (2), who showed clinical manifestations in the form of symmetrical edema, stiffness, pain, and limited motion of muscles, especially arm muscles. Lung affection and skin involvement were also described. PM had already been established at the time when DM was reported in 1891 by Unverricht (3). In 1916 for the first time DM was shown in association with a malignancy (4), although a casual relationship between the two was not hypothesized until 1935 (5). Chou was the first to describe a sporadic case of IBM (s-IBM) in 1967 in a 66-year-old man with PM (6). A muscle biopsy showed that the patient had distinctive intra-nuclear and cytoplasmic filaments, inclusions, and vacuoles. The term “IBM” was not used until established in 1971 by Yunis and Samaha (7). In 1991 Mendell and colleagues (8) identified the presence of amyloid β deposits in muscle fibers by using Congo red staining, which is a characteristic histopathological sign in muscle tissue of IBM patients.

4.1. Diagnostic criteria

There are no officially agreed criteria for the diagnosis of IIMs, but the most often used and generally accepted criteria for PM and DM were proposed in 1975 by Bohan and Peter (9, 10). They consist of five major points to define PM and DM (Table 1).

Table 1

Diagnostic criteria for polymyositis and dermatomyositis by Bohan and Peter (1975)

I.	Symmetrical muscle weakness of limb-girdle and anterior neck flexors
II.	Muscle biopsy revealing evidence of necrosis of type I and II fibers, phagocytosis, regeneration with basophilia, large vesicular sarcolemmal nuclei and prominent nucleoli, atrophy in a perifascicular distribution, variation in fiber size, and inflammatory exudates, often perivascular
III.	Elevated serum levels of enzymes derived from skeletal muscle
IV.	Electromyography must show characteristics features
V.	Characteristic cutaneous manifestations of DM, including heliotrope rash or Gottron's sign

The diagnosis of PM is considered as *definite* when four criteria (not including rash) are met. With three fulfilled criteria (without the rash), the diagnosis is considered as *probable* whereas it is *possible* when two out of five criteria (without the rash) are fulfilled. The diagnosis of DM is considered as *definite* when three or four of these criteria (including the rash) can be found. With two criteria (plus the rash), the

diagnosis is *probable*, and when an individual fulfils one criterion only (plus the rash) the diagnosis is *possible*. Moreover, Bohan and Peter classified patients with PM and DM into five subclasses, see Table 2.

Table 2

Subclasses of polymyositis and dermatomyositis by Bohan and Peter (1975)	
I.	Primary, idiopathic polymyositis
II.	Primary, idiopathic dermatomyositis
III.	Polymyositis/dermatomyositis associated with malignancy
IV.	Childhood polymyositis/dermatomyositis
V.	Polymyositis/dermatomyositis associated with other connective tissue disease

Importantly, the presence of circulating myositis-specific autoantibodies (11) and radiological findings in muscle tissue to localize inflammation, as it can be seen with magnetic resonance imaging (MRI), were not included in Bohan and Peter’s criteria, but would certainly be useful for exact disease classification. Therefore, these additional characteristic features of PM and DM have recently been included in a proposed revision (12). Furthermore, disease designation can also be assigned based on the patient’s age, additional clinical findings, and/or the coexistence of another disease (13).

Several diagnostic criteria for IBM have been proposed, first by Calabrese and colleagues in 1987 (14), and then by Dalakas in 1991 (15). More recent and more frequently used criteria for IBM have been proposed by Griggs and colleagues (16), who separated sporadic IBM (s-IBM) from familial or hereditary IBM (h-IBM). The main characteristics that IBM patients should display for diagnosis are listed in Table 3.

Table 3

Diagnostic criteria for inclusion body myositis by Griggs and colleagues (1995)	
I.	Duration of illness for at least 6 months
II.	Age at disease onset must be at least 30 years of age
III.	Muscle weakness, must affect proximal and distal muscles of arms and legs and patient must exhibit at least one of the following features: <ul style="list-style-type: none"> I. Finger flexor weakness II. Wrist flexor > wrist extensor weakness III. Quadriceps muscle weakness
IV.	Serum creatine kinase <12 times normal
V.	Muscle biopsy with the following features: <ul style="list-style-type: none"> I. Inflammatory myopathy characterized by mononuclear cell invasion of non-necrotic muscle fibers II. Vacuolated muscle fibers III. Intracellular amyloid deposits or tubulofilaments by electron microscopy
VI.	Electromyography must be consistent with features of an inflammatory myopathies

Definite IBM is established upon a diagnostic muscle biopsy, irrespective of other clinical features. In contrast, if the muscle biopsy specimen fails to demonstrate the

characteristic histology, the patient can still be diagnosed with *possible* IBM by the presence of other characteristic clinical and laboratory features.

5. Clinical features of IIMs

5.1. Polymyositis (PM)

PM usually begins insidiously over 3 to 6 months, with no identifiable precipitant, and typically presents with weakness of the proximal muscles. Distal muscles may be involved late in the disease onset with impairment of fine motor tasks. Although the shoulder and pelvic girdle muscles are affected most severely, weakness of neck muscles, particularly the flexors, occurs in about 50% of all patients while ocular and facial muscles are almost never affected. Dysphagia may develop secondary to esophageal dysfunction or cricopharyngeal obstruction. Muscle- and joint pains are not unusual, but severe tenderness and synovitis are relatively uncommon. Pulmonary and cardiac manifestations, the latter usually restricted to asymptomatic electrocardiographic (ECG) abnormalities, can occur at any time during the course of disease. Normal creatine kinase (CK) levels may be found very early in the course of the disease and in advanced cases with significant muscle atrophy. Nevertheless, elevation of serum CK levels is still a reasonable indicator of disease activity and severity in most patients. The erythrocyte sedimentation rate (ESR) is normal in 50% of patients with PM, and is elevated above 50mm/1hour in only 20%. Autoantibodies have been detected in 60-70% of PM patients. Electromyography (EMG) classically reveals an increased insertion activity, fibrillations, and sharp positive waves. In addition spontaneous, bizarre, high-frequency discharges and polyphasic motor-unit potentials of low amplitude and short duration can be found in EMG. Alterations indicating muscular atrophy, inflammation, and substitution of muscle with adipose tissue are frequently seen in PM patients with MRI (17-19) (Table 4).

Table 4

Clinical and laboratory features of PM	
I.	Gradual onset of weeks to some months (subacute)
II.	Insidious yet progressive proximal (early) and distal (late) muscle atrophy and weakness
III.	Affects predominantly middle-age to elderly population (M/F=1:2)
IV.	Rare association with malignancy but associated with other connective tissue diseases
V.	Creatine kinase normal or elevated (usually >2 or as high as >50 times normal)
VI.	Presence of autoantibodies (Jo-1)
VII.	Mixed myopathic electromyographic features
VIII.	Muscle pain is fairly common
IX.	Respond partly to glucocorticoids and immunosuppressive drugs

One of the most common histopathological signs in muscle tissue in PM is the expression of CD8+ lymphocytes which can be found near myofibers that are expressing major histocompatibility complex (MHC) class I. Furthermore, evidence that auto-reactive T lymphocytes contribute to the muscle pathology is supported by the presence of a restricted T cell receptor (TCR) repertoire in a subset of patients, an

oligoclonal expansion of muscle-infiltration T lymphocytes, and the long-term presence of clonally expanded T lymphocytes.

5.2. *Dermatomyositis (DM)*

The typical clinical features of DM include all those described for PM, plus a variety of cutaneous manifestations that can widely differ from person to person. Rashes may precede the onset of muscle weakness by a year or more. Pink or lilaceous areas found symmetrically on the dorsal aspect of interphalangeal joints, elbows, patellae, and medial malleoli, are considered as pathognomonic. Characteristic changes also include discoloration of the eyelids, often associated with periorbital edema, erythema on the shoulders, neck, upper chest, face, and forehead (for more information see “cutaneous manifestations”). Generally, no alterations indicating an absence of fat infiltration and muscular atrophy but symmetric edematous alterations are frequently seen with MRI in DM patients (18-20) (Table 5).

Table 5

Clinical and laboratory features of DM

I.	Disease onset of days (acute) or gradual onset over weeks to some months (subacute)
II.	Insidious yet progressive proximal (early) and distal (late) muscle atrophy and weakness
III.	Affects predominantly young (juvenile DM) and middle-age to elderly (M/F=1:2)
IV.	Common association with malignancy or other connective tissue diseases
V.	Common with cutaneous involvement of the skin
VI.	Creatine kinase normal or elevated (usually >2 or as high as >50 times normal)
VII.	Presence of autoantibodies (Jo-1, Mi-2, Ro 52)
VIII.	Mixed myopathic electromyographic features
IX.	Muscle pain is common
X.	Responds partly to glucocorticoids and immunosuppressive drugs

The muscle histopathology of classic adult DM may be like that of PM, but more commonly shows a perivascular and perimysial infiltration of inflammatory cells composed of a high percentage of CD4+ T lymphocytes, suggesting a MHC class II restricted immune response. A large proportion of these CD4+ cells were recently found to be plasmacytoid dendritic cells (pDCs) (21), and not T lymphocytes. Occasionally, B cells can be found. Biopsies may also reveal perifascicular atrophy, which may be diagnostic for DM. There is also an activation of the complement system that leads to a formation and deposition of membranolytic attack complex on the microvasculature, which may cause lysis of endomysial capillaries and in rare cases muscle ischemia.

5.3. *Inclusion body myositis (IBM)*

IBM patients are mainly from the elderly segment of the general population over the age of 50 years, and the symptoms begin insidiously and progress slowly (14, 22). In its fully developed form, IBM has distinctive clinical features but the frequently slow progression often delays its diagnosis. It is not uncommon for IBM to be misdiagnosed or to be confused with treatment-resistant PM (14). The clinical picture in some patients differs from that of typical PM in that it may include focal, distal, asymmetric

weakness, as well as neurogenic or mixed neurogenic/myopathic changes on EMG. Autoantibodies are less frequently found in sera of IBM patients compared to PM and DM patients. Distal muscles are typically involved early in the disease course, especially wrist and finger flexors. Dysphagia is more frequent in this IIM-type since it can be noted in more than 60% of patients (23, 24). As the muscle weakness becomes more and more severe, it is also accompanied by atrophy and diminished tendon reflexes. Fatty replacement and hypo-/atrophy of the muscular tissue are often reported subsequent to inflammation by MRI (25). In some patients, the disease continues a slow, steady progression while in others it seems to plateau, leaving permanent weakness and atrophy of the involved musculature. The interaction among these various pathological changes remains unknown and there is a continuing debate as to whether IBM is primarily a T-cell-mediated inflammatory myopathy or a pure myodegenerative disorder (26, 27) (Table 6).

Table 6

Clinical and laboratory features of IBM

I.	Gradual disease onset over months to years
II.	Progressive proximal (early) and distal (early) muscle atrophy and weakness
III.	Affects predominantly middle-age and elderly population (M/F=2:1)
IV.	Rare association with malignancy or other connective tissue disease
V.	Creatine kinase normal or only low-level elevation (usually <5-6 times normal)
VI.	Unusual with autoantibodies
VII.	Mixed myopathic and neuropathic electromyographic features
VIII.	Muscle pain is uncommon
IX.	Resistant to treatment with glucocorticoids and immunosuppressive drugs

As the name “inclusion body” implies, the characteristic change in this disease is the presence of intracellular inclusion seen on electron microscopy (14). Electron microscopy reveals either intracytoplasmatic or intranuclear inclusions, which may be tubular or filamentous. These structures are straight and rigid-appearing, with periodic transverse and longitudinal striations. On histological muscle sections, vacuoles lined with eosinophilic material are a typical finding, so called rimmed vacuoles. These can be missed on formalin fixed tissue and require staining on frozen preparations where basophilic granules lining the vacuoles may be evident. The pathological characteristics of IBM are composed of a unique triad of (i) inflammatory changes with invasion of CD8+ lymphocytes in muscle fibers which appear to be non-necrotic and express MHC class I (resembling the picture for PM), (ii) rimmed vacuoles, (iii) cytoplasmic and intranuclear inclusions containing amyloid β and several Alzheimer-type proteins, and (iiii) segmental loss of cytochrome *c* oxidase (COX) activity in muscle fibers, which is associated with the presence of clonally expanded somatic mitochondrial DNA mutations.

5.4. Brief histopathological similarities and differences in IIMs

The molecular basis of IIMs in humans, as in many other autoimmune rheumatic diseases, is heterogeneous involving several complex cellular compartments that contribute to differences in disease susceptibility, onset, and severity. We have only just

started to understand the orchestrated life of T lymphocytes, B lymphocytes, and DCs in IIMs and still many questions how this usually effective system can go awry and result in false immune-mediated reactions remain unanswered. Based on detailed immunohistochemical studies on muscle biopsies, two types of inflammatory infiltrates can be observed: endomysial and perivascular/perimysial. In endomysial infiltrates, mostly seen in PM and IBM patients, there is a striking dominance of CD8⁺ T lymphocytes which can even be the predominating infiltrating cell type, followed by macrophages and CD4⁺ T lymphocytes. These infiltrates are often surrounding non-necrotic fibers and sometimes appear to invade the fibers (Figure 1). This observation suggests an immune reaction that targets muscle fibers. The perivascular infiltrates, on the other hand, mostly seen in DM patients, is predominated by CD4⁺ T lymphocytes and macrophages and sometimes with the presence of B lymphocytes. Another characteristic histopathological finding in muscle tissue of IIM patients is the widespread expression on MHC class I on non-necrotic fibers (28-30). However, there are cases with a less distinct localization of infiltrates or with combined endomysial and perivascular cellular infiltrates (31, 32). Moreover, in some cases the inflammatory cell infiltrates are diffusely spread in the tissue, whereas in other cases the infiltrates are very small or are not found at all. In addition, the perivascular changes may be seen in patients without a skin rash, whereas endomysial infiltrates are occasionally seen in cases with a skin rash. This complexity of T lymphocyte populations in muscle tissue in clinical subsets of myositis was demonstrated already in the original observations of different cellular subsets in muscle tissue by Arahata and Engel (29, 32, 33) and is exemplified in Figure 2 and Figure 3. These observations make it necessary to revise the “old historical” hypothesis regarding the pathogenesis of IIMs. Recently a dispute over the most appropriate and accurate diagnostic criteria, including the importance of the histopathological and the localization of immune cells, has erupted (34).

The presence of T lymphocytes in all subsets of IIMs indicates a permanent immune response that requires the presence of APC, a mechanism shared by the three major subsets of IIMs. DCs are central in the development of innate and adaptive immune responses. Two main classes of DCs have been classified, myeloid and pDCs. Myeloid DCs are potent APCs and play a role in the adaptive immune system. Until recently, few data were available on DCs in IIMs, but the results of some very recent studies have partly revealed important insights on this issue. Physiologically, DCs do not appear in normal muscle tissue whereas local DCs have recently been demonstrated in all subsets of IIMs (35). In muscle specimens of patients with IBM and PM, myeloid DCs were present in substantial numbers, frequently surrounding and sometimes invading intact myofibers. They were part of a dense collection of cells that also included T lymphocytes. In DM muscles, an increased number of pDCs was found when compared to the amount of myeloid DCs (35). Furthermore, immature DCs have been detected in lymphocytic infiltrates in both PM and DM muscle tissue samples (36). Interestingly, sera from positive anti-Jo-1 or anti-Ro 52/anti-Ro 60 patients have been found to act as endogenous interferon (IFN)- α inducers which could activate IFN- α production in pDCs (pDCs are the major cell type which produce type I IFN) (37). IFN- α/β inducible protein (MxA) expression has been demonstrated in capillaries and in perifascicular myofibers, characteristic sites of dermatomyositis pathology (21). Taken together, muscle tissue of IIMs patients demonstrates a complex involvement

of the immune system in which both the innate and adaptive immune systems are involved. Some features are common to all IIM patients suggesting that some mechanisms are shared by the subsets whereas other features seem to be specific for certain subsets suggesting that some molecular mechanisms may differ between IIM patients.

Figure 1

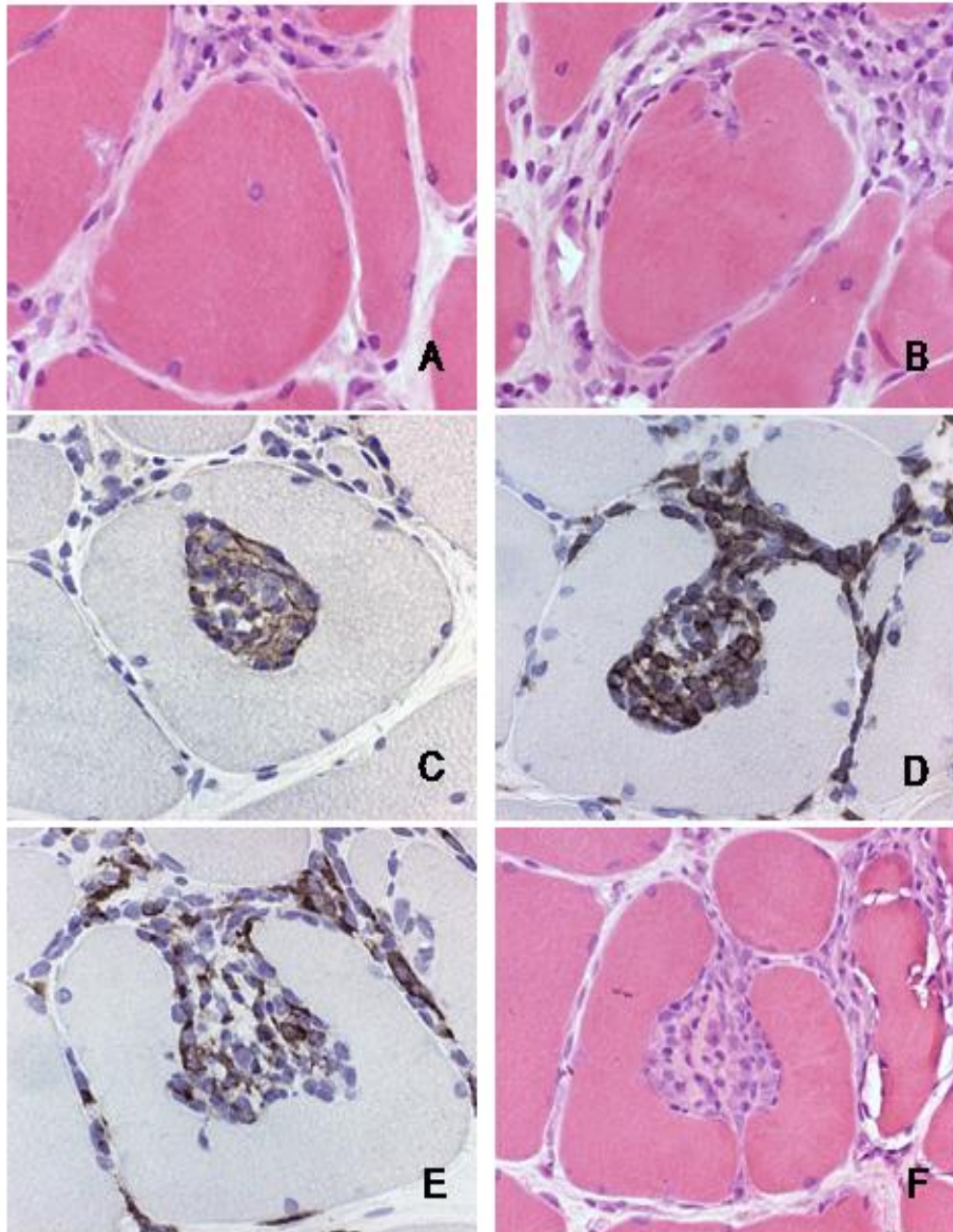


Figure 1. Immunohistochemical stainings of mononuclear cells invading a non-necrotic muscle fiber in a PM patient. These stainings illustrate how a non-necrotic fiber (this fiber has centralized muscle nuclei) can be attacked by mononuclear cells. A: Section 1, hematoxylin and eosin (H&E) staining to localize inflammatory cell infiltrates. B: Section 5, H&E staining. C: Section 10, IL-1 β staining. D: Section 14, CD3+ T lymphocyte staining. E: Section 20, CD163+ macrophage staining. F: Section 60, H&E staining, the invaded muscle fiber has now divided into two fibers and the fiber at the right hand side seems to be “necrotic”. Original magnification x 500.

Figure 2

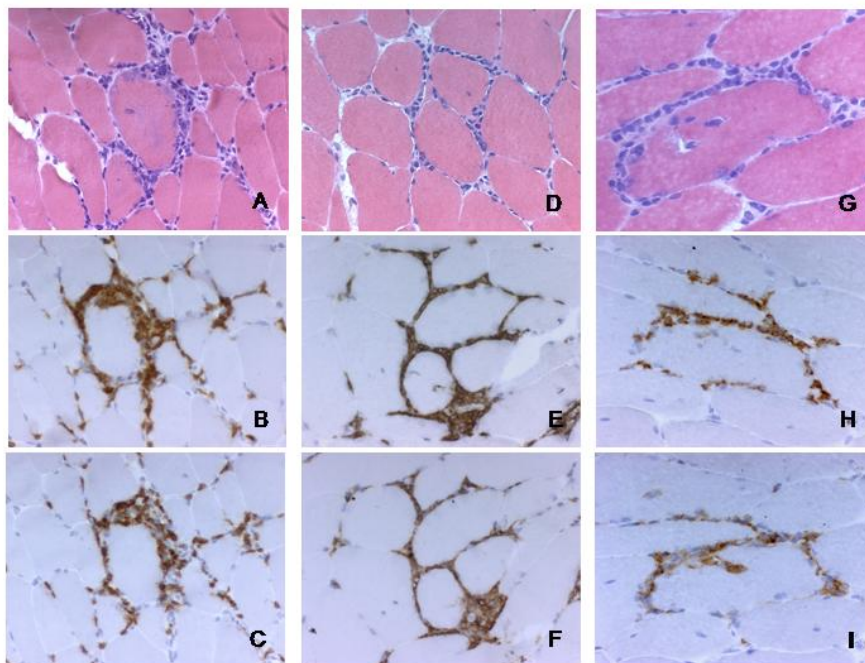


Figure 2. Endomysial localization of CD4+ and CD8+ T lymphocytes with immunohistochemical stainings of PM (A-C), DM (D-F), and IBM (G-I) patient. A: hematoxylin and eosin (H&E) staining to localize inflammatory cell infiltrates in a PM patient. B: CD4+ T lymphocytes in the same area as in A but further down in the biopsy. C: CD8+ T lymphocytes in a consecutive section to B. D: H&E staining to localize inflammatory cell infiltrates in a DM patient. E: CD4+ T lymphocytes in the same area as in D but further down in the biopsy. F: CD8+ T lymphocytes in a consecutive section to E. G: H&E staining to localize inflammatory cell infiltrates in an IBM patient. H: CD4+ T lymphocytes in the same area as in G but further down in the biopsy. I: CD8+ T lymphocytes in a consecutive section to H. Original magnification $\times 250$ in A-F and $\times 312.5$ in G-I. The figure is modified from a review, Grundtman C *et al.* (38).

Figure 3

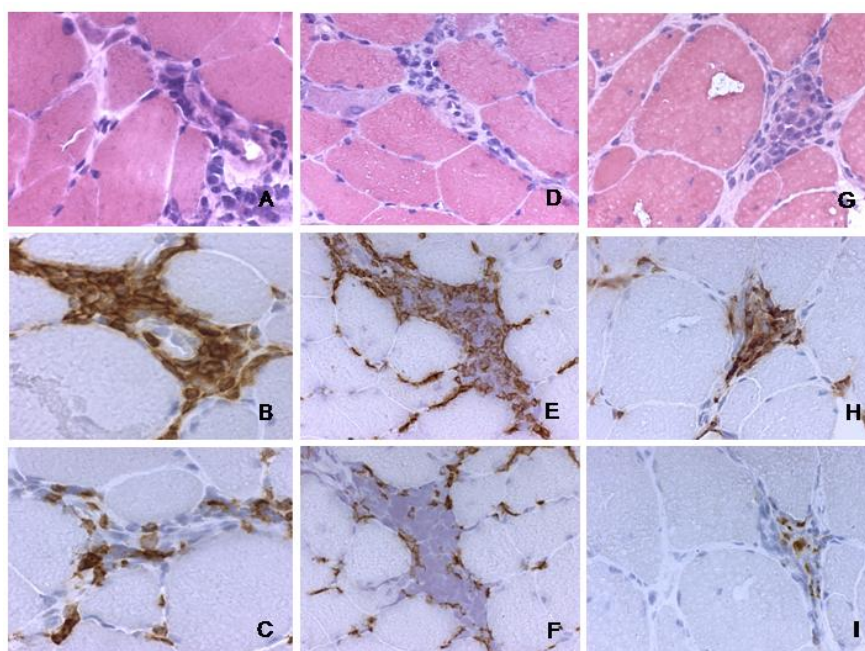


Figure 3. Perivascular localization of CD4+ and CD8+ T lymphocytes with immunohistochemical stainings of PM (A-C), DM (D-F), and IBM (G-I) patient. For description see figure legend in Figure 2.

Original magnification $\times 312.5$ in A-C and G-I and $\times 250$ in D-F. The figure is modified from a review, Grundtman C *et al.* (38).

6. Epidemiology and etiology of IIMs

6.1. Epidemiology

The exact incidence and prevalence of IIMs is difficult to estimate since the diseases are relatively rare and standardized agreed diagnostic criteria are lacking. Therefore, estimates of the incidence and prevalence of IIMs range in different studies and countries. Furthermore, many of these studies are based on a few numbers of patients which make it even more difficult to get an exact number of the incidence and prevalence in IIMs. The incidence of PM and DM, using Bohan and Peters criteria (9, 10), in a US population of Allegheny County, Pennsylvania between 1963 and 1983 was determined to be 0.55 per 100,000 (39). An annual incidence of 0.76 cases per 100,000 was estimated in a Swedish county-based population study including definite or probable PM, DM, and IBM (40). In another Swedish study, an estimated annual incidence rate of 0.22 per 100,000 (41) has been observed for IBM patients. Prevalence rates of 0.49 per 100,000 in The Netherlands (IBM) (42), 0.218 in Israel (PM and DM) (43), 0.107 in Connecticut, USA (IBM) (44), 0.93 in Western Australia (IBM) (45), and 0.41 for PM and 0.79 for IBM patients in Olmsted County, USA (46) have been reported. However, it appears that the incidence is increasing, which might be partially due to a generally increased clinical awareness and knowledge about the disease among physicians, higher referral rates to hospitals, and more accurate diagnostic possibilities (39, 40, 43, 47). Larger, multi-centre trials are clearly needed and could certainly clarify the true epidemiology of IIMs.

The age of disease-onset for IIMs has a bimodal distribution, with an initial peak between 10 and 15 years in children (39, 43, 48), and a later peak between 45 and 69 years in adults (39, 43, 47). In general, women are affected twice as often as men in PM and DM (39, 47); however, IBM affects men twice as often (42). Racial differences are apparent, since in adults the lowest rates are reported in the Japanese and the highest in African Americans (39, 47).

6.2. Genetic predisposition

Although the exact pathogenesis in IIMs is still unknown, the importance of genetic factors becomes evident from several observations of certain polymorphic genes of the human MHC which have been associated with IIMs. These include HLA class I genes (HLA-A, B, and Cw) and HLA class II genes (HLA-DR, DQ, and DP), which code for antigen-presenting molecules that play important regulatory roles in immune activation. HLA genes are among the strongest and most consistently identified genetic factors associated with the development of human autoimmune diseases, including IIMs (11, 49-53), especially in patients who present with anti-Jo-1 autoantibodies and/or patients with interstitial lung disease (ILD) (52, 54-59). The relationship between MHC genetic variability and autoimmune disease may be explained, at least in part, by influences of the MHC molecules on T cell receptor development, peripheral tolerance, and immune responses to environmental agents (53, 60, 61). The alleles of the 8.1 ancestral haplotype (8.1 AH, containing HLA-A*0101, B*0801, Cw*0701, DRB1*0301, and

DQA1*0501) are important risk factors for the development of IIMs in patients producing anti-synthetase/anti-Jo-1, anti-La, anti-PM/Scl, and anti-Ro autoantibodies. The detection of shared and distinct HLA susceptibility factors for IIM patients further exemplifies the complex polygenic and multifactorial nature of disease susceptibility. Taken together, it is most likely that genetic and environmental risk and protective factors influence the development of IIMs among subgroups of patients displaying particular clinical and serological phenotypes.

As pro-inflammatory cytokines seem to be effectively involved in the pathogenesis of IIMs, several researchers have elaborated the genetic predisposition for these susceptibility genes. A possible non-MHC candidate gene is tumor necrosis factor (TNF), located in the HLA class II region, and associations of TNF-308A polymorphism have been found in small studies of juvenile and adult onset myositis (62-65). In individuals of European ancestry, it is known that a strong linkage disequilibrium exists between the TNF-308A allele and the HLA-DRB1*03-DQA1*05-DQB1*02 common ancestral haplotype, as has been demonstrated in adult onset myositis patients (64). Furthermore, several studies have suggested a genetic contribution of TNF regulation, while possession of the ancestral haplotype or TNF-308A is associated with higher circulating levels of serum TNF (66-68). Genetic markers in the IFN- γ and interleukin (IL)-4 genes have demonstrated allelic associations with IIMs in a UK Caucasian population (69). Another specific allele is the IL-1 A1, which has been found to be a risk factor for juvenile IIMs (70). Although all of these factors can be involved in the development of the disease, the primary cause of IIMs still remains obscure.

6.3. UV-light

There is increasing evidence that autoimmune diseases result from environmental exposure in genetically predisposed individuals. One such environmental factor of increasing interest in the pathogenesis of immune-mediated disorders is ultraviolet (UV) radiation. UV radiation, besides inducing accelerated skin aging and skin cancer, has a number of immunomodulatory effects (71). It triggers cytokine production (72), regulates surface expression of adhesion molecules (73), affects cellular mitosis (74), and induces apoptotic cell death (75). UV radiation may also alter the expression, cellular location, or immune responses to autoantigens (76, 77). Although little is known about the role of UV radiation in the development of autoimmune diseases, it has been associated with the development of some disorders and is known to increase the clinical picture of conditions that are characterized by photosensitive rashes, such as skin lesions in systemic lupus erythematosus (SLE) and in DM (78). The mechanisms by which UV-light exerts these effects, however, remain poorly understood. The seasonal occurrence of IIMs onset and relapses has been analyzed in a retrospective study in a group of 53 patients with PM or DM (79). In DM, the incidence of both myositic and cutaneous relapses was highest in the summer whereas in the PM group relapses were more evidently distributed throughout all four seasons, although lowest in summer. These findings suggest that environmental factors, such as intermittent infections and light exposure, may be involved in reactivating the disease processes, e.g. relapses in DM (79). A high correlation with the intensity of world surface UV irradiation (irradiance) and the relative proportion of DM has been found, and a latitudinal gradient (in Europe) in the relative proportion of DM was observed (80). In a

global study, UV irradiance again showed the strongest correlation with the relative proportion of DM (81), implying that UV intensity is primarily responsible for the different proportions of patients with DM and that other environmental variables, including latitude, derive their association with DM largely from their correlation with UV intensity.

6.4. Infections

At their first clinical presentation, a significant proportion of patients coming to our clinic report that their onset of clinical symptoms of IIMs appeared after a common cold or flu and reports about relapses of the disease after an infectious episode are not uncommon. Viral infections that have been suggested to be associated with IIMs are Hepatitis C (82-85), retroviruses such as human immunodeficiency virus (HIV) and human T lymphocytic virus type I (HTLV-1) (86-90), parvovirus B19 (91), mumps viruses (92), but also influenza viruses (93). It has also been occasionally reported that associations between IIMs and bacterial infections, such as *Staphylococcus aureus* and streptococci (94) exist. Furthermore, mouse models for human IIMs, where mice have been infected with different viruses or parasites have been developed; for details see chapter “Different experimental models to study IIMs”.

It has been considered that IBM might be caused by a chronic viral infection, partly because of the muscular histopathology of filaments. Moreover, it has been speculated that the filaments within the inclusions resemble paramyxovirus nucleocapsids (6, 95). An isolation of adenovirus type II from muscle biopsy specimen from IBM patients has also been reported (96). Despite the observed positive immunostaining for the inclusions by antibodies to mumps virus antigens, no molecular or serological techniques have yet succeeded to amplify viruses from patients’ muscle or sera (92). The closest connection with viruses has been the observation that occasionally HIV and HTLV-1 infection patients also develop IBM (89, 90, 97, 98). Because these retroviruses are not detected within the muscle, it has been attractive for many years to conclude that they do not directly infect the muscle, but rather trigger an inflammatory response against muscle. However, new data suggest that HIV positive IBM patients have a subset of CD8⁺ T cells surrounding the muscle fibers that are viral specific, and may therefore play a role in the disease mechanisms by cross-reacting with antigens on the surface of muscle fibers (99).

7. Autoantibodies in IIMs

It appears that the disease is, at least partly, driven by a loss of self-tolerance with the production of autoantibodies. Autoantibodies directed against various cellular constituents have been detected in 60-70% of PM and DM patients, but less often in IBM. Myositis-specific autoantibodies (MSAs) are closely associated with characteristic clinical features and therefore can provide useful information for diagnosis, patient classification, as well as the prediction of signs and symptoms of myositis, response to treatment, and prognosis. The most commonly found MSAs in IIMs is the anti-synthase antibody, anti-Jo-1 (histidyl tRNA synthetase) autoantibody. Other MSAs autoantibodies found in IIMs are anti-Mi-2 and anti-SRP autoantibodies. Although also myositis-associated autoantibodies (MAAs), such as anti-SSA

autoantibodies (anti-Ro 52, anti-Ro 60) and anti-SSB (anti-La) autoantibodies can often be present in IIMs, they are found with much less specificity. The MSAs are often associated with distinct clinical manifestations, e.g. the anti-synthetase syndrome, which is characterized by myositis, ILD, arthritis, Raynaud's phenomenon, and skin changes called "mechanic's hands" (100). MSAs and MAAs seem to have specific associations with HLA-DR haplotypes, irrespective of the myositis subtype (58, 59). The role of autoantibodies in IIMs is not fully elucidated and further analysis of the molecular structure and biological function of target autoantigens recognized by these MSAs and/or MAAs might provide another key to the understanding of the etiology and pathogenesis of IIMs.

7.1. Anti-Jo-1 (histidyl tRNA synthetase)

The anti-Jo-1 autoantibody was firstly described in 1980 (101) and initially thought to be an exclusive marker of inflammatory myopathy. Now, anti-Jo-1 autoantibody is known to be associated with a distinct clinical entity, known as the anti-synthetase syndrome (100). Especially ILD is often found in the anti-synthetase syndrome, occurring in around 75% of patients with anti-Jo-1 autoantibodies, compared to around 30% of patients with IIMs without anti-synthetase antibodies (102-104). Anti-Jo-1 autoantibodies are more common in patients with PM but may also be present in DM (11, 105). The anti-Jo-1 autoantibody recognizes different epitopes of histidyl-tRNA synthetase (Jo-1) (100, 106, 107). Moderate correlations between anti-Jo-1 autoantibody titers and clinical indicators of disease activity in myositis, including an elevation of CK levels, muscle dysfunction, and articular involvement have been found (108). Furthermore, levels of IgG1 anti-Jo-1 or recombinant human Jo-1 have been found to vary in relation to disease activity (109, 110). These data suggest that anti-Jo-1 autoantibodies could be useful as prognosticators for disease activity and severity. However, this is at least partially contradicted by another study where no difference in the overall survival between anti-Jo-1 positive and negative patients could be detected (102). Anti-Jo-1 autoantibodies are usually present at the time of diagnosis and may even precede the development of myositis symptoms (109). Based on a range of immunologic and immunogenetic data, it is likely that Jo-1 plays a direct role in the induction and maintenance of autoimmunity in the anti-synthetase syndrome. For example, the antibody response to histidyl tRNA synthetase undergoes class switching, spectrotype broadening, and affinity maturation, all of which are indicators of a T cell dependent, antigen-driven process (106, 107, 109, 110). This indicates that an underlying T cell response directed against Jo-1 might drive autoantibody formation and tissue damage.

7.2. Other autoantibodies in IIMs

Recently, a new study illustrated that the prevalence of autoantibodies in IIM patients is much higher than predicted; this might be due to an increased sensitivity of detection methods used (111). High levels of anti-Mi-2 autoantigen have been found in PM and DM muscle lysates and have also been connected with malignancy in DM (112). Anti-Mi-2 autoantibodies are particularly evident in DM patients (113), of which almost 20% are positive (54, 81). Anti-Mi-2 autoantibodies are associated with acute onset of prominent skin changes in patients who respond well to therapy (11, 114). The newly discovered autoantibody anti-p155 seems to be more often associated with DM and para-neoplastic DM and its frequency is similarly high in children (29%) and adults

(21%) (with para-neoplasia 75%) (115). Anti-Ro 52 antibodies are strongly correlated with Sjögren's syndrome (SS) and SLE patients, but IIM patients with anti-Ro 52 autoantibodies often co-present with anti-Jo-1 autoantibodies (116, 117). There are also reports showing the presence of autoantibodies in patients with IBM, and an increased frequency of serum monoclonal antibodies reactive to a muscle constituent (118).

Autoantibodies, in particular anti-Jo-1 autoantibodies, seem to have specific associations with specific clinical phenotypes and disease mechanisms, irrespective of myositis subtype. This would support the concept that IIM patients with anti-Jo-1 autoantibodies may define a specific myositis subtype.

8. Extra-muscular manifestations in IIMs

Before the “glucocorticoids era”, more than 50% of IIM patients died within 5 years of diagnosis due to different disease complications. In two recently investigated cohorts followed over 20 years, the 5 year survival rate was 95% in both and 10 year survival rates were 84% and 89% (119, 120). Occurrence of extra-muscular organ involvement, such as ILD (104, 121, 122) and cardiac involvement (120), is associated with worse prognosis for survival. Therefore, it is important not to underestimate the importance of the extra-muscular involvement in IIMs.

8.1. Cutaneous manifestations

DM is at least partially defined by the presence of highly characteristic inflammatory skin changes that are integral to the illness. This set of hallmarks includes Gottron's papules, periorbital (heliotrope) violaceous erythema, periungual telangiectasia, confluent macular violaceous erythema, and poikiloderma atrophicum vasculare. The color and regional anatomical distribution of these skin changes are of considerable diagnostic importance and in DM often includes the periorbital regions, malar aspects of the face, V-area of the neck, scalp, posterior neck, and shoulders (shawl sign), and extensor aspects of the shoulders, arms, forearms, hands, and fingers (123).

In approximately 60% of patients with classical DM, the skin and muscle changes appear concurrently, whereas approximately 30% of classical DM patients have skin manifestations that precede the muscle symptoms by a period of several weeks or months. There are also approximately 10-20% of the DM population that does not develop classical skin manifestations for 6 months or even longer, called “dermatomyositis sine myositis” (123). It has been demonstrated that active skin disease in DM is associated with reduced quality of life (124). Therapy for cutaneous disease in patients with DM is often difficult because, although the myositis may respond to treatment with glucocorticoids and/or immunosuppressive drugs, the cutaneous lesions often persist despite therapy (125).

8.2. Lung involvement

In 1956, Mills and Matthews reported the first cases of ILD in association with DM (126). Since then, it has been well recognized that many patients with IIMs also suffer from different lung manifestations. Pulmonary involvement in PM and DM includes respiratory muscle weakness, aspiration pneumonia, ILD, infections, and drug-induced

pneumonia. ILD is now recognized as a direct manifestation of PM and DM and occurs in 23-65% of all patients (103, 127, 128). Pulmonary manifestations in IBM patients are rare and the frequencies of subclinical alveolitis are not known. ILD has been reported to be a major cause of death in patients with PM and DM and contributes substantially to morbidity and mortality (104, 121, 122). The clinical manifestations of ILD in patients may vary from asymptomatic to severe rapidly progressive dyspnoea with eventual fatal outcome.

Dyspnoea and cough are the two most common initial clinical presentations for ILD (103, 127-129) and the presence of anti-Jo-1 autoantibodies is more commonly seen in patients with ILD (129, 130). The underlying mechanisms of ILD in IIM patients could be multifactorial due to the various histological features (131) of ILD in myositis although lung biopsies are seldom performed. A subset of DM patients with ILD has been shown to have a markedly poorer prognosis compared to PM patients with ILD, because of greater resistance towards glucocorticoid therapy (132). It is likely that the reported incidence of ILD will increase with more frequent use of diagnostic methods such as high resolution computerized tomography (HRCT) and broncho-alveolar lavage (BAL), as has been used in patients with other connective diseases (133, 134). ILD may appear concomitantly with, before, or after the onset of the skin or muscle manifestations of IIMs (103, 135, 136). There are even case-reports of ILD and dermatomyositis sine myositis with an acute onset of rapidly progressive ILD (137-139).

8.3. Cardiac involvement

Cardiac involvement is common in IIMs but seldom symptomatic until far advanced. The frequency of cardiac dysfunction depends on the diagnostic method that is used. A persistently elevated CK-MB fraction is believed to predict or indicate extensive cardiac disease (140, 141), but a rising MB fraction to total CK ratio may be more specific for cardiac involvement (142). However, an elevated CK-MB fraction does not necessarily signify cardiac involvement since it could also be elevated because of its presence in regenerating skeletal muscle fibers. Cardiac troponin I is a more useful marker to distinguish whether CK-MB elevations are due to myocardial or skeletal muscle injury (143). Clinically manifest heart problems are relative infrequent in IIM patients varying in frequency between less than 10% and 25% (144-149). Although, in a small Finnish study including 16 cases of PM and DM found that 62% had features of cardiac involvement, most of the patients showed clinically apparent heart problems such as congestive heart failure and coronary heart disease (150). One of the most frequently reported clinically manifest cardiac problem in IIM patients is congestive heart failure, between 3% and 45%, and in some patients the heart involvement may have a fatal outcome (144, 146, 150, 151). Subclinical cardiac involvement is much more common and frequency varies depending on methods used. Abnormalities on ECG were observed in 32.5-72% (145, 146, 149, 151-153). These asymptomatic ECG abnormalities include, atrial and ventricular arrhythmias, conduction abnormalities including bundle branch block, and A-V block to mention a few. Cardiac involvement as a cause of death in PM is reported to be 10-20% (119, 120, 144, 148). However, this figure is relatively uncertain since large epidemiological studies of what factors are the causes of death registered in PM and DM is scarce. In a 6-year follow-up study of PM patients, a four-fold increased overall mortality was recorded (153). A 16-time

increased death rate due to myocardial infarction was reported in the same patient cohort, with an even higher risk for females (32 times) than for males (9 times) (153). In two other studies, the leading cause of death was cardiovascular disease (120, 144). Less common but serious features of cardiac involvement include myocarditis leading to congestive heart failure (154), endomyocardial fibrosis (155), or pericardial effusion with tamponade (156).

8.4. Malignancy

Although the relationship between cancer and myositis was proposed as early as 1916 (4), this area has been controversially discussed for decades and its meaning and significance still remains unclear. However, several recent population surveys support an association between cancer and IIMs and significant progress has been made in our understanding of the link between cancer and IIMs. Robust epidemiological studies have not only confirmed the overall association, but a temporal relationship between the two entities has been emphasized. One epidemiological study in 2001 (157) revealed that cancer was detectable in 15% of PM patients and 30% of DM patients, with over 60% of these tumors diagnosed after the diagnosis of myositis. Furthermore, both PM and DM patients had an increased risk to develop cancer compared to the normal population and DM patients were at the highest risk. The majority of cancers were diagnosed within one year after the development of myositis; however, PM patients had an increased risk the first 5 years and thereafter dropped substantially and the risk in DM patients never return to expected population values for most cancers. As in previous observational studies, the most common cancer types were adenocarcinomas, which accounted for 70% of all associated tumors in both PM and DM patients. Another population survey, older but also with a large cohort of patients, in Sweden from Sigurgeirsson and colleagues has demonstrated a significantly increased risk of cancer in patients with either PM or DM, with most but not all cancers developing within two years of the onset of myositis; cancer was stronger linked with DM than PM (158). Buchbinder and colleagues confirmed these findings in a study in 2001 (159). Further evidence for the coincident expression of myositis and cancer comes from a recent study examining the prevalence of tumor-marker positivity in newly diagnosed PM and DM patients. An elevated carcino-embryonic antigen (CA)-125 (a standard tumor marker) level at time of diagnosis was associated with markedly increased risk of developing a solid malignancy during the follow-up period (160), suggesting that unapparent malignancies in this group of patients were already present before or at the time of the myositis diagnosis. An increased risk of cancer has been found in IIM patients with autoantibodies to different fragments of the Mi-2 beta autoantigen (114). Furthermore, high levels of Mi-2 autoantigen have been found in lung adenocarcinomas and hepatocellular carcinoma, which implies that the Mi-2 autoantigen could be a link between malignancy and DM (112).

While many types of malignancies have been associated with IIMs, it appears that gastric, ovarian, lung, and non-Hodgkin lymphoma cancers may be over-represented (157, 161, 162), and that hematological cancers (e.g. leukemia) and lymphomas are more common in children with IIMs (163). Most cancer therapies result in rapid improvement in the muscle and skin conditions suggesting that these forms of IIMs are paramalignant phenomenon.

9. Therapy regimes in IIMs

9.1. Glucocorticoid therapy

Glucocorticoids are the primary agents for the initial treatment of IIMs. Although their efficacies in IIMs have not been fully established in randomized, placebo-controlled trials, their clinical efficacy is recognized in most cases, especially in newly diagnosed patients (164-166). Several regimens have been studied (167): at the start, daily doses are high in single (normally around 0.75 mg/kg/day prednisolone) or divided dosages (around 40-60 mg/day) often concomitantly with a disease modifying anti-rheumatic drug (DMARD) such as methotrexate and azathioprine. Although prednisolone may reduce the disease symptoms for some patients, its use may lead to unwanted glucocorticoid-related side effects (168). In an attempt to reduce the side-effects, Nzeusseu and colleagues studied the functional outcome in a small group of patients receiving a low dose regimen (≤ 0.5 mg/kg/day) compared with another group of patients who were receiving higher doses (> 0.5 mg/kg/day) (169). There were no statistically significant clinical differences between the two groups (169), although others queried the statistical analysis in that study (166).

Up to 40% of patients have glucocorticoid-resistant disease, and patients with muscle symptoms longer than 9 months before start of treatment are less likely to respond completely to immunosuppressive treatment (170). “Refractory myositis” has not been adequately defined or uniformly agreed on.

Most IBM patients do not respond to anti-inflammatory immunosuppressant or immunomodulatory drugs that are available, and there is no established therapy to stop the progression of the disease (171). Most of these patients require a walking aid already after about 5 years and the use of wheelchair after 10 years of disease (172, 173). However, a small proportion of IBM patients do, for unknown reasons, respond at least initially to treatment, but so far there are no reliable markers to prospectively identify them. The treatment of newly diagnosed cases of IBM is therefore largely empirical and varies considerably in different centers (174).

9.2. Other therapies

Immunosuppressive agents, especially methotrexate or azathioprine, are often used as second line agents in patients with PM or DM but again their use in IIMs is mostly based on clinical experience rather than on results from systematic randomized controlled trials. If patients who do not respond satisfactorily or do not tolerate these agents other therapies that have been used are cyclosporine, high dose intravenous immunoglobulins (IVIGs), cyclophosphamide or more recently mycophenylate

As a significant number of IIM patients do not adequately respond to treatment with glucocorticoids and immunosuppressive agents, or IVIGs, and side-effects are common more effective drugs are needed. However, development of improved treatment with more targeted therapies requires increased knowledge on key molecular pathways in IIMs, which has been the focus of my studies.

9.3. Physical exercise

Until recently, patients with IIMs were encouraged to avoid physical activity and exercise because of the irrational fear that exercise would aggravate muscle inflammation. Since the first case reports of safety and beneficial effects of exercise in PM and DM patients in 1993 (175, 176), additional studies have suggested that IIM patients benefit from physical training. Furthermore, one training program was used to investigate the effects of exercise on molecular expression in muscle tissue including muscle fiber types and cross-sectional areas. Before training, the participating patients had a lower proportion of oxidative type I fibers and higher proportion of the intermediate type IIc fibers compared to healthy controls. However, after training the fiber composition was closer to normal (177). This change in fiber type compositions to more oxidative fibers after training supports a hypothesis that hypoxia in muscle tissue may contribute to muscle weakness and fatigue, however this could be partly defeated with physical exercise.

10. Different experimental models to study IIMs

The exact elucidation of the pathogenic mechanisms in IIMs in humans is not only hindered by the heterogeneity of the population, the complexity, chronicity, and rareness of the disease, but also by the necessary types of interventions for research purposes which cannot be performed in humans because of ethical reasons. Therefore, the availability of appropriate experimental animal models to study IIMs is extremely important for the understanding of the underlying disease mechanisms. Animal models have proven to be very useful in a number of muscle diseases, and several animal models that resemble human IIMs have been developed. Animal models of IIMs can be divided into three major categories: (i) animal models of spontaneously occurring disease, (ii) animal models where the disease has to be induced, and (iii) transgenic animal models, where the disease develops in genetically susceptible animals due to a lack of certain inhibiting genes (178). Another experimental system that can be used is muscle cell cultures where myoblasts, myotubes, and differentiated muscle fibers can be analyzed. Muscle cultures are potentially a good alternative, since muscle cells from both patients and healthy donors could be studied.

10.1. Animal models of spontaneously occurring disease

IIMs can spontaneously occur in Collies and Shetland dogs (179, 180) and the disease in canines is well described (181, 182). The clinical illness in canines bears many similarities to human PM and DM, including the presence of symmetric muscle atrophy, histologic myositis, myopathic EMG changes, and dermatitis (DM). Moreover, in 2 models for SLE in mice (the NZW x NZW F1 hybrid and MRL ipr/ipr), IIMs can spontaneously occur as a part of their immune-mediated syndromes (183).

10.2. Animal models of induced disease

Induced animal models are used when a specific condition that should be investigated can be induced experimentally. Usually the condition is induced with antigens, tissue cells, viruses, or drugs. IIMs can be induced in guinea pigs, rats, mice, cats, and monkeys through injection of a variety of agents. Different viruses that have been used

for immunization are: coxsackievirus B1 (184, 185), encephalomyocarditis virus (186), and getah virus (187), only to mention a few. Mice infected with the protozoan parasite *Trypanosoma cruzii* also develop PM-like disease with similar histopathology (188). Autologous or heterologous muscle homogenates have also been used, but the results of these experiments have varied and a generalized reproducible myositis is infrequently seen (189-194).

10.3. Transgenic animal models

Transgenic animal models have revolutionized the understanding of single gene disorders, but their promise has not yet been fully realized for studying complex multi-genetic disorders such as autoimmune diseases. A major advantage is that a single molecule/protein could be studied. The early widespread appearance of MHC class I in muscle fibers of IIM patients is such a striking feature that it led to the development of a transgenic mouse model mirroring human IIMs, where MHC class I is specifically up-regulated in skeletal muscle. These mice make it possible to study the role of MHC class I expression in muscle fibers and interestingly these mice developed clinical, biochemical, histological, and immunological features that are very similar to human IIMs. The disease is inflammatory, limited to skeletal muscles, self-sustaining, chronic, more severe in females, and often accompanied by autoantibodies, like anti-Jo-1 autoantibodies (195). Studies so far suggest that MHC class I expression induces ER-stress that may affect muscle strength and performance (31), however, it is unclear if MHC class I could induce muscle weakness. Two transgenic mouse models for IBM have also been generated (196, 197) where the majority of the 24-month old mice showed myopathic changes, and approximately one third of them had degenerating fibers with sarcoplasmic vacuoles and thioflavin-S-positive deposits (197).

10.4. Muscle cultures

It is possible to culture cells from almost any organ of the body under conditions in which they continue to express at least some of their differentiated traits. A muscle cell culture could mainly be made in three different stages of muscle development, as myoblasts, myotubes, and as adult muscle fibers (differentiated muscle fibers). Although a myoblast is a muscle precursor exhibiting reasonably similar metabolic characteristics to adult skeletal muscle, it does not physically resemble a differentiated muscle fiber or myotube because of a single nucleus. The process of differentiation into adult skeletal muscle cells (myogenesis) is characterized by the fusing of myoblasts into multinucleated myotubes. As they fuse, they undergo a dramatic switch of phenotype that depends on the coordinated activation of a whole battery of muscle-specific genes and increased expression of skeletal muscle specific proteins such as myosin and MyoD. Once fusion has occurred, the nuclei never again replicate their DNA. Fusion involves specific mutual recognition between myoblasts; they do not fuse with adjacent non-muscle cells. In culture, these processes can be partially spontaneous because the proximity of myoblasts to each other. However, changing the contents of the culture medium can more robustly induce myogenesis. Both myoblasts and myotubes express MHC class I but neither has the capacity to contract like adult muscle fibers. Furthermore, mature differentiated skeletal muscle fibers do not have the capacity to divide like myoblasts and myotubes. Several muscle cell lines exist on the market; the most often used are C₂C₁₂ (from mouse muscle) and L6 (from rat muscle). There are also some primary cell cultures raised from human biopsy samples to study

mechanisms that regulate skeletal muscle metabolism (198). It should be noted, that it is important to exactly know the stage of differentiation of the muscle cells used to study skeletal muscle in health and disease using muscle cultures, because of the huge variation in their character.

11. Summary of what is known about pathogenesis of IIMs today

Although IIMs were first described for more than a century ago, major questions concerning the etiology and the pathogenesis of the disease remain elusive. IIMs are heterogeneous muscle disorders; histopathological characterized by the common finding of the presence of inflammatory cell infiltrates mainly composed of a mixed population of macrophages and T lymphocytes. Muscle tissue from PM and IBM patients are historically characterized by presence of endomysial inflammatory infiltrates consisting of CD8+ T lymphocytes and macrophages invading non-necrotic muscle fibers that express MHC class I molecules on sarcolemma. The mononuclear cell infiltrations in the skeletal muscle of DM patients are reported to be predominated of CD4+ T lymphocytes and B cells mainly localized to the perivascular regions, furthermore a disturbed microvasculature have been reported. For a schematic overview of the hypothetic involvement of mononuclear cells in muscle tissue in IIMs see Figure 4. Another common histopathological finding is the presence of degenerating and regenerating muscle fibers. As well as muscle symptoms, DM patients also show characteristic skin rashes. Another characteristic finding is the presence of specific autoantibodies, the presence of both autoantibodies and T lymphocytes in muscle tissue suggest that IIMs are autoimmune disorders but the specificity of the immune reactions is unknown. Inflammatory cell infiltrates could possible also contribute to the clinically predominating symptom of reduced muscle endurance. Different experimental models can be used to study IIMs like, spontaneously occurring animal models, induced animal models, transgenic animal models, and muscle cell cultures. The primary agents for the initial treatment of IIMs are usually glucocorticoids but the effect of treatment on muscle performance is slow and incomplete and a substantial number of patients do not respond at all. Although the pathogenesis of IIMs remains obscure, great strides over the past years have placed us closer to understanding the etiologies of the diverse disease entities. The knowledge as to why especially muscles are the major targets of the immune system and why the immune reaction cause impaired muscle function is however still sparse.

Figure 4

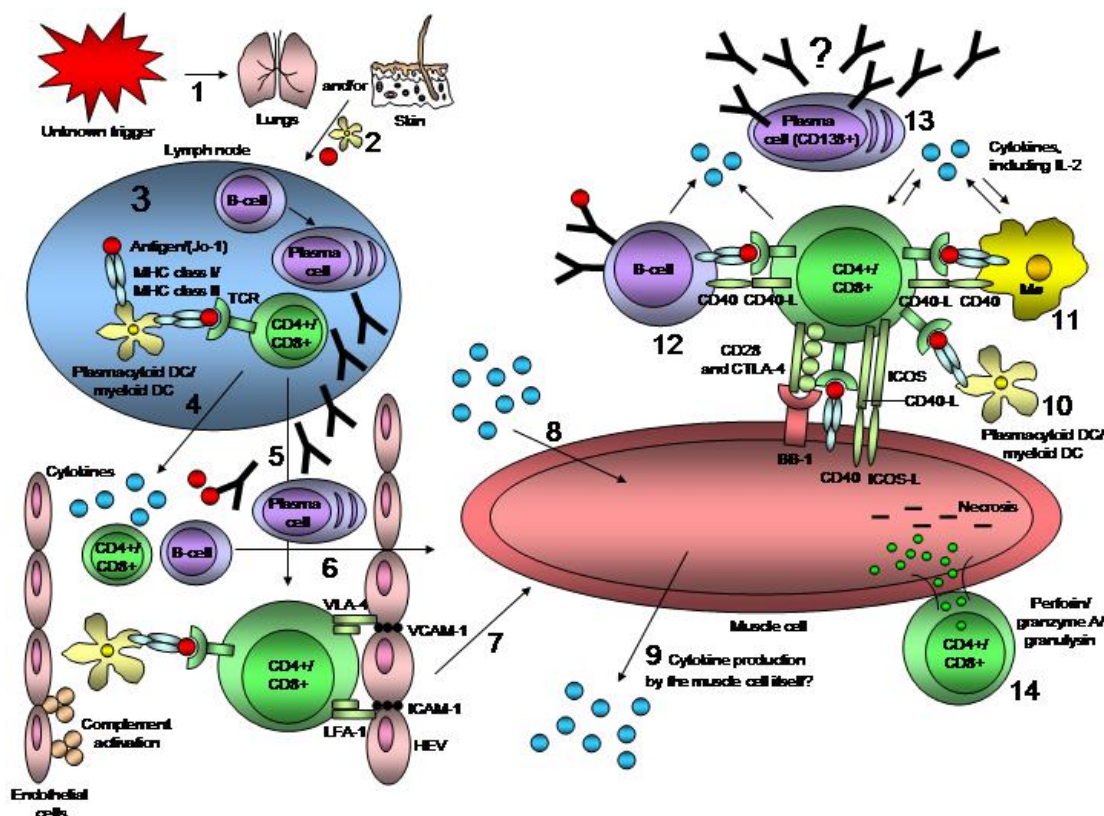


Figure 4. Schematic overview of the hypothetical involvement of T lymphocytes, B lymphocytes, and dendritic cells (DCs) in IIMs. 1: an unknown trigger (viral infection, UV-radiation, or something else) in the respiratory tract or through the skin leads to granzyme B cleavage of histidyl-tRNA synthetase through antiviral CD8⁺ T lymphocytes in the lungs. 2: immature DCs carry receptors on its surface that recognize common features of many pathogens. When a DC takes up a pathogen in infected tissue it becomes activated and migrates to the lymph node. 3: upon activation, the DC matures into a highly effective antigen-presenting cell (APC) changes that enable it to activate pathogen specific lymphocytes in the lymph node. T lymphocytes are activated and B lymphocytes, with active help from CD4⁺ T lymphocytes, will proliferate and differentiate into plasma cells. 4: activated DCs, T- and B lymphocytes could release cytokines into the bloodstream. 5: the activated T lymphocyte, on which the DC-MHC-antigen-complex is bound, itself binds to specialized endothelial cells, called high endothelial venules (HEV). For this purpose it uses the VLA-4 and LFA-1 molecules on its surface to interact with adhesion molecules (VCAM-1 and ICAM-1) on HEVs, where they could penetrate into peripheral lymphoid tissues. 6-7: naïve T- and B lymphocytes that have not yet encountered their specific antigen continuously circulate from the blood into the peripheral lymphoid tissues, 8-9: various cytokines from the bloodstream or locally produced can affect the muscle tissue/cell in many different ways. However, it is not clear if the muscle cell itself can produce and release cytokines. 10-12: DCs, macrophages (M ϕ), and B lymphocytes can interact with T lymphocytes in various ways. T lymphocytes could possibly also bind to muscle cells through inducible co-stimulators (ICOS), CD40-L, CD28, and CTLA-4 (CD152) on T lymphocytes to ICOS ligand (ICOS-L), CD40, and BB-1 on the muscle cell. In this fashion, the muscle cell would function as an APC. 13: plasma cells (CD138⁺) can be found in the muscle tissue of certain subgroups of IIM patients, but whether these cells can locally produce autoantibodies is not yet elucidated. 14: T lymphocytes have been shown to bind in close contact to muscle cells and release perforin, granzyme A, and granulysin, which may cause necrosis of muscle tissue/cells. The figure is modified from a review, Grundtman C *et al.* (38).

12. Healthy skeletal muscle

A solid knowledge about the natural histopathology of muscles, physical concepts, and behavior is important for understanding the clinical disease state. Such fundamental ground is provided in the Swedish textbook entitled “Fysiologi (Physiology)” from Jan Lännergren and colleagues (199), which will form the basis for the description given in the following pages.

12.1. The histopathological appearance of a healthy skeletal muscle

Skeletal muscles represent around 45% of the body weight and can therefore be seen as the largest organ in the body. Typically skeletal muscle is connected to a tendon at each end. The purpose of the tendon is to connect the muscle to a bone and elastically transfer the muscle's force production; for a general overview see Figure 5. In principle, a skeletal muscle consists of hundreds of multinucleated muscle cells. It is surrounded by a strong fascia, which separates muscles from their adjacent structures and provides optimal conditions for sliding against each other. Just underneath the fascia is the epimysium, a tight connection to the perimysium which surrounds bundle of fibers. Such a bundle consists of 10-100 muscle fibers and is called a fascicle. Both blood vessels and nerves (motor and sensory nerves) can be found in the perimysium. Sometimes, single muscle fibers can reach from one tendon to the other and therefore be several decimeters long, but mostly they are much shorter. A cross-section of the fiber is almost circular in shape with a diameter of 40-80 μm . The muscle fiber is enclosed by a cell membrane. A thin mesh of connective tissue, the glycoalyx, strengthens the outer layer of the cell membrane. The nuclei are situated peripherally and immediately below the sarcolemma, each fiber is also surrounded by the endomysium. Inside the sarcolemma is the cytoplasm, which is called the sarcoplasm or the myoplasm. Every muscle fiber is build up of around 1000 contractive myofibrils with an average diameter of 1 μm .

When looking at the myofibril in light microscopy, one can see light and dark bands. This appearance is because the myofibril consists of two different types of filaments, the thin actin filament (7 nm in diameter) and the thicker myosin filament (11 nm in diameter). Together, they create the sarcomer, which is the smallest functional unit of the skeletal muscle. The actin filaments are connected to the Z-bands and the myosin filaments to the A-bands.

12.2. Fiber types

Several distinct types of skeletal muscle fibers, each with different sets of protein isoforms, can be found side by side in a single muscle. In adults, two of these are easily recognized even macroscopically with the naked eye: “red fibers” (type I or slow-twitch) (more myoglobin) and “white fibers” (type II or fast-twitch) (less myoglobin). The different content of myoglobin reflects different oxygen requirements. While type I fibers are specialized for oxidative phosphorylation, they are more resistant to fatigue and are most efficient for generating sustained force/endurance work. Type II fibers are specialized for anaerobic glycolysis; they fatigue more easily, and are most efficient for explosive muscle work for short periods of time/intermittent movements.

Figure 5

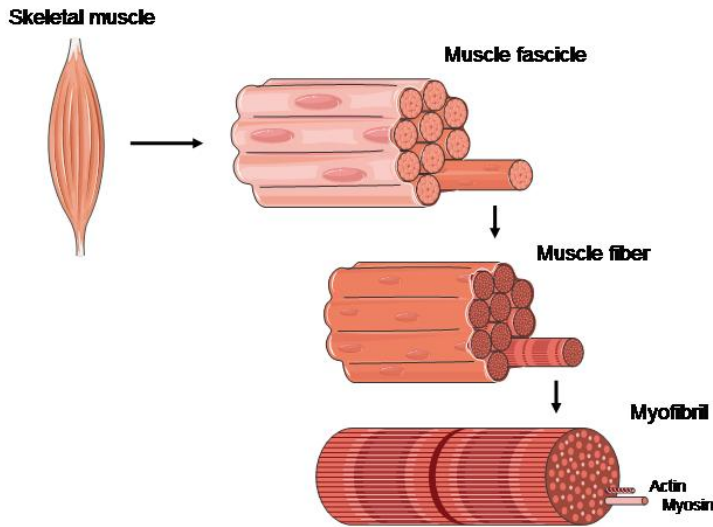


Figure 5. Overview of the main components in a skeletal muscle, parts of the figure are provided by courtesy of Servier.

12.3. Muscle contraction

The actin and the myosin filaments freely slide against each other during rest. However, an activation of a muscle cell requires a release of Ca^{2+} from the SR, which makes it possible for the myosin to bind to actin. A series of different cascades will lead to muscle contraction. This requires energy in the form of adenosine triphosphate (ATP).

12.4. Isometric contraction

Isometric, which means constant length, indicates that a muscle length is constant due to the fixation of the tendons. Activation of the muscle fiber gives rise only to force production and repeated activation of a skeletal muscle will lead to an increased force production. This occurs because the force in single twitches will sum up, as seen in Figure 6.

Figure 6

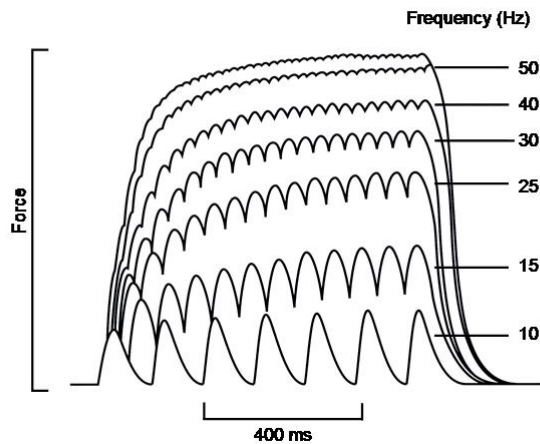


Figure 6. When a skeletal muscle fiber is repeatedly activated, summation of the force of single twitches (15 Hz) can be seen. Higher stimulation rates (e.g. 50 Hz) result in higher force production and single twitches can no longer be identified. This state is called “tetanus”. The figure is modified from the textbook “Fysiologi” (199).

Every action potential will give a release of Ca^{2+} from the SR. Sequential motor nerve impulses lead to frequent action potentials in the muscle fiber. One consequence is, however, that the re-uptake of Ca^{2+} into the SR is incomplete before the next Ca^{2+} release occurs and thus single twitches are added on to each other. If the action potentials are very frequent (around 50 Hz), single twitches will no longer be visible. Instead, you will get a smooth force development called tetanus (Figure 6 and Figure 7). The muscle develops its maximal isometric force during a maximal tetanus and the size of the force depends on the muscle cross-sectional area. The frequency of an action potential to generate a maximal tetanus is lower for slow-twitch fibers (type I) than for fast-twitch fibers (type II).

Figure 7

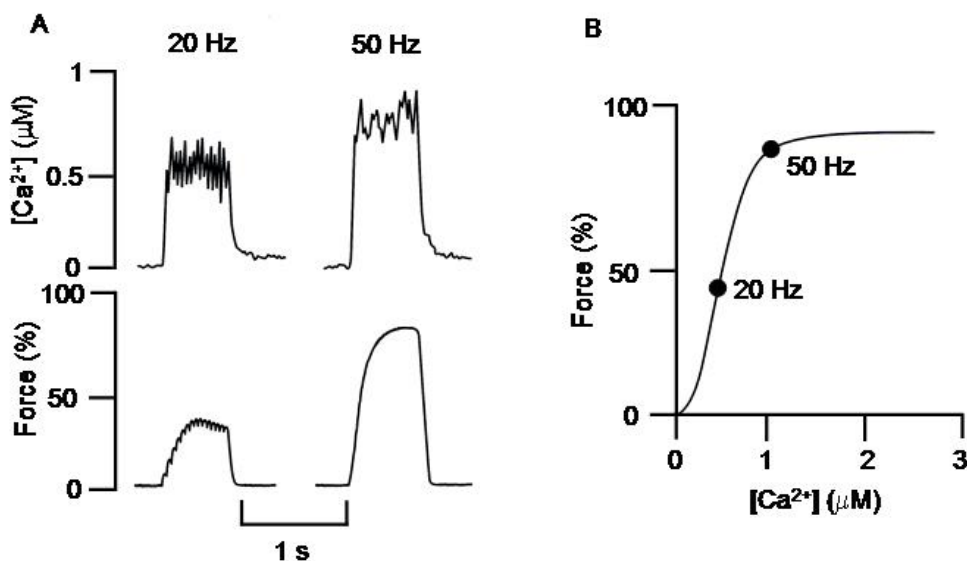


Figure 7. (A) The phenomenon that single twitches at higher work can not be seen, is a consequence of the fact that Ca^{2+} is not fully transported back into the SR after every action potential, and the concentration of Ca^{2+} ($[\text{Ca}^{2+}]_i$) in the myoplasm increases. (B) The relationship between force and $[\text{Ca}^{2+}]_i$ at rest is shallow and the cross bridges are inactive. With activation, Ca^{2+} is released from the SR, leading to increased $[\text{Ca}^{2+}]_i$ and an increased activation of cross bridges. Hence, the force rapidly increases. At high stimulation frequencies, the levels of $[\text{Ca}^{2+}]_i$ are high and all cross bridges are activated and thus maximal force is produced. The figure is modified from the textbook "Fysiologi" (199).

12.5. Isotonic contraction

Isotonic means that the same tonus or loading capacity is used. The muscle will shorten if the loading is less than the force that the muscle produces. But if the loading is greater than the muscle's force, the muscle will be stretched (eccentric contraction). There is a direct function between the loading (force) and the maximal speed with which a muscle can shorten; the higher the load, the smaller is the shortening of the muscle. If the muscle does not have any load, the maximal shortening velocity (V_{\max}) can be reached. Power is equal to force x speed. Maximum power is obtained with loading around 30-40% of the maximal isometric force (Figure 8).

Figure 8

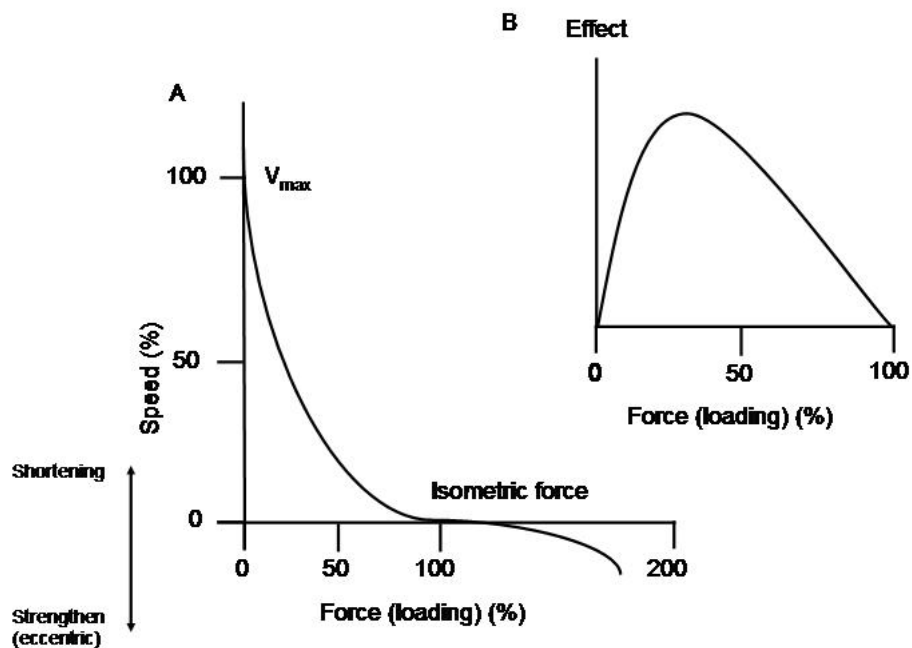


Figure 8. The relationship between force and speed: (A) In an isometric condition, the speed = 0. Maximal shortening (V_{max}) is observed when the force (loading) = 0. The highest force development is when the skeletal muscle is strengthening (eccentric contraction). (B) The power (force x speed) in a muscle is maximal at about 30-40% of the maximal isometric force. The figure is modified from the textbook “Fysiologi” (199).

12.6. Muscle fatigue

Prolonged or repeated contractions of skeletal muscles lead to impaired muscle function and therefore to the development of fatigue. Fatigue is defined as the point when a muscle fails to maintain the required or expected force to perform a given activity. Thus, fatigue is not the total exhaustion or a complete inability to exert force, but rather more a subtle phenomenon where impaired muscle function is manifest. Although the development of fatigue is a hindrance to being physically active, it may serve as a protective function against muscle damage. Fatigue may set in acutely during high-intensity exercise, and then is mainly caused by factors related to increased energy metabolism. There are also other long-lasting types of fatigue in which metabolic factors appear to be only of little importance.

Historically, discussions concerning the locus of fatigue have been focused on three possible sites; the central nervous system (CNS), the neuromuscular junctions, and the muscle cells. At present, failure at the CNS level (central fatigue) is not considered to be a major cause behind the force decline in skeletal muscles seen in fatigue, especially not during high-intensity exercise. In support of this, several investigators have shown that when repeated voluntary contractions are compared with supramaximal nerve stimulation, similar forces are generated (200-202). This would obviously not be the case if failure within the CNS was the major cause of fatigue, because then the artificial elicited nerve stimulation would produce markedly more force than the voluntary force. Failure at the neuromuscular junction is probably also of limited importance during fatiguing voluntary contractions (203-205). Consequently, the reduced force caused by prolonged or repeated contractions seems to be mainly localized in the muscle fibers themselves. Three different factors can be identified when muscle fatigue has occurred:

first there is a reduced capacity to produce force, secondly there is a reduced shortening of the muscle, and thirdly the relaxation time is prolonged.

13. Muscle weakness in IIMs

13.1. Muscle weakness

Although proximal muscle weakness is the most common symptom of a patient with IIM at disease onset, the clinical features at disease presentation vary considerably from patient to patient. In PM and DM, the weakness is most frequently insidious, bilateral, symmetric, progressive, and painless over the course of months. Usually, the lower extremities are affected first, recognized when individuals complain about difficulties when getting up from a chair or walking up stairs. Walking may become clumsy with a “waddling gait”. People may fall and may be unable to get up without assistance; this occurs more often in IBM patients. Upper extremity symptoms often follow, with patients unable to raise their arms up or having difficulty combing their hair. Proximal weakness may also be manifested by the inability to raise one’s head from a pillow, due to severe neck flexor involvement. Although less common, muscle pain may occur (206).

Muscle weakness can either result from a loss of muscle fibers, as the consequence of the attack of cytotoxic T cells, MHC class I expression, or from a loss of vascular supply. In many patients, the actual degree of muscle weakness exceeds that expected from the extent of inflammation and damage seen in biopsies. These observations suggest that other factors must also play a role in producing muscle weakness. One possibility is that mediators released by inflammatory cells interfere with membrane dynamics that are crucial to muscle function. Peripheral blood mononuclear cells (PBMCs) from IIM patients have been reported to release mediators that inhibit calcium binding to SR (207). An abnormality in the SR of patients, leading to less viability to accumulate calcium, is also possible. The maintenance of the membrane integrity is essential for muscle contraction, and contraction itself requires energy (via ATP). It has been recorded that the ATP pools are depleted more rapidly in PM and DM patients and also that it takes longer to return to baseline ATP levels compared to healthy individuals (208). Mitochondrial abnormalities, measured as COX, COX negative fibers are a frequent finding in DM and IBM patients (209, 210). In DM patients, this increase is correlated to capillary loss (211). The combination of abnormal indices of energy state at rest with impaired recovery of high-energy phosphates after exercise strongly suggests abnormal mitochondrial function in IBM patients (212, 213). Impaired recovery of ATP is a more sensitive and specific index of impaired muscle oxidative metabolism, because recovery only depends on mitochondrial function (212, 213). Furthermore, another study found that IBM patients show a normal recovery rate of ATP, despite the presence of an abnormal resting nuclear magnetic resonance (NMR) spectroscopy (214) which indicates that the IBM pathophysiology of muscle weakness is not associated with defective oxidative metabolism. Muscle inflammation in DM patients is diminished with treatment along with an improvement in strength (215), suggesting that an altered energy metabolism is also an important component to the weakness. To date, it is not clear if the inflammation, abnormalities of the SR, capillary loss, MHC class I expression in muscle fibers, and/or mitochondrial

abnormalities are primary or secondary phenomenon leading to muscle weakness in IIM patients. This issue will be further discussed in the chapter “Different hypothesis of how and why muscle weakness is induced in IIMs”.

13.2. Muscle fatigue

Many of the above-mentioned factors, like capillary loss, ATP, SR, MHC class I expression in muscle fibers, and/or mitochondrial abnormalities, could also play a role in muscle fatigue in IIM patients. Of note, the skeletal muscle cells in an individual who displays muscle weakness have to be used closer to their maximum capacity during normal activity. Thus, while acute muscle fatigue only limits performance during normal extensive activities in young healthy individuals, it may severely hinder everyday physical activities in IIM patients.

14. Patients and methods used in my studies

The following section describes the main features of the methods used in the studies. Detailed protocols are described in the respective papers. All studies were approved by the Karolinska Institutet/Karolinska University Hospital, Solna and the Stockholm North local ethics committee. All patients gave their informed consent to participate.

14.1. Patients and healthy controls

Most patients included in this thesis were recruited from the Rheumatology Unit at Karolinska University Hospital, Solna. This is a non-selected referral center for the Stockholm area and nearby counties. PM and DM patients were diagnosed according to Bohan and Peters criteria (9, 10). IBM patients, who were diagnosed according to Griggs and colleagues criteria (16), were recruited from the Department of Neurology, Karolinska University Hospital, Solna.

Several groups of patients have been investigated in this thesis (Figure 9):

Group 1: This group of patients (8 PM and 6 DM) consisted of patients with a short duration of clinical symptoms and no detectable infiltration of inflammatory cells were found in muscle tissue (Paper III and VI).

Group 2: This group of patients (7 PM and 3 DM) had a short duration of clinical symptoms but muscle biopsies contained infiltration of inflammatory cells. This group of patients was treated with high doses of glucocorticoids. Muscle biopsies were obtained before and after the study period of 3 to 6 months (Paper III and V).

Group 3: This group of patients (8 PM and 2 DM) had a short disease duration. Muscle specimens were obtained from symptomatic and non-symptomatic muscle; the biopsy site was based on the patients' subjective symptoms of muscle weakness or tenderness. Both biopsies contained equally amounts of inflammatory cell infiltrates (Paper I).

Group 4: This group of treatment-refractory patients (5 PM, 4 DM, and 4 IBM) had a long disease duration of clinical symptoms and the muscle specimens contained inflammatory cell infiltrates. This group of patients was treated with infliximab at a

dose of 5 mg/kg body weight four times and biopsies were obtained before and after the study period of 16 weeks (Paper II and before biopsies in Paper VI).

Group 5: This group of chronic patients (5 PM and 4 DM) had a long disease duration and persisting muscle weakness despite effective therapy in diminution of inflammatory cell infiltration (Paper III and VI).

Figure 9

Early phase of disease	Early, active phase of disease	Active phase of disease	Late, chronic phase of disease
Patient group 1	Patient groups 2-3	Patient group 4	Patient group 5
Muscle weakness	Muscle weakness	Muscle weakness	Muscle weakness
Histopathological changes	Histopathological changes	Histopathological changes	No inflammation
No inflammation	With inflammation	With inflammation	

Figure 9. Schematic figure of the patient groups used in this thesis.

Muscle tissue with normal histopathological appearance from individuals without muscle weakness served as healthy controls. All controls were age and sex matched volunteers. Most of these biopsies were retrieved from a biobank through collaboration with professor Kristian Borg, Rehabilitation medicine, Danderyds Hospital, Sweden.

14.2. Muscle biopsy

Of all the laboratory investigations methods, the muscle biopsy is the most important tool for diagnosis. Obtaining a muscle specimen for histopathological study is an important component of the diagnostic evaluation for most suspected myopathies. Histopathological evaluation of muscle biopsies is also important to exclude other myopathies and to identify subsets of IIMs. To assess the effect of treatment on tissue levels it is important to use a muscle biopsy technique that allows for repeated biopsies. One such muscle biopsy technique is the percutaneous conchotome biopsy or the semi-open muscle biopsy (216). These methods have become widely used in Sweden and other Scandinavian countries during the last 20 years for both diagnostic and research purposes, but have received little attention outside of Scandinavia. The percutaneous conchotome muscle biopsy technique used in our clinic gives a good sized sample that allows for diagnostic evaluation in IIM patients (217). It is a simple procedure, easy to learn and to perform, with a low complication rate and minimum discomfort for the patient (216, 218). The method can preferably be used as a diagnostic tool and to perform repeated biopsies to assess the effect of a given therapy for both clinical and research purposes. The muscle biopsy is 100% specific for detecting inflammation in tissue but the sensitivity is low (66%) due to the patchy and focal nature of the infiltrates (18). Two ways to overcome the problem with “skip lesions” are to take

several biopsies from the same inclusion or to use MRI to direct the biopsy sampling (217, 219, 220).

Various methods for sampling muscle tissue are available, and each has advantages and disadvantages. An open biopsy generally harvests the largest amount of tissue and can be performed on numerous muscles, but this technique is invasive and gives a large scar. Conversely, needle biopsy has become or is becoming the standard method of muscle biopsy at some institutions in North America, Europe, and elsewhere in the world (221). Needle biopsy can be performed rapidly and is less invasive, but the disadvantage is a small amount of tissue per sample and a small cross sectional area of the muscle sample.

In my investigations I have mainly used muscles from the thigh muscle, m. vastus lateralis as this is often clinically involved in patients with IIM. I have also included some samples from m. deltoideus or m. tibialis anterior.

14.3. Immunohistochemistry and immunofluorescence techniques

Immunohistochemistry and immunofluorescence techniques are good tools to investigate molecules on the protein level. The immunohistochemistry technique alone, however, does not always distinguish staining to different cellular structures but together with immunofluorescence and confocal microscopy with high magnifications this issue could be resolved. These techniques, in combination with double staining and confocal microscopy, allow localization of protein expression to cellular structures such as sarcolemma and nuclei. However, the key to success using these methods concerns critical steps in tissue preparation and fixation, also the handling of the biopsy specimen is crucial and it is important to freeze the tissue sample as quickly as possible. The morphology of the cut specimen will highly depend on optimal handling during freezing and fixation. In our lab, we use formaldehyde for intracellular staining protocols and fixation with acetone is used for detection of cell surface markers. Permeabilization of cell membranes allows the penetration of antibodies, an important step to enable intracellular staining. In this thesis I have used saponin as a permeabilizing detergent. Saponin intercalates in the membrane and reversibly replaces cholesterol.

Conventional microscopic measurement is used for descriptive purposes, to visualize the staining, and to manually describe it using a scoring system. Although immunohistochemistry is a descriptive technique a computerized image analysis system can be used to perform repetitive visual evaluations. Thus, the use of computerized image analysis reduces subjectivity by applying automated routines, however, the computer cannot always “see” what should be counted as true positive staining.

14.4. Whole muscle measurements

In order to investigate muscle force in whole muscles I also investigated mouse muscles in my thesis. From these both the extensor digitorum longus (EDL) a glycolytic (with dominating type II fibers) and soleus muscles, oxidative (with dominating type I fibers) were dissected from both hindlimbs. Small stainless steel loops were tied, using thin nylon thread, to the tendon of the muscle. The muscle was mounted between a force transducer and an adjustable hook, which allowed the muscle

to be stretched to the length giving maximum tetanic force. Tetanic stimulation was produced by applying supramaximal current pulses (0.5 ms duration) via plate electrodes lying on each side of the muscle. The stimulation frequency and tetanic duration were 70 Hz and 300 ms for EDL muscles and 50 Hz and 600 ms for soleus muscles.

After mounting, muscles were allowed to rest for 30 minutes before fatiguing stimulation was started. Fatigue was produced by giving a tetanus every 2 second and the total number of tetani given was 50 for EDL and 100 for soleus. These fatiguing protocols were designed to give a force reduction in whole muscles. Recovery of force was followed by a single 70 Hz (EDL) or 50 Hz (soleus) tetanic contraction at 1, 2, 5, 10, 20 and 30 minutes after the end of fatiguing stimulation.

14.5. $[Ca^{2+}]_i$ measurements

Muscle weakness can also indirectly be measured in single muscle fibers by measure the myoplasmic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ was measured with the fluorescent Ca^{2+} indicator indo-1 in flexor digitorum brevis (FDB) fibers. Indo-1 was excited with light at 360 nm, and light emission at 405 ± 5 and 495 ± 5 nm was measured with two photomultiplier tubes. After correction for background and inherent fluorescence of the fiber the ratio (R) of the light emission at 405nm to that at 495 nm was translated to $[Ca^{2+}]_i$ using the following equation (222):

$$[Ca^{2+}]_i = K_D \beta (R - R_{min})(R_{max} - R)^{-1}$$

where K_D is the apparent dissociation constant of the dye, β is the ratio of the 495 nm signals at very low and saturating $[Ca^{2+}]_i$ and R_{min} and R_{max} are the ratios at very low and at saturating $[Ca^{2+}]_i$ respectively (223). Although force is not directly measured with this technique, a reduced tetanic $[Ca^{2+}]_i$ during fatigue is accompanied by decreased force production (224).

14.6. Clinical outcome measurement (used in paper II)

An international consensus of outcome measures for IIM patients has been developed and validated by the International Myositis Assessment and Clinical Studies Group (IMACS) (225-227). The core set of disease activity consists of six domains: physician and patient/parent global assessments of disease activity, muscle strength, physical function, serum activity of muscle enzymes, and an assessment tool to capture extra-skeletal muscle disease activity. To be rated as improved, three or more of the core set parameters must be increased $\geq 20\%$ and no more than two, excluding the manual muscle test (MMT), can be worsened. Worsening is defined by $\geq 30\%$ reduction in any three of the six variables of the IMACS core set disease activity (228).

15. Different hypothesis of how and why muscle weakness is induced in IIMs

My thesis is focused on the pathophysiology of IIMs with the overall aim to improve the molecular mechanisms of muscle weakness and muscle fatigue. More specifically I have postulated the following hypotheses that have been addressed in my studies.

- I. Muscle weakness could be induced by pro-inflammatory cytokines
- II. Muscle weakness could be induced by hypoxia
- III. Muscle weakness could be induced by MHC class I expression

15.1. Paper I. The pro-inflammatory cytokine hypothesis (IL-1)

This hypothesis is based on the assumption that pro-inflammatory cytokines have a direct effect on muscle contractility. Cytokines, which function as inter- and intracellular signaling molecules, are potent mediators of a number of cell functions and are essential in coordinating inflammatory responses. They can be produced by a large variety of cells and exhibit both anti- and pro-inflammatory effects, as they act in cascaded networks in a hierarchic fashion. A key role in chronic inflammatory diseases has been well documented by the often strikingly good response to therapies targeting pro-inflammatory cytokines. Recent findings suggested that cytokines are important key molecules in the pathogenic mechanisms of IIMs. As mentioned, a common histopathological finding in skeletal muscles of patients is the infiltration of mononuclear effectors cells, mainly consisting of T lymphocytes and macrophages. Several pro-inflammatory cytokines, e.g. IL-1, IFN- α , and TNF have been demonstrated in muscle tissue and sera from patients with PM or DM (229-232). The pro-inflammatory cytokines can induce proliferation and activation of immune cells as well as an enhancement of MHC molecule expression on immuno-competent cells and on human myoblasts and myotubes (233). Various pro-inflammatory cytokines, including IL-1, TNF, and IL-15, also have metabolic effects, which have been demonstrated to affect muscle cell metabolism and regeneration (234-236), indicating that pro-inflammatory cytokines can induce mechanisms leading to muscle dysfunction in patients with IIM. IL-1 is one of the most abundantly found cytokines in muscle tissue of patients with IIMs. IL-1 has several effects on muscle tissue, it can be toxic to human muscle cells in *in vitro* systems (237) and IL-1 α plays a role in muscle fiber regeneration, in which it can suppress myoblast proliferation as well as myoblast fusion, leading to poor muscle cell regeneration (238). IL-1 is mainly produced by activated macrophages but also by other cells, including endothelial cells (229, 231, 232). IL-1 possesses several biological properties resulting in the increased expression of pro-inflammatory genes. Maybe the most salient and relevant property is the ability to increase the expression of adhesion molecules such as intracellular adhesion molecule (ICAM)-1 on mesenchymal cells and vascular cell adhesion molecule (VCAM)-1 on endothelial cells, both of which have been found in muscle tissue of IIM patients (239). This latter property promotes the infiltration of inflammatory and immuno-competent cells into the extravascular space. The synthesis of IL-1 is induced by several other cytokines, such as TNF, IFN, and IL-1 itself (240).

There are two functional forms of IL-1: IL-1 α and IL-1 β . The IL-1 α precursor (proIL-1 α) is synthesized in association with cytoskeletal structures, unlike most proteins which are translated in endoplasmic reticulum (ER). ProIL-1 α is fully active as a precursor and remains active intracellularly. Even under conditions of cell stimulation, human PBMCs do not process or secrete mature IL-1 α . The opposite is the case with the IL-1 β precursor (proIL-1 β), which is not fully active and of which a considerable amount is secreted as mature IL-1 β following cleavage by a specific, intracellular cysteine protease, IL-1 β converting enzyme (ICE) (241). Because of the lack of leader peptide, proIL-1 α remains in the cytosol after translation, and there is no appreciable accumulation of IL-1 in any specific organelles. In studies of experimental inflammatory bowel disease, there is a better correlation of disease severity with colonic tissue levels of IL-1 α than of IL-1 β (242), presumably due to the cell-associated nature of IL-1 α . In contrast to IL-1 β , IL-1 α is not commonly found in the circulation or in body fluids except during severe disease, in which case the cytokine may be released from dying cells. Unlike IL-1 α , IL-1 β is frequently found in the circulation and levels of IL-1 β often, but not always, correlates with disease severity (243). IL-1 receptor antagonist (IL-1Ra) inhibits the activity of IL-1 by binding to the IL-1 receptor I (IL-1RI) without inducing a signal transduction (244). Therefore, IL-1Ra can act as an anti-inflammatory competitive inhibitor of IL-1. IL-1 has two unique receptors, IL-1RI and IL-1RII, which both can bind IL-1 α and IL-1 β . Only IL-1RI can associate with the IL-1R accessory protein (IL-1RAcP), which is a necessary step to induce a transduction signal. IL-1RII works as a decoy receptor and is more likely to bind to IL-1 β than IL-1 α , which in practice can result in a diminished response to IL-1 β (245, 246).

Based on the above mentioned observations, IL-1 might affect muscle fiber function and thus contribute to muscle impairment by mechanisms other than muscle fiber damage. A direct molecular effect of IL-1 on muscle fibers presumably would require an expression of IL-1 receptor on the muscle fibers' membrane. However, whether muscle fibers in human subjects' express IL-1 receptors was not known when I started my thesis work. Thus, the objective of my studies published in Paper I was to investigate if muscle fibers express IL-1RI and/or IL-1RII, and if so, whether there was a qualitative and quantitative difference in their expression between muscle from healthy individuals and PM or DM patients. Furthermore, we wanted to investigate whether IL-1 receptor expression was co-localized with that of IL-1 α , IL-1 β , and IL-1Ra.

Muscle biopsies from ten patients with PM or DM (patient group 3) and seven healthy controls were investigated by immunohistochemistry using antibodies against IL-1RI, IL-1RII, IL-1 α , IL-1 β , and IL-1Ra. Quantification was performed by computerized image analysis and localization of expression was determined by double staining using immunofluorescence and confocal microscopy.

Our results in Paper I showed that both IL-1RI and IL-1RII were expressed in muscle fibers, in inflammatory cells, and in endothelial cells. The muscle fiber expression was localized to the sarcolemma and to the nuclei. The nuclear expression was further verified by the observation that both IL-1Rs were detected in the nuclei of stimulated PBMCs. IL-1 α was expressed in endothelial cells and inflammatory cells whereas IL-1 β and IL-1Ra were only expressed in inflammatory cells. The expression of the two

IL-1Rs and their ligands was significantly higher in patients compared to controls. The IL-1Rs expression on muscle fibers was most pronounced in the vicinity of IL-1 α and IL-1 β expressing cells.

Both receptors were expressed on the membrane of muscle cells. A surface expression is of course likely on most IL-1 responsive cells. The biological activity of IL-1 has been found to be a better assessment for receptor expression than ligand binding to cell surface (247). The expression of IL-1 α and IL-1 β in muscle nuclei and its receptors raises the interesting possibility that IL-1 might function as a transcription factor; however this possibility was not elucidated in Paper I. It is known from data by Werman A. and colleagues that proIL-1 α can function as an intracrine pro-inflammatory activator of transcription (248). It also appears that one of the functions of intracellular IL-1 α is to decrease the threshold of NF- κ B and AP-1 dependent gene expression (248). NF- κ B is a major transcription factor modulating the cellular immune, inflammatory, and proliferative responses. Furthermore, NF- κ B has been shown to be activated in all subsets of IIMs (26, 31, 249-251). This indicates that IL-1 α might act upstream, leading to the activation of NF- κ B and AP-1. The fact that intracellular IL-1 α activates these transcription factors by an IL-1R-independent mechanism has not been previously a characterized role for cytokines in general. However, Werman A. and colleagues did not study intracellular levels of IL-1Rs in their cell line, instead they blocked possible receptors with IL-1Ra. Moreover, high mobility group box chromosomal protein (HMGB)-1 has also been found to act thru IL-1RI and IL-1RII (Zetterström et al., Thesis 2001), which indicates that IL-1 is not the only ligands for these receptors.

In Paper I, we found that IL-1 α , IL-1 β , IL-1Ra, and their receptors were expressed and co-localized in muscle cell nuclei. However, in another study IL-1 α and IL-1 β were found being expressed by muscle fibers with a sarcoplasmic appearance (252). This was in agreement with a study predominantly of DM patients where IL-1 was demonstrated to be expressed by muscle fibers undergoing ischemic damage and the expression by muscle fibers was associated with myofibrillar protein breakdown and regeneration (253). In line with other studies, we found that endothelial cells expressed IL-1 α and mononuclear inflammatory cells expressed IL-1 α , IL-1 β , and IL-1Ra (231, 232, 252). However we did not find sarcoplasmic expression of IL-1 in “normal” or atrophic fibers of PM or DM patients. An elevation of both IL-1Ra mRNA and protein has been found in sera and in culture supernatants of unstimulated monocytes from patients with active-stage PM and DM (254). This suggests that higher serum levels of IL-1Ra in myositis may reflect an increased production of IL-1Ra, and that IL-1Ra may regulate IL-1-mediated muscle fiber damage in PM and DM patients. Protein expression of IL-1 α and IL-1 β in PM and DM muscle and serum levels of IL-1Ra have been shown to be decreased in response to steroid treatment (232, 255). However, in our study of Paper I, we could not find any difference in protein levels of IL-1 or their receptors in muscle tissue between steroid-treated compared to untreated patients.

In conclusion, the increased expression of IL-1Rs and the co-localization with reciprocal ligands in patients but not in healthy controls supports the hypothesis of a crucial role of IL-1 in the pathogenesis of PM and DM. This could be mediated through

direct effects on muscle fibers and might affect muscle fiber metabolism and function. This makes IL-1 a possible target for treatment of these disorders.

15.2. Paper II. The effect of TNF-blockade in IIMs

This hypothesis is based on the assumption that by using TNF-blockade the harmful effects of IL-1 and TNF could be reduced. With the information of IL-1 as a consistently expressed cytokine in muscle tissue from patients with IIMs we were interested in using IL-1 blockade to investigate the role of IL-1 in disease mechanisms of IIMs. When we started this study there was no IL-1 blocking agent available for clinical use. However, TNF-blockade had successfully been used in patients with other chronic inflammatory diseases like rheumatoid arthritis (RA) and Crohn's disease. Additionally, there were *in vitro* studies that clearly suggested that blocking TNF could lead to reduction of IL-1 expression (256). There are also data which supports a role for TNF itself in the pathogenesis of PM and DM. TNF is present both at the mRNA and protein level in muscle tissue as well as increased serum levels of soluble TNF receptors (64, 257-260). Furthermore, one genetic study proposed an association between DM and the -308A TNF polymorphism (62). TNF has many physiological functions, some of which are important in the skeletal muscular system. TNF can induce, or augment, the production of other pro-inflammatory cytokines such as IL-1, IL-6, and IL-8 (261). Moreover, TNF is believed to be important in the pathogenesis of muscle wasting, with acute administration causing severe transient weight loss in rodents (262), and prolonged exposure resulting in profound wasting (262, 263). Site-specific TNF production also alters the patterns of tissue wasting (263), suggesting that local production may be sufficient to induce degradation. In addition, TNF can enhance its own secretion in myoblasts (261). Interestingly, TNF has also been found to have direct effects on muscle contractility (264).

Based on the molecular and histological observations described above, we concluded that TNF could be an attractive target for therapeutic interventions in IIMs. Experiences on the use of TNF-inhibitors in IIMs have been limited to date, and restricted to case reports and small case series.

Thirteen patients with treatment-refractory PM, DM, or IBM (patient group 4) were treated with 4 infliximab infusions (5mg/kg body weight) during a period of 14 weeks. Outcome measures included a myositis disease activity score with improvement defined according to IMACS (225-227), functional index (FI) of myositis (265), and MRI of thigh muscle. Repeated muscle biopsies were investigated by immunohistochemistry for cellular infiltrates, MHC class I and II, endothelial cells, and pro-inflammatory cytokines including TNF, IL-1, IL-6, HMGB-1, IFN- γ , MxA, and membrane attack complex (MAC) expression.

After completion of our trial, we concluded that infliximab treatment had only limited, if any, transient clinical effects in nine refractory IIMs patients who finished the study. Three patients discontinued due to adverse events and one due to a discovered malignancy. Three of the completers improved ($\geq 20\%$) in three or more variables of the disease activity core set, four were unchanged and two worsened ($\geq 30\%$). Importantly, no patient improved in muscle strength. The improvement was mainly seen in non-muscular variables, e.g. the number of tender joints. The absence of clinical

improvement in muscle strength was associated with persistent signs of muscle inflammation in muscle biopsies including expression of IL-1 and in MRI of thigh muscles. Furthermore we recorded a systemic increased type I IFN activity.

In contrast to our study, in another retrospective data analysis where eight patients with similar refractory PM and DM were treated with different TNF-blockade, a favorable outcome was reported in six cases (266). Notably, in this study, improvement was only assessed as reduced serum CK levels as well as by the physician's global assessment of improvement. No outcome measure that reflected muscle performance or patient perception was used. Another difference to our study was that in this retrospective case series six patients were treated with etanercept, one with infliximab, and one patient was sequentially treated with both agents. Of the six responders, five were treated with etanercept and one was treated with both agents. It cannot be excluded, that etanercept may have a more advantageous effect in IIM patients than infliximab, but this still needs to be tested with validated outcome measures. These results are consistent with several other previous reports on favorable outcomes with the use of TNF-blockers in smaller series of patients (267-271). The long-term follow-up report of one of those studies was less encouraging (269), as both patients in the study developed a relapse characterized by myalgia, symmetric muscle weakness of the proximal muscles, and an increase in serum CK. Another open-label controlled trial in which six drug-naïve recent-onset patients were enrolled, had to be stopped due to low inclusion rate but more importantly due to the high drop-out rate because of disease progression (272). The disease progression presented as progressive muscle weakness with a decrease in muscle strength of 20% or more when compared to the baseline value in two and progression of pulmonary fibrosis in one patient. The authors advised against the use of anti-TNF treatment in drug-naïve patients with PM and DM. In conclusion, the effect of etanercept is still controversial, it might be beneficial in some patients but so far the studies undertaken were limited and consisted of relatively few patients.

As some patients got worse in their muscle inflammation we searched for a possible molecular explanation. Interestingly we found increased type I IFN activity in sera that was associated with clinical worsening. Although the number of patients in our trial was relatively small, the lack of improvement in association with increased type I IFN activity was evident. A similar increased type I IFN activity was recorded after TNF-blockade using etanercept in patients with primary SS, likewise without any clinical improvement (273). Type I IFN activation has been implicated to have a role in the pathogenesis in DM and PM (21, 37). IFN- α and IFN- β are members of the type I IFNs, which are produced by pDCs (274-276). pDCs have been found to be expressed in muscle tissue of patients with IIMs (21, 35). Both IFN- α and IFN- β have several functions including, for example, the stimulation of cells to specialized protein production as a defense against pathogens. IFN- α can transform healthy monocytes into cells with properties of DCs, which was seen in sera from patients with active SLE (277). IFN- α can also contribute to plasma cell differentiation and hence may be important for the generation and sustenance of antibody responses (278). In concordance with other studies, we observed in our study an increased expression of the IFN- α/β inducible protein MxA in muscle tissue of IIM patients, predominantly localized to endothelial cells of capillaries (21). A significantly increased expression of BDCA-2 in muscle tissue of PM patients with anti-Jo-1 autoantibodies compared to

healthy controls has been found (37). Similar to our results, the expression of MxA in these patients was localized to both mononuclear inflammatory cells and capillaries (37). It has also recently been shown that type I IFN inducible gene is expressed in blood in PM and DM patients and reflects disease activity (279). Interestingly, we did not find any expression of IFN- γ in the muscle tissue of our included patients. IFN- γ is a member of type II IFNs and is believed to be able to generate tolerogenic DCs (280). It seems most likely that type I IFNs and IFN- γ exert opposite effects on DC function.

In conclusion, the absence of clinical improvement, the persisting inflammatory infiltrates in muscle tissue, and the activation of the type I IFN system in the circulation upon anti-TNF treatment suggests that TNF is unlikely to be a key molecule in the disease mechanism in cases of treatment-resistant IIMs. Whether IL-1 has a role in disease mechanism could not be answered by our study as the expression of this molecule was not affected by the TNF-blockade. From a clinical perspective, the clear radiological and clinical worsening in several cases suggests that infliximab is not a drug to be used in IIM patients.

15.3. Paper III. The hypoxia hypothesis

This hypothesis is based on the assumption that hypoxia could induce effects on muscle tissue leading to muscle impairment. One of the mechanisms leading to the impaired muscle function seen in IIM patients could be a loss of capillaries, which has been reported in patients with DM, even in early cases without detectable inflammatory infiltrates (281, 282). Another observation that suggests a disturbed microcirculation in muscle tissue is the presence of morphologically changed endothelial cells resembling of high endothelium venules (HEV) indicating that they are activated (283). Notably, such phenotypically changed endothelial cells were observed in muscle tissue even without detectable inflammatory cell infiltrates in newly diagnosed cases. This, together with the loss of capillaries in early cases of DM, suggests that endothelial cells may be a primary target of the immune response, at least in a subset of myositis patients (231).

Capillaries are important for the microenvironment in muscle tissue, for the recirculation of nutrients, as well as for the homing of lymphocytes that is accomplished via an interaction with endothelial cells. Phenotypically altered microvessels might affect the local circulation of the muscle and hence lead to the development of tissue hypoxia and metabolic alterations, such as reduced levels of ATP and phosphocreatine (PCr) reported in PM and DM. These compounds are important energy sources for muscle contractions and low levels of these could cause muscle weakness and fatigue. Interestingly, in a subgroup of DM patients without clinical myositis - amyopathic DM - normal ATP and PCr levels were observed at rest, but low levels were recorded after exercise, suggesting a subclinical metabolic disturbance in muscles of these patients. The reported increased urinary secretion of PCr might likewise reflect a dysfunctional muscle metabolism (284). Interestingly, the low levels of ATP and PCr that can be induced by tissue hypoxia has also been confirmed to dynamically modulate endothelial functions (285). IIM patients have an increased endothelial expression of ICAM-1 and VCAM-1 (232). Binding to these molecules enables effector cells to migrate through blood vessels walls. Both ICAM-1 and VCAM-1 are known to be up-regulated by hypoxia, which is also the case for cytokines

like IL-1 α , IL-1 β , and transforming growth factor (TGF)- β , all highly expressed in muscle tissue of patients with PM and DM (232, 286-289). From this perspective, it is attractive to speculate that involvement of micro-vessels with morphologically and/or reduced numbers of capillaries, increased expression of adhesion molecules as well as cytokines, could result in local tissue hypoxia in muscle tissue and subsequently lead to muscle weakness in patients with IIMs. This hypothesis is supported by the findings in an earlier small pilot study (290). Also the reported benefits of exercise in patients with IIM support the hypoxia hypothesis (291).

Vascular endothelium growth factor (VEGF) is induced by hypoxia and could function as an angiogenic growth factor *in vivo* that also has pro-inflammatory properties (292, 293). To test the hypothesis that IIMs patients have a hypoxic state in their skeletal muscles, we investigated the number of capillaries and larger vessels and the expression of VEGF in muscle biopsies and serum samples of PM and DM patients and healthy controls.

Muscle biopsies (from patient groups 1, 2, and 5) and serum samples from 56 patients with PM or DM and 15 muscle biopsies as well as serum samples from 56 healthy controls were analyzed. Depending on disease-duration and the presence or absence of inflammatory infiltrates, patients were divided into three groups: (i) Patients with clinical signs of active PM or DM without inflammatory cell infiltrates (patient group 1); (ii) Patients with clinically active PM or DM with inflammatory cells infiltrates, before and after corticosteroid treatment (patient group 2); (iii) Patients with chronic PM or DM without inflammatory cells infiltrates (patient group 5). VEGF and the vessel marker CD31 expression in skeletal muscle were analyzed by immunohistochemistry, VEGF mRNA-expression by *in situ* hybridization, and VEGF serum levels by ELISA.

We found a low total number of capillaries patients with short duration of symptoms and without inflammation in muscle tissue. The number of capillaries was equally low in PM and DM patients, indicating that a loss of capillaries is an early event in both subsets of myositis. The reduced numbers of capillaries were associated with an increased VEGF expression in muscle fibers. Furthermore, the muscle cell itself most likely produces VEGF expression in muscle fibers. This is supported by the observation that mRNA levels of VEGF were expressed adjacent to muscle nuclei. In an active phase of the disease with inflammation, an increased number of capillaries could be seen together with an increased VEGF expression in endothelial cells, muscle fibers, and inflammatory cells. VEGF was not down-regulated by high levels of glucocorticoids treatment for 3 to 6 months. In the chronic phase of the disease, a reduced number of capillaries compared to healthy individuals' were evident but no increase of VEGF could be found. Significantly increased serum levels of VEGF were found in untreated patients compared to treated patients and controls. We could identify muscle fibers as a source of VEGF, by mRNA and protein level both in healthy individuals and patients.

The results of Paper III indicate that early in the disease, even before inflammation is detectable in muscle tissue there is a hypoxic state in skeletal muscle of both PM and DM patients. Furthermore, this phenomenon is not restricted to DM patients only as

earlier believed (281, 282). Moreover, in patients with established disease with inflammatory infiltrates, total VEGF-expression was high compared to healthy controls, and no difference in the number of capillaries could be found. Thus, an increased VEGF expression in muscle fibers was determined in early phases of disease. This expression could be explained by the loss of capillaries, which would then lead to tissue hypoxia and consequently an induction of VEGF production would begin presumably in muscle fibers. The higher number of capillaries with inflammatory infiltrates present could suggest a VEGF induced angiogenesis, which is supported by a previous paper in patients with DM (294).

In conclusion, our data supports an important role of VEGF in the pathophysiology in the early phases of disease and supports our hypothesis that local muscle hypoxia is a contributing factor to reduced muscle endurance and fatigue.

15.4. Paper IV. The MHC class I hypothesis

This hypothesis is based on the assumption that MHC class I could mediate muscle weakness. The study in Paper IV aimed at investigating and characterizing muscle function in a transgenic mouse model mimicking human IIMs. While differentiated skeletal muscle fibers do not constitutively express or display MHC class I molecules under physiological conditions, it is a characteristic early event in patients with IIMs (28-30). MHC class I expression in muscle fibers is such a common early finding in IIM patients that this observation has even been considered specific enough for use as a diagnostic criterion (29, 295, 296). MHC class I expression in muscle can be induced by several pro-inflammatory cytokines (29, 33, 233, 297) and many of these are expressed in muscle tissue of IIM patients (231, 258). Several findings indicated that MHC class I itself can mediate muscle weakness in both clinical and experimental settings. For instance, gene transfer of MHC class I plasmids can attenuate muscle regeneration and differentiation (298). One suggested mechanism for a non-immune dysfunction of muscle fibers is the so-called “ER stress response”. The folding, exporting, and processing of newly synthesized proteins occurs in the ER, including the processing of MHC class I molecules. ER stress response could be induced as a protective mechanism when newly formed proteins overload ER, e.g. due to an infection, hypoxia or other causes. Two major components of the ER stress response pathway, the unfolded protein response (glucose-regulated protein 78 pathway) and the ER overload response (NF- κ B pathway), are highly activated in muscle tissue in both human DM and in a transgenic MHC class I mouse model (31). This indicates that ER stress response might be a major non-immune mechanism responsible for skeletal muscle damage and muscle dysfunction without muscle fiber damage. The over-expression of MHC class I could trigger the NF- κ B pathway through an ER overload response and NF- κ B consecutively activate several pro-inflammatory target genes as well as endogenous MHC class I production and thus initiate a self-sustaining loop (31). In a transgenic mouse model, MHC class I expression is specifically induced in skeletal muscle fibers (195). These mice develop clinical, biochemical, histological, and immunological features of human IIMs. Even though the myopathic mice showed decreased activity in open field behavioral activity measures (195), it has not been determined whether these mice have any specific impairment in muscle function. Thus, the pathological expression of MHC class I molecules could have a major impact in chronic immune mediated diseases and could hypothetically be a possible cause of

muscle impairment in IIMs. Furthermore, it is not clear if the induction of MHC class I could directly lead to muscle impairment.

In study IV we used the above described transgenic mouse model (195) to investigate whether MHC class I expression could induce muscle impairment. Muscle force development, fatigue, and recovery was studied in isolated EDL (fast-twitch) and soleus (slow-twitch) muscles of both myopathic (n=8) and control mice (n=8). Immunohistochemical stainings for Mayer's haematoxylin and eosin and different fiber types and cross-sectional area, enzymatic assays for mitochondrial and glycogenolytic enzymes, and western blots for HMGB-1 were performed.

We characterized muscle physiology properties in this model and found a reduction in force production in myopathic mice compared to controls. This reduction was associated with a decrease in cross-sectional area in EDL muscles whereas it was due to a decrease in the intrinsic force-generating capacity in soleus muscles. The pathologic significance of these two observations is (i) that slow-twitch fibers may have marked defects in their force-generating capacity despite relatively normal appearance in histochemical analyses of muscle biopsies, and (ii) muscle weakness can be severe despite relatively small changes in muscle cross-sectional area and fiber type composition. EDL muscle was composed of type II fibers and soleus muscle contained a mix between type I and II fibers, as expected. Furthermore, the fiber cross-sectional area was smaller in myopathic compared to control EDL muscles. This is in accordance with the smaller size of the isolated myopathic EDL muscles, and was in contrast to the soleus muscle where we saw an increase in muscle size. Inflammation measured by HMGB-1 was markedly increased with western blotting. In addition, a localization of mononuclear infiltrating cells in muscle tissue of myopathic mice was seen. However no changes were found in the activities of key mitochondrial and glycogenolytic enzymes in myopathic mice.

It is not clear from our study if it is the MHC class I up-regulation *per se* or if it is the inflammatory stimuli which MHC class I induce that leads to the observed muscle weakness. It has been shown in several studies that pro-inflammatory cytokines can lead to muscle weakness (264) and it is also well documented that fast-twitch fibers are more susceptible to inflammation-induced atrophy than slow-twitch fibers (299-304). Therefore, we cannot fully exclude the possibility that the muscle weakness seen in this model is solely dependent on MHC class I up-regulation or on the inflammatory state induced by the induction of MHC class I.

Seeking support for the latter possibility, we investigated muscle force production, with the same experimental set up as used in Paper IV, in an arthritis model (collagen induced arthritis (CIA) mouse model). The muscle force production in this arthritis model was reduced in a similar way as in the MHC class I model; with a force reduction that was associated with a decrease in cross-sectional area in EDL muscles and a decrease in the intrinsic force-generating capacity in soleus muscles. However, an even more obvious force reduction was distinguished in the highly affected paws compared to low affected paws (unpublished results). This indicates that reductions in muscle force in this arthritis model and possible in the MHC class I model are strongly correlated with inflammation.

In conclusion, MHC class I expression on muscle fibers might have a more central role in mediating muscle weakness in muscle disease than previously assumed. The muscle weakness observed could possibly depend on the inflammatory state induced by MHC class I and not solely on MHC class I up-regulation *per se*.

15.5. Paper V-VI. The pro-inflammatory cytokine hypothesis (HMGB-1)

This hypothesis is based on the assumption that pro-inflammatory cytokines have a direct effect on muscle contractility. As described above, several pro-inflammatory cytokines have been demonstrated in muscle tissue from patients with PM or DM (229-232) but blocking TNF was not successful in the treatment of muscle impairment in patients with IIMs, as demonstrated in Paper II. These findings suggested that other cytokines or mediators could be of higher functional importance. HMGB-1 is a non-histone nuclear protein that displays potent pro-inflammatory activity when released from cells, serving as an alarmin. Upon release from cells, HMGB-1 can stimulate monocytes to produce pro-inflammatory molecules in a downstream cascade fashion (305) and exert effects on other cell types in a manner similar to conventional cytokines. In this context HMGB-1, a ubiquitous eukaryotic nuclear protein, emerges as a possible candidate in the pathogenesis since HMGB-1 can be released from any eukaryotic nuclei and exert extracellular pro-inflammatory functions (306-309). HMGB-1 is thought to act mainly through the receptor for advanced glycosylated end products (RAGE) (310) and toll like receptors (TLR) (311). Furthermore, HMGB-1 has also been found in several inflammatory and autoimmune diseases like cancer (312), sepsis (313, 314), RA (315, 316), acute lung inflammation (317), and heart failure (318).

The aim of Paper V was to assess if HMGB-1 was expressed in muscle tissue of PM or DM patients and, if so, whether such expression could be modulated by glucocorticoid treatment.

Muscle biopsies of patients with active inflammation in muscle tissue obtained before and after glucocorticoid treatment (patient group 2) and healthy controls were immunohistochemically stained for HMGB-1 expression. We found that HMGB-1 was expressed in infiltrating mononuclear cells, endothelial cells, and interestingly also by the muscle fibers itself both in the muscle cell nuclei and in the myoplasm. The expression was found intranuclearly, cytosolic, and extracellularly. In muscle biopsies of patients after prednisolone treatment, both the cytoplasmic and extracellular expression was reduced, coinciding mainly with a decreased number of infiltrating mononuclear inflammatory cells. Cytoplasmic expression of HMGB-1 was still evident in endothelial cells and muscle fibers. We could not find any expression of HMGB-1 in healthy controls. Extracellular release of HMGB-1 from inflammatory cells has been found in several other rheumatic conditions e.g. RA (315, 316). Interestingly, intra-articular therapy with triaminolone hexacetonide did not affect HMGB-1 expression in endothelial cells in RA patients (316). Another study found that the cytoplasmic and extracellular expression of HMGB-1 was decreased in five RA patients, remained unchanged in one patient, and increased in three patients after nine weeks of treatment with infliximab, rendering the overall change in HMGB-1 protein expression not significant. No correlation between the clinical response (DAS28 and ACR20, 50, and 70) could be found (319). These results suggest that HMGB-1 might

serve as a possible TNF-independent target molecule for biological therapy. To our knowledge, no pro-inflammatory cytokine has been shown with such a myoplasmic expression in muscle fibers previously. This could indicate that HMGB-1 is an early inducer of disease, even earlier than local muscle inflammation. Furthermore, we could not detect any reduction of HMGB-1 in muscle fibers after therapy, which could indicate that HMGB-1 expression is resistant to therapy and could therefore sustain the disease progression.

In Paper VI, we investigated the role of HMGB-1 in the pathogenesis of IIM by testing the hypothesis that HMGB-1 can induce MHC class I expression in muscle fibers and mediate the inflammatory cascade in IIMs that causes impaired muscle function.

In Paper VI we investigated patients at different phases of disease with or without inflammatory infiltrates (patient groups 1, 4 (before treatment only), and 5). In addition to immunohistochemical stainings of HMGB-1, MHC class I, and regeneration markers, we performed *in vitro* experiments in which differentiated mouse muscle fibers were stimulated with recombinant IFN- γ to elucidate if the endogenous expression of HMGB-1 could be translocated from the nucleus to the myoplasm. Various concentrations of recombinant HMGB-1 was used to assess the effect on MHC class I expression and if HMGB-1 could influence muscle performance assessed by measuring $[Ca^{2+}]_i$. RAGE^{-/-} mice were used to examine if HMGB-1 was acting through this receptor in skeletal muscles.

In this study we confirmed our earlier results from Paper V where we found that HMGB-1 was expressed in the myoplasm and in the nuclei of muscle fibers. However, we also found that HMGB-1 was co-localized with MHC class I expression in muscle fibers. Furthermore, in early disease without inflammation, fibers with HMGB-1 outnumbered fibers expressing MHC class I. HMGB-1 is known to act as a “necrotic marker” when found in the extracellular milieu (320) but we could not find any increased amounts of HMGB-1 in or adjacent to a necrotic area. In addition, no local “active secretion” from immune cells (321, 322) could be found in the two groups of patients without detectable inflammatory cell infiltrates. How HMGB-1 could be released without the “passive release” from necrotic cells or through the “active secretion” from immune cells is an intriguing question for us, which is still unanswered. Extracellular HMGB-1 is known to be able to orchestrate a defensive inflammatory response to ischemia, burn, infection, or sepsis and initiate tissue regeneration. Both HMGB-1 and MHC class I have been found expressed in regenerating fibers (323, 324). In agreement with earlier findings we found that a number of the HMGB-1 and MHC class I expressing fibers were regenerating fibers, however the majority were non-regenerating fibers.

Stimulation of wild-type mouse muscle fibers with recombinant HMGB-1 induced an up-regulation of MHC class I and impaired Ca^{2+} release from SR during induction of fatigue. After removal of HMGB-1 from the *in vitro* cultures, the MHC class I expression was reversed but the fibers still showed premature development of fatigue. This observation suggests that the impaired Ca^{2+} release from SR induced by HMGB-1 is independent of MHC class I expression. Because of the sigmoid relation between calcium concentration and force, it is most likely that the decrease in calcium, which

we observed, has a disproportionate effect on contraction and therefore will result in less force, especially during the induction of fatigue, as reduced tetanic $[Ca^{2+}]_i$ accompanied by decreased force production during fatigue has been previously reported (224). By using the same experimental set up, we have found that both IL-1 β and TNF could induce a similar impaired Ca^{2+} release from SR during fatigue (unpublished results). In line with our study, another group found that HMGB-1 depresses L-type calcium current in cardiac myocytes (318). These authors also observed that HMGB-1 resulted in a decreased fluorescence of fluo-3 suggesting a reduced release of calcium from SR. The same study found that the negative inotropic effects of HMGB-1 were partly mediated via RAGE and TLR4 in cardiac myocytes (318). In contrast, we found that the effects of HMGB-1 were not mediated through RAGE receptors and we did not identify RAGE expression in skeletal muscle of wild-type mice. Interestingly, both RAGE and TLR4 have been found to be decreased in cultured myoblasts after five days in differentiation medium (323). Interestingly, HMGB-1 has been found to act via IL-1RI and IL-1RII (Zetterström et al., Thesis 2001). Since in Paper I, we found that IL-1Rs and their ligands were expressed in muscle fiber nuclei and in Paper V-VI we found that HMGB-1 is also expressed in muscle fiber nuclei, this could indicate that HMGB-1 might partly act through IL-1Rs in muscle fibers. Subsequently, this could indicate that IL-1 and HMGB-1 could work together and regulate its own expression in muscle fibers and consequently regulate muscle fiber weakness.

We found that HMGB-1 was translocated from the muscle nuclei into the muscle fiber cytoplasm after stimulation with recombinant IFN- γ . Thus, HMGB-1 can serve as an endogenous pro-inflammatory stimulus to muscle fibers. Although HMGB-1 was originally described as a late mediator of inflammation and endotoxin lethality (313), more recent studies have shown that HMGB-1 plays an important role as an early inflammatory marker following acute tissue injury. Increased HMGB-1 expression has for example been demonstrated in lungs within 4 hours of inducing hemorrhagic shock (325). Similarly, HMGB-1 levels in hepatocytes in ischemia-reperfusion injury of the liver were increased within 1 hour (326). Taken together, these findings suggest that HMGB-1 could induce acute effects as well as inducing deleterious effects later in the disease phase.

In conclusion, the results of the studies in Paper V and VI suggest that HMGB-1 could directly promote muscle weakness in IIMs. In our experimental setting, HMGB-1 irreversibly initiated muscle weakness, and endogenous HMGB-1 could be translocated from the nuclei into the cytosol of differentiated muscle fibers. Since HMGB-1 is widely expressed in the myoplasm of IIM patients without inflammatory cell infiltrates with short duration of symptoms, these findings point to reduction of myoplasmic HMGB-1 as a potential target for therapy in these disorders.

16. Concluding remarks

During my work within this thesis, I was able to elucidate different pathogenic mechanisms in IIMs that could be responsible for muscle impairment. The results presented herein support our hypothesis that a decreased number of capillaries and certain immunological molecules expressed in muscle tissue of IIM patients, in particular MHC class I, IL-1, and HMGB-1, might be connected to the clinical symptom of decreased muscle function.

In this thesis I have found that muscle weakness could be a cause from either (i) hypoxia as a consequence of low number of capillaries, (ii) an inflammatory state in muscle fibers or (iii) a MHC class I expression on muscle fibers. Early in the disease course, before inflammatory cells are infiltrating the muscle tissue we found both a low number of capillaries and an increased expression of VEGF and HMGB-1. Thus, even if we can not find any inflammatory cells in the muscle tissue the muscle fibers seem to be capable to express pro-inflammatory cytokines themselves. Another important finding in this thesis is that muscle weakness could be induced by MHC class I upregulation in fibers but is not dependent of MHC class I expression *per se*. Our data indicate that MHC class I expression in muscle fibers may be capable to induce other signals which could lead to muscle weakness and that these mechanisms may be different and vary depending on the oxidative capacity of muscle fibers. Moreover, we found that IL-1 receptors and their ligands were expressed in muscle fiber nuclei which could imply that IL-1 might regulate its own expression in muscle fibers. The other consistently expressed pro-inflammatory cytokine in muscle tissue, HMGB-1 has by others (Zetterström et al., Thesis 2001) been found to interact with IL-1RI and IL-1RII, indicating that HMGB-1 can utilize IL-1Rs to regulate its own expression in muscle fibers. Thus, intracellular functions of IL-1 and HMGB-1 might play an unforeseen role in the pathogenesis of inflammation in muscle fibers. Both these molecules are potential targets for treatment of IIMs (Figure 10).

In conclusion, both immune-mediated and non-immune mediated mechanisms seem to be involved in the pathogenic mechanisms that causes impaired muscle performance in patients with IIMs, this needs to be recognized in the development of new therapies for patients with IIMs.

Figure 10

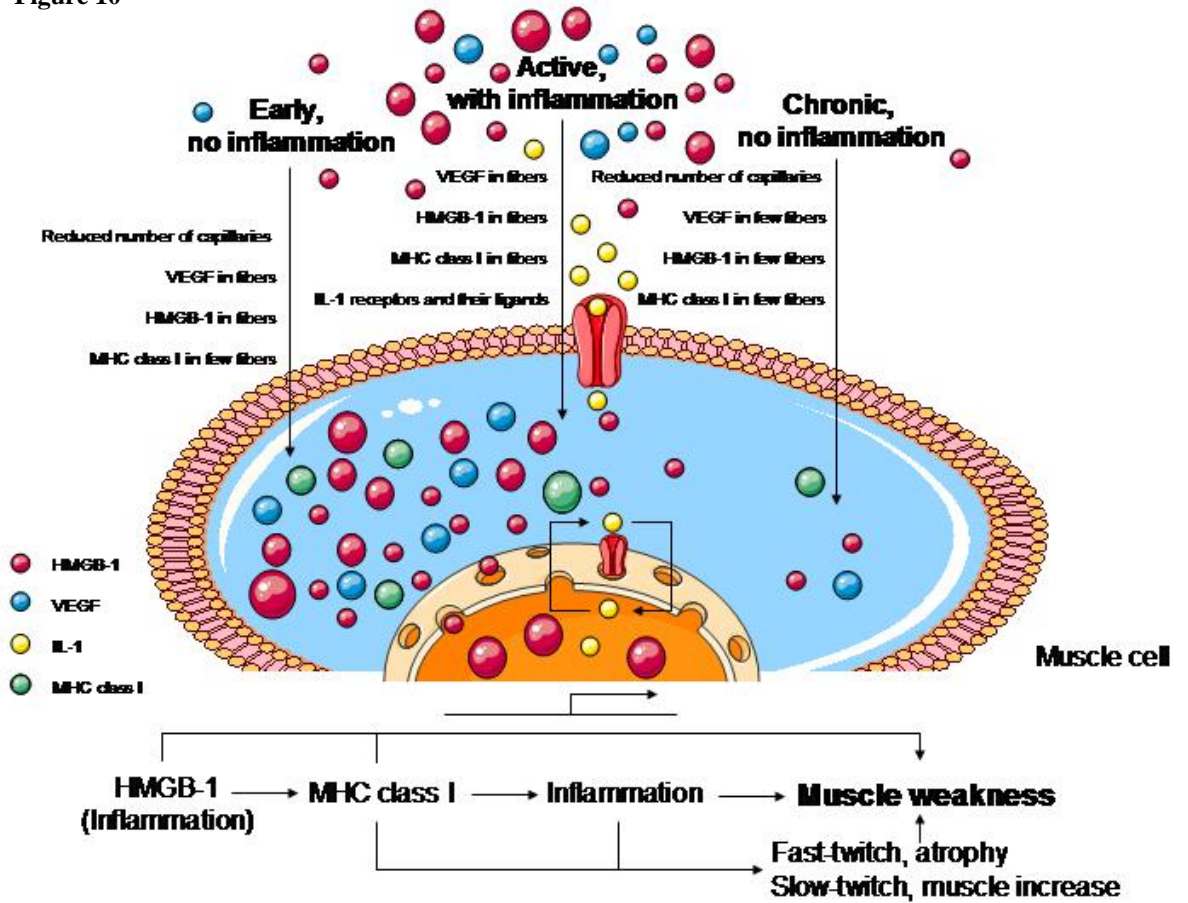


Figure 10. Schematic illustration of the major findings in my thesis. Parts of the figure are provided by courtesy of Servier.

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