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IMMUNE MECHANISMS IN CHRONIC RHEUMATIC MUSCLE INFLAMMATION, MYOSITIS

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Immune mechanisms in chronic rheumatic muscle inflammation, myositis

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To my Family

ABSTRACT

The idiopathic inflammatory myopathies (IIM), shortly named myositis, is a group of heterogeneous and rare autoimmune diseases with the target tissue of skeletal muscle. It is not curable and affects the daily life of patients. The treatment of myositis is mainly glucocorticoids in combination with immunosuppressive agents. The incomplete response and common side effects of conventional treatment requires new therapies. The pathological mechanisms of myositis are still not known, however, it is widely accepted that both innate immunity and adaptive immunity contribute to the pathogenesis of myositis.

T cells play an important role in the pathogenesis of myositis, which is also a promising target to develop novel treatment. The main aim of **Project 1 and 2** was to investigate the effects of abatacept (CTLA4-Ig), a costimulatory modulator of T cells, on disease activity, expression of different molecules in muscle biopsies, and phenotypes of T and B cells in blood samples of adult patients with refractory dermatomyositis (DM) or polymyositis (PM). In this pilot study, we found that almost half of the DM and PM patients obtained improvement of disease activity after abatacept treatment and few side effects. The elevated number of FOXP3$^+$ Tregs in repeated muscle biopsies indicates a positive effect of treatment in muscle tissue. CyTOF technology requires a larger patient cohort for discovery research and for immune-monitoring if the patients are clinically heterogeneous. Furthermore, CD4/CD8 ratio in circulation at time of active disease may be a predictor of response to abatacept treatment in patients with DM and PM.

Type I IFN is also thought to be an important pathway involved in the pathogenesis of patients with DM and PM. In **Project 3**, we found evidence to support our hypothesis that CD66b$^+$ neutrophils express LL-37, which may stimulate BDCA2$^+$ pDCs to produce type I IFN (MxA) in muscle and skin samples of patients with DM and PM, regardless of short or long disease duration. The higher number of CD66b$^+$ neutrophils and the association between neutrophils and MxA in patients negative for autoantibodies targeting RNA-binding proteins may suggest that our hypothesis is a potential alternative pathway to induce the elevated type I IFN in myositis patients without these autoantibodies.

In **project 4**, we aimed to identify biomarkers in repeated muscle biopsies or blood samples, taken before and after conventional immunosuppressive therapy, to predict therapeutic response in patients with myositis. In this pilot study, we conclude that baseline biopsy, or monocyte profile did not predict long-term treatment response, but in the repeated biopsy taken within 1 year of immunosuppressive treatment, the lower number of macrophages (CD68$^+$) seemed to predict a more favorable long-term response with regard to improved muscle strength. Furthermore, T cells in muscle biopsies were not affected by the conventional immunosuppressive therapy. This may indicate that repeated muscle biopsies provide additional information to guide immunosuppressive treatment.

In conclusion, our findings provide more information about effects of treatment and pathologic immune mechanisms of myositis and strengthen the critical role of innate and adaptive immune response in the pathogenesis of myositis by investigation from different perspectives, which may provide basis to develop novel and effective therapies for patients with myositis.

LIST OF SCIENTIFIC PAPERS

I. Tjärnlund A*, **Tang Q***, Wick C, Dastmalchi M, Mann H, Tomasová Studýnková J, Chura R, Gullick NJ, Salerno R, Rönnelid J, Alexanderson H, Lindroos E, Aggarwal R, Gordon P, Vencovsky J, Lundberg IE. Abatacept in the treatment of adult dermatomyositis and polymyositis: a randomised, phase IIb treatment delayed-start trial. Ann Rheum Dis. 2018;77(1):55-62.

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LIST OF ABBREVIATIONS

ABC	Avidin-biotin-peroxidase complex
ACR	American College of Rheumatology
AH	Ancestral hapolotype
ALT	Alanine transaminase
ASS	Anti-synthetase syndrome
AST	Asparate transaminase
APCs	Antigen presenting cells
ARS	Aminoacyl tRNA synthetases
CADM	Clinical amyopathic dermatomyositis
Citrus	Cluster identification, characterization, and regression
CK	Creatine kinase
cN-1A	Cytosolic 5′-nucleotidase 1A
CoA	Coenzyme
CTLA-4	Cytotoxic T-lymphocyte-associated protein-4
C5b	Complement protein 5b
C9	Complement protein 9
CyTOF	Time of Flight Mass Cytometry
DAB	3, 3’-diaminobenzidine
DAMPs	Damage associated molecular patterns
DAPI	4’, 6-diamidino-2-phenylindole
DCs	Dendritic cells
DLE	Discoid lupus erythematosus
DM	Dermatomyositis
DMSO	Dimethyl sulfoxide
DOI	Definition of Improvement
dsRNA	Double-stranded RNA
EBV	Epstein-Barr virus
EJ	Glycyl tRNA synthetase

ER	Endoplasmic reticulum
EULAR	European League Against Rheumatism
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FHL1	Four-and-a-half LIM domain 1
FI-2	Functional index-2
GC	Glucocorticoid
GWAS	Genome-wide association study
HAQ	Health Assessment Questionnaire
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCMV	Human cytomegalovirus
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
HMGCR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
HRP	Horseradish peroxidase
HTLV-1	Human T-cell lymphotropic Virus Type I
ICAM-1	Intercellular adhesion molecule-1
Ig	Immunoglobulin
IFN	Interferon
IIM	Idiopathic inflammatory myopathies
IL	Interleukin
ILD	Interstitial lung disease
IL-1R	Interleukin-1 receptor
IMACS	International Myositis Assessment and Clinical Studies Group
IMNM	Immune mediated necrotizing myopathy
iNOS	Inducible nitric oxide synthetase
IRFs	Interferon regulator factors
ISG	Interferon-stimulated gene
IVIGs	Intravenous immunoglobulins

JDM	Juvenile dermatomyositis
Jo-1	Hystidyl tRNA synthetase
KS	Asparaginyl tRNA synthetase
LDH	Lactate dehydrogenase
MAAs	Myositis-associated autoantibodies
MAC	Membrane attack complex
MDA5	Melanoma differentiation-associated protein 5
MDAAT	Myositis Disease Activity Assessment Tool
MHC	Major histocompatibility complex
MHCn	Myosin heavy chain neonatal
MITAX	Myositis Intention to treat Activity Index
MMF	Mycophenolate mofetil
MMT-8	Manual muscle test-8
MS	Multiple sclerosis
MSAs	Myositis-specific autoantibodies
MST	Minimum spanning tree
MxA	Myxovirus resistance protein A
MYOACT	Myositis Disease Activity Assessment Visual Analogue Scales
NF-κB	Nuclear factor kappa B
NHS	National health service
NK	Natural killer
NuRD	Nucleosome remodeling-deacetylase
NXP-2	Nuclear matrix protein-2
OJ	Isoleucyl tRNA synthetase
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pDCs	Plasmacytoid dendritic cells
PL-7	Threonyl tRNA synthetase
PL-12	Alanyl tRNA synthetase

PM	Polymyositis
PRRs	Pathogen recognition receptors
RA	Rheumatoid arthritis
RIG	Retinoic acid inducible gene
RIM	Rituximab in Myositis
SAE	SUMO-1 activating enzyme
SCLE	Subacute cutaneous lupus erythematosus
sIBM	Sporadic inclusion body myositis
SLE	Systemic lupus erythematosus
SPADE	Spanning-tree progression analysis of density-normalized events
SRP	Single recognition particle
SUMO-1	Small ubiquitin-like modifier-1
TCR	T cell receptor
TGF	Tumor growth factor
T_{FH} cells	T follicular helper cells
T_H cells	T helper cells
TIF1γ/α	Transcription intermediary factor 1γ/α
TLR	Toll like receptor
TNF	Tumor necrosis factor
Treg cells	T regulatory cells
Tregs	T regulatory cells
t-SNE	t-Distributed Stochastic Neighbor Embedding
UV	Ultraviolet
VAS	Visual analogue scale
V_{O2} max	Maximal oxygen uptake
YPS/HA	Tyrosyl tRNA synthetase
ZO	Phenylalanyl tRNA synthetase

1 INTRODUCTION

The idiopathic inflammatory myopathies (IIM), briefly named myositis, is a group of chronic inflammatory diseases, with an incidence around 11 cases per million people per year [1, 2]. Muscle weakness is the main clinical manifestation, however, other organs are often involved like skin, lung, and the heart. Cardiovascular complications, interstitial lung disease (ILD), and cancer are common causes of death of myositis patients [3, 4]. Myositis in adults has for several years mainly included three subsets based on distinct clinical and laboratory characteristics. Dermatomyositis (DM) that occurs in both adults and children (juvenile dermatomyositis, JDM), with skin involvement and may be associated with cancer. Polymyositis (PM) mainly affects adults and is more common in women than men. Patients with sporadic inclusion body myositis (sIBM) are usually men over 50 years of age at disease onset and the myopathy is treatment resistant [1]. However, there are also other more recently suggested subgroups of myositis, such as clinically amyopathic dermatomyositis (CADM), immune mediated necrotizing myopathy (IMNM), anti-synthetase syndrome (ASS) and myositis with overlap syndrome [5].

1.1 CLINICAL AND LABORATORY FEATURES

Proximal muscle weakness and low muscle endurance are common features of most patients with myositis. These symptoms can exist for weeks or months and in some individuals for years before diagnosis. The patients usually experience difficulties to conduct daily activities using the proximal muscle, like climbing stairs and combing hair. Swallowing problems may also be present. Some patients have difficulties lifting their heads while lying down due to weakness of the neck flexors. Ocular and facial muscles often remain normal [1, 6]. As the disorder progresses, the fine-motor movements using distal muscles can also be affected, which translates into difficulties with knitting and sewing, for instance. Muscle enzymes are released when muscle fibers suffer from degeneration and damage [1]. Creatine kinase (CK) as the most sensitive muscle enzyme could increase up to 50-fold (DM and PM) and below 12-fold (sIBM) compared to normal levels. Elevated levels of other enzymes like lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) may also be observed [1]. Except for skeletal muscle, other organs like skin, lung, gastrointestinal tract, pharyngeal muscle, heart, and joints may also be involved and further cause limitations/disabilities of the patients.

The histological characterization of most patients with myositis is the presence of inflammatory cells in skeletal muscle like T cells, B cells, macrophages, and dendritic cells (DCs) [7, 8]. Both $CD4^+$ and $CD8^+$ T cells can be present in muscle tissue of myositis patients but different patterns and localizations of T cells have been reported in different subgroups as described below. Figure 1 is a representative picture of immunohistochemistry staining of $CD3^+$, $CD4^+$, $CD8^+$ T cells, and $CD68^+$ macrophages in muscle biopsies from one patient with PM diagnosis. The distinct clinical and laboratory features of myositis are presented in Table 1. $CD28^{null}$ T cells of both CD4 and CD8 phenotypes are observed to dominate in the muscle biopsies of some patients with myositis [9]. The cytotoxicity targeting muscle fibers and resistance to apoptosis may contribute to the pathogenesis of myositis. Circulating autoantibodies are found in more than 50% of patients with myositis [10], including myositis specific autoantibodies (MSAs) and myositis associated autoantibodies (MAAs). Autoantibodies, especially MSAs, could correlate to specific clinical symptoms like anti-Jo-1 antibodies and the occurrence of ILD, and anti-Mi-2 antibodies with skin rash [11, 12], suggesting that autoantibodies are critical

in the development of clinical manifestations and immune pathogenesis of myositis. The presence of T cells in muscle biopsies, the involvement of specific HLA-DR genotype [13, 14], and the existence of autoantibodies in myositis patients indicate important roles of the adaptive immune system in the pathological mechanisms underlying myositis.

1.1.1 Dermatomyositis (DM)

Skin rash makes DM distinct from PM and sIBM. The typical skin rashes in DM patients are as follows: heliotrope discoloration rash on the eyelids; Gottron´s signs or papules on the body surface of joints e.g. fingers, knees, elbows. Other typical skin rash includes redness over the neck, upper chest (often in a V shape), shoulders (shawl sign) or the back [1]. Sunshine exposure may aggravate the skin rash [15]. Thickened and distorted cuticles and dilated capillary loops in fingernails may also be found in patients with DM and an increased incidence of cancer is also observed in patients with DM [1].

The muscle biopsies of DM patients are in typical cases characterized by inflammatory infiltrates surrounding blood vessels in perivascular and perimysial area, constituted primarily of $CD4^+$ T cells, macrophages, and plasmacytoid dendritic cells (pDCs) [16]. Perifascicular atrophic muscle fibers are also observed in the muscle biopsies of patients with DM [1, 16]. Deposits of complement C5b-C9 and membrane attack complex (MAC) in vessel walls is also a distinct histopathological feature of DM [5]. In some cases the biopsy may look normal, and a normal muscle pathology does not exclude DM diagnosis. These histopathological features in muscle biopsies suggest involvement of immune mechanisms in patients with DM where micro-vessels could be a target of the immune reaction.

1.1.2 Polymyositis (PM)

Polymyositis (PM) is mostly seen in adult patients. By definition they do not have the DM skin rash. PM may have lasted for a period up to months before the patients realize they need the help from physicians.

Invasion of inflammatory cells into non-necrotic muscle fibers is often regarded as one distinct feature of muscle biopsies from patients with PM. The inflammatory cells surrounding the non-necrotic muscle fibers are primarily $CD8^+$ T cells, $CD4^+$ T cells, macrophages, and DCs [16]. Compared to DM, cytotoxic $CD8^+$ T cells seem to contribute to the injury of muscle tissue of patients with PM as the perforin (protein involved in cell membrane pore formation) in cytotoxic $CD8^+$ T cells of patients with PM has been reported to be distributed towards the muscle fibers whereas the perforin in $CD8^+$ T cells of patients with DM was distributed evenly in the cytoplasm [17]. The different observations of histopathological features suggest different immune mechanisms involved in patients with DM and PM.

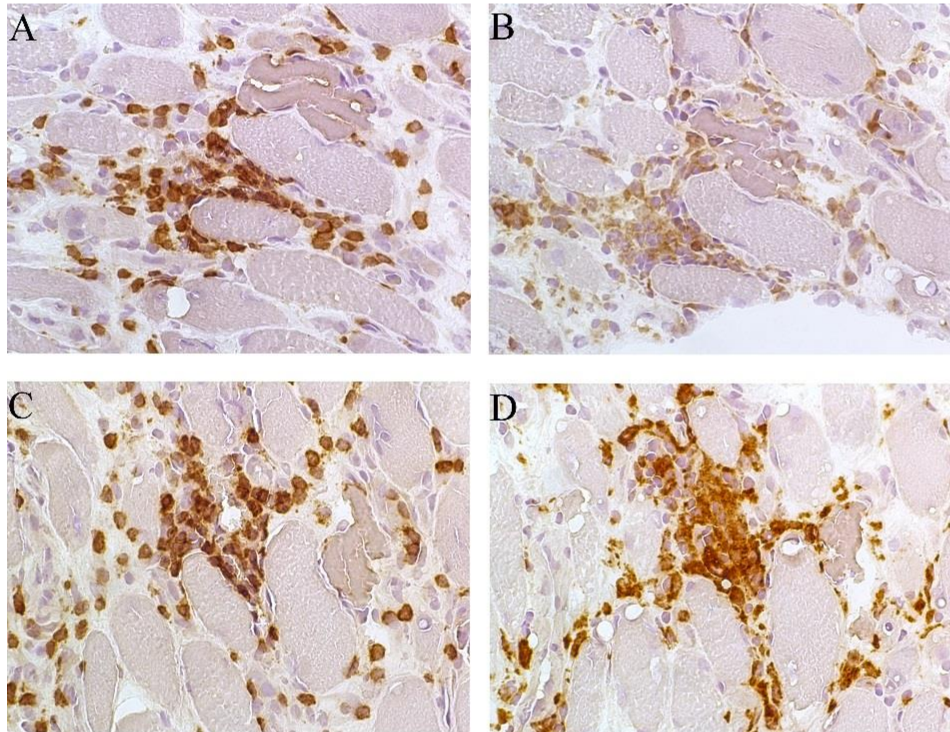

Figure 1. Immunohistochemistry staining of inflammatory infiltrates in consecutive muscle biopsy sections of a patient with diagnosis of PM. The staining shows inflammatory cell infiltrates consisting of T cells and macrophages surrounding muscle fibers. (A) CD3 staining, (B) CD4 staining, (C) CD8 staining, and (D) CD68 staining. Original magnification is 250×.

1.1.3 Sporadic inclusion body myositis (sIBM)

Patients with sIBM are typically above 50 years old (more males than females, the ratio is 3:1) at onset with a slowly progressive muscle weakness distributed asymmetrically in not only proximal muscle but also distal muscle [1]. sIBM patients are liable to fall due to the weakness of quadriceps muscle in the early stage [1]. Besides the thigh muscle, finger flexor muscles, neck flexors, and pharyngeal muscles are also often affected [14]. sIBM progresses more slowly than DM and PM patients, up to years instead of months [1]. sIBM patients are easily to be misdiagnosed initially as PM due to the shared pattern of muscle weakness, endomysial inflammation in muscle biopsies, and absence of skin rash [14]. However, patients with sIBM are in general resistant to immunosuppressive therapy, which is a distinct feature of sIBM.

Patients can be diagnosed as sIBM based on typical clinical features, but muscle biopsies are helpful in the diagnosis [14]. There are slit-like vacuoles (rimmed vacuoles) with basophilic granular inclusions inside muscle fibers. There may be also accumulated amyloid deposits inside muscle fibers, which is one histological characteristic of muscle biopsies from sIBM patients. The endomysial inflammatory cells and the degenerative features indicate the involvement of both immune and non-immune mechanisms in the pathogenesis of sIBM.

Table 1. The distinct clinical and laboratory features of myositis.

	DM	PM	sIBM
Age	Adults and children	Adults > 18 years old	Adults > 50 years old
Gender	Females:males (3:1)	Females:males (3:1)	Females:males (1:3)
Skin rash	yes	no	no
Affected muscle	Proximal; symmetrical	Proximal; symmetrical	Distal and proximal; asymmetrical
Progress period	Weeks to months	Weeks to months	Months to years
CK level	Above 50-fold	Above 50-fold	Below 12-fold
Resistant to IS treatment	no	no	yes
Histological features in muscle biopsy	Perimysial and perivascular inflammation; Perifascicular atrophy; Deposits of C5b-C9 and MAC in vessel walls; MHC class I expressed in muscle fibers	Endomysial infiltrates surrounding non-necrotic muscle fibers; MHC class I expressed in muscle fibers	Endomysial infiltrates surrounding non-necrotic muscle fibers; Rimmed vacuoles; β-amyloid deposits inside muscle fibers; MHC class I expressed in muscle fibers

CK: creatine kinase; C5b: Complement protein 5b; C9: Complement protein 9; MAC: membrane attack complex; IS: immunosuppressive treatment; MHC: major histocompatibility complex

1.2 DIAGNOSITIC AND CLASSIFICATION CRITERIA

1.2.1 Bohan and Peter criteria

There are no generally accepted criteria for diagnosis of DM and PM, while there are generally accepted principles. The most often used criteria were proposed by Bohan and Peter [18, 19]. Bohan and Peter criteria were developed to be both diagnostic and classification criteria.

The Bohan and Peter criteria for DM and PM consist of five items.

1. Symmetrically distributed muscle weakness of limb-girdle and anterior neck flexors.

2. Characteristic histological features in muscle biopsies: necrosis, phagocytosis, regeneration features, perifascicular atrophy and degeneration of muscle fibers, different sizes of muscle fibers, perivascular inflammatory infiltrates, and increased connective tissue.

3. Increased muscle enzymes especially CK in serum.

4. Electromyography shows characteristic features.

5. Typical skin manifestations of DM like the heliotrope discoloration on the eyelids and Gottron's sign.

Notably other causes of myopathies need to be excluded.

There are 3 subgroups classified from DM and PM, including *definite DM/PM, probable DM/PM, and possible DM/PM*.

For DM classification skin rash is mandatory and then DM is considered as *definite* when three of criteria 1-4 above are achieved. With two criteria met, it is considered as *probable DM* and as *possible DM* when one criterion in addition to skin rash is present. The diagnosis is confirmed as *definite PM* when the criteria 1-4 are met. The diagnosis of *probable PM* is considered when three criteria are fulfilled. With only two criteria fulfilled, the diagnosis is *possible PM*. For the diagnosis of PM, the skin manifestations (rash) are not included.

1.2.2 Griggs criteria

Diagnostic criteria for sIBM were first proposed by Calabrese and colleagues in 1987 [20]. After that, different researchers suggested other different criteria. However, the more frequently used diagnostic criteria were proposed by Griggs' group [21]. These criteria have a strong emphasis on muscle biopsy features with a high specificity but low sensitivity.

The criteria include 6 main points:

1. Duration of illness must last at least 6 months.

2. Age at disease onset at least 30-year-old.

3. Muscle weakness must have effect on proximal and distal muscles of arms and legs and patients must display at least one of the following features:

    1) Finger flexor weakness.
    2) Wrist flexor weakness is more serious than wrist extensor weakness.
    3) Quadriceps muscle weakness.

4. Serum CK less than 12 times normal

5. Muscle biopsy with the following features:

    1) Inflammatory myopathy characterized by mononuclear cell invasion of non-necrotic muscle fibers.
    2) Vacuolated muscle fibers.
    3) Intracellular amyloid deposits or tubulofilaments by electron microscopy.

6. Electromyography must display consistent features of inflammatory myopathies.

When there are typical findings in muscle biopsy (criterion 5 above), *definite sIBM* is diagnosed irrespective of other clinical features. In contrast, the diagnosis of *possible sIBM* is considered when the characteristic clinical and laboratory features are present even with absence of the typical histopathological characteristics in muscle biopsies.

1.2.3 2017 EULAR-ACR classification criteria

The Bohan and Peter criteria were published in 1975 and Griggs' criteria for sIBM were proposed in 1995 [18, 19, 21]. During the last decades, much progress has been made in the field of myositis and has offered some new perspectives to classify myositis, like inclusion of extramuscular manifestations other than skin rash such as ILD and autoantibodies [5]. The increased knowledge and the limitations of old criteria prompted myositis experts to work together to issue new classification criteria. 2017 European League Against Rheumatism/American College of Rheumatology Classification Criteria for adult and juvenile idiopathic inflammatory myopathies and their major subgroups were proposed [22, 23]. There are several merits of the new criteria [22, 23]. Firstly, the variables that classify a patient as having myositis are data driven based on comparisons between cases and comparator cases. Secondly, each variable is given a score based on statistical analysis, and the total value of adding all the scores will decide the probability of myositis. This quantitative way is easier to be put into practice. Thirdly, the score system includes two options - with muscle biopsies or not, which is more flexible than previous criteria. Fourthly, subgrouping of patients classified as having myositis is based on a classification tree. This criteria could divide myositis patients into JDM, DM, CADM, PM, and sIBM. Fifthly, a web-calculator has been developed to help investigators to easily calculate the values. In conclusion, the new classification criteria are flexible and easily to use for investigators.

1.3 IMPROVEMENT CRITERIA

1.3.1 IMACS criteria

Two improvement criteria will be introduced here and both of them are based on the International Myositis Assessment and Clinical Studies Group (IMACS) core set measures [24].

The core set measures of IMACS include six points:

1. Physician´s global disease activity, measured by Likert or visual analogue scale (VAS).

2. Patient´s global disease activity, measured by Likert or VAS.

3. Manual muscle test (MMT)-8, which is used to test muscle strength.

4. Health Assessment Questionnaire of activities of daily living (HAQ)

5. Serum levels of muscle enzymes. At least two of the following muscle enzyme activities: CK, LDH, ALT, and AST.

6. Extra muscular score: evaluation of constitutional, cutaneous, gastrointestinal, articular, cardiac, and pulmonary activity by using Myositis Disease Activity Assessment Tool (MDAAT). MDAAT include two methods: Myositis Disease Activity Assessment Visual Analogue Scales (MYOACT) and Myositis Intention to treat Activity Index (MITAX).

The generally accepted improvement criteria are IMACS preliminary Definition of Improvement (DOI). DOI is defined as any 3 of 6 core set measures improved ≥ 20%, with no more than 2 (not including MMT-8) worsening by ≥25% [24].

1.3.2 2016 IMACS Improvement Criteria

A new set of improvement criteria, 2016 American College of Rheumatology/European League Against Rheumatism criteria for minimal, moderate, and major clinical response in adult dermatomyositis and polymyositis, were published based on the core set measures of IMACS describing myositis disease activity [24, 25]. Total improvement score (range 1-100) is calculated by adding scores of the six core set disease activities of IMACS, and ≥ 20, ≥ 40, ≥ 60 represents minimal, moderate, and major improvement, respectively. This improvement criteria use a continuous quantitative definition, which not only defines patients qualitatively, but also quantitatively [25]. The exact scores representing the improvement of patients could also be used to calculate the correlations between the improvement and other clinical or molecular variables. Myositis experts voted to agree on the new definition also to lay the foundation of general application of these criteria in the future. These criteria are also suitable for both adult DM/PM and also JDM patients except for different thresholds, which help the parallel comparisons of different studies [25].

1.4 TREATMENT

The primary treatment of myositis patients is glucocorticoids in combination with immunosuppressive agents. However, few randomized controlled studies have been performed in patients with myositis, and treatment recommendations are mainly based on open studies and case reports due to the rarity of the disease, the heterogeneity of myositis and the up to recently lack of widely accepted classification criteria and improvement criteria. In general, an incomplete response and unwanted side effects are often observed. Thus, more effective and safer therapy is clearly needed. Exercise was introduced due to the beneficial effects and minor adverse effects [26]. In addition, biological treatments targeting specific pathogenic cells and molecules have recently been tested but few studies are available to support treatment recommendations. Most of the exercise studies have shown efficacy and been well tolerated by patients with myositis. Whereas the reports of biological therapies often are small and have shown inconsistent results. Further larger, randomized, controlled, and homogeneous studies are clearly required to confirm the results. Moreover, the unclear molecular mechanism of myositis also put up obstacles to develop novel and effective therapies.

1.4.1 Conventional treatment

Glucocorticoids, first applied in 1950s, are still first line agents but treatment outcome is not satisfactory and side effects are common [27]. Hence, immunosuppressants (methotrexate or azathioprine as first line therapy) are recommended in combination with glucocorticoids either to reduce the dose of glucocorticoids or to enhance the immunosuppressive effect [28]. Some patients with DM and PM could be responsive to the treatment and get improvement of muscle performance, but most patients fail to recover their muscle strength. In some patients the inflammatory infiltrates in muscle tissue may persist over several years despite high doses of immunosuppressive treatment. If methotrexate or azathioprine are not tolerated or if patients do not improve, cyclosporine A, high dose intravenous immunoglobulins (IVIGs) [29], cyclophosphamide, or mycophenolate mofetil (MMF) could be applied (second line therapy) [28]. But still not all patients are responsive to these therapeutic options. Several explanations

could be considered. Firstly, the application of the therapies is mainly according to the case reports from literature due to lack of large, randomized, and controlled trials. Secondly, due to limited understanding of the molecular mechanisms of the disease conditions, the therapies are not specific which increases the risk of unspecific adverse events such as bone marrow depression and increased risk of infections. Lastly, several factors could affect the response of patients to the treatment like the autoantibody profiles and the duration since time of diagnosis [30].

sIBM is even more difficult to treat and there are no data about beneficial effects of pharmacological treatment in sIBM [28]. Randomized placebo-controlled trials of large patient groups, long duration of treatment, more useful validation, better outcome measures are highly warranted to investigate the effects of different therapies on the progression of sIBM [31].

1.4.2 Biological treatment

Biological therapies have been introduced with great success to treat patients with rheumatoid arthritis (RA) [32]. Furthermore, through evaluating the effects of biological treatments on myositis patients, we could also investigate the pathogenesis of myositis at the same time. As most of the biological therapies specifically target distinct cells or cytokines present in the disease conditions, the administration of these treatments could block or interrupt the functions of cells and cytokines. If the interruption or blocking of a specific molecule is followed by clinical improvement, this might support that the associated molecular pathways are associated with the pathogenesis of myositis. The predominant roles of $CD28^{null}$ T cells and the association with HLA-DR phenotypes suggests the importance of T cells in the disease pathogenesis, which makes specific therapy targeting T cells activity very interesting. Other interesting targets include B cells and type I IFN. A detailed introduction is given based on the molecular targets of the biological treatments. Several biological treatments to myositis are listed in Table 2.

1.4.2.1 B cells

Rituximab specifically depletes $CD20^+$ B cells [28]. Reasons for using B cell deletion therapy in patients with myositis are the observed B cells and plasma cells in muscle tissue and the presence of autoantibodies in about over 50% of patients with myositis [10, 16]. A randomized, double-blind, placebo-controlled trial, “Rituximab in Myositis” (RIM), has been conducted to investigate the effect of rituximab in 200 patients with refractory DM, JDM, and PM. Patients were randomized into early treatment or 8 weeks later treatment. Although no significant difference was observed between the two groups, still 83% of the patients improved as defined by IMACS improvement criteria [33]. A post hoc analysis of these patients revealed that patients with anti-Jo-1 or anti-Mi-2 antibodies were more liable to respond to rituximab compared to the patients without these autoantibodies [34]. Rituximab has been suggested by some investigators as one of options of third-line therapies [28] but further investigations are recommended to demonstrate significant efficacy compared to placebo.

1.4.2.2 T cells

T cells account for a large proportion of the inflammatory infiltrates in muscle tissue of patients with myositis [16], which makes T cell blockade a logically potential therapy. Abatacept (CTLA-4Ig) is a recombinant fusion protein composed of the extracellular part of human CTLA-4 and a fragment of the Fc region of human IgG1, which binds specifically to

costimulatory molecules B7 on antigen presenting cells (APCs) and hence block the CD28-mediated activation of T cells. Abatacept has been approved to be used in RA patients. Abatacept proved useful in one female patient with PM resistant to conventional treatment [35]. In another case report, abatacept also showed efficacy in a female patient with severe myositis overlap syndrome, who was refractory to immunosuppressive treatment and two biological (rituximab and tocilizumab) agents [36].

1.4.2.3 Lymphocyte cells

Alemtuzumab is a human anti-CD52 antibody, also named as CAMPATH-1. CD52 is a polyprotein on the surface of T cells, B cells, monocytes, and natural killer (NK) cells [37]. Alemtuzumab has shown promising effects on patients with RA and multiple sclerosis (MS) [38]. One 48-year-old female patient with refractory PM received treatment of alemtuzumab and got a relief of her muscle weakness for several weeks, and the patient could thereafter respond to a combination of MMF and prednisolone which was not useful previously [39]. Thirteen sIBM patients who received alemtuzumab for four days experienced improved muscle strength after six months follow-up but returned to conditions before treatment after twelve months. These data are interesting in this refractory subset of myositis suggesting that alemtuzumab may be a potential drug for sIBM patients [38].

1.4.2.4 TNF-α and TNF-α receptor

The presence of tumor necrosis factor (TNF)-α mRNA and protein in muscle biopsies of myositis patients imply the possibility that TNF-α is involved in the pathogenesis of myositis [40, 41], further suggesting that TNF-α is a potential target to design specific therapy for myositis. However, several case studies reported that TNF-inhibitor could also induce the occurrence of DM in patients with RA [36]. Thus, the efficacy and safety of TNF-inhibitor therapy in patients with myositis is controversial. Three biological drugs available to block TNF-α are discussed in patients with myositis here: infliximab, adalimumab, and etanercept.

Infliximab is a chimeric monoclonal antibody targeting TNF-α. Infliximab has shown beneficial effect in patients with RA [42]. A few early case reports suggested that infliximab could be an effective treatment for patients with myositis [42, 43]. However, an open label pilot study with 13 refractory myositis patients did not show efficacy of infliximab [41]. On the contrary, some patients flared and the flares were associated with increased type I interferon (IFN) activity in peripheral blood, suggesting that anti-TNF blockade using antibodies may activate the innate immune system leading to IFN-α activity which could induce increased muscle inflammation in patients with myositis [41]. An open-label trial cooperated by several European centers was stopped preterm due to difficulties to include target myositis patients, high termination rate because of disease progression, and the development of unwanted adverse effects [44]. These studies indicate that application of infliximab for treating myositis might only be suitable for a minority of patients with myositis.

Adalimumab is a human monoclonal anti-TNF-α antibody. A 48-year-old female DM patient with ILD was treated by adalimumab due to the minor improvement after the treatment of glucocorticoids and immunosuppressive agents. After three months, the muscle weakness, skin rashes, and ILD were improved [45]. Adalimumab could therefore be a useful drug for DM patients with ILD and resistant to conventional treatment, but larger trials are needed.

Etanercept is a fusion protein of TNF receptor with a constant portion of human IgG1. Etanercept was efficient in a 50-year-old female patient with PM leading to decreased serum level of LDH and increased muscle strength [46]. In a pilot study including new onset patients with DM, etanercept or placebo was added in combination with prednisone. There was no recorded difference in adverse events between treatment-group and placebo-group, and half of the patients in the treatment-group successfully decreased the dose of prednisone which was the primary endpoint in this study [47]. Another pilot study including sIBM patients treated by etanercept for an average time of 17 months showed improved grip strength after 12 months [48]. Considering the potential effects and the few side effects in new onset patients with DM and with the observed distal muscle improvement in sIBM patients, etanercept may be worth to be tested in a larger clinical trial.

1.4.2.5 Type I IFN

The overexpression of type I IFN signature in peripheral blood of patients with DM and PM and the correlation between type I IFN-inducible gene signature score and clinical disease activity render type I IFN an attractive candidate drug target [49]. A phase 1b randomized, double-blinded, multicenter trial including fifty-one patients with DM and PM has been conducted, showing that sifalimumab (anti-IFN-α monoclonal antibody) successfully decreased around 50% of the IFN gene signature in blood and muscle compared to placebo group, and patients with ≥ 15% improvement of MMT-8 had higher level of neutralization of type I IFN gene signature compared to patients with < 15% MMT-8 improvement [50]. However, a larger randomized blinded study is required to confirm its efficacy in patients with myositis or subgroups of myositis.

1.4.2.6 IL-6

Interleukin (IL)-6 is a multifunctional cytokine involved in many different immune responses, like stimulating the production of antibodies and the development of effector T cells. It is secreted by immune cells, endothelial cells, and also muscle fibers [51, 52]. In chronic inflammatory conditions, elevated IL-6 in circulation could result in muscle atrophy [52]. Tocilizumab is an IL-6 receptor antagonist, which has been approved for use in patients with RA in about 70 countries [53]. IL-6 expression levels in serum of DM patients correlated significantly with disease activity in one study [54]. Thus, there is data to support a role of IL-6 in the pathogenesis of myositis, in particular in the subset with DM. A randomized, multi-center, placebo-controlled pilot trial is in the stage of recruiting participants with the sponsorship of the University of Pittsburgh (Clinical tirals.gov).

1.4.2.7 IL-1

IL-1α and IL-1β are consistently expressed in muscle tissue by inflammatory cells of patients with myositis but not in healthy controls, and IL-1 receptors are overexpressed in the sarcolemma and nuclei of muscle fibers, endothelial cells, and inflammatory cells of patients with myositis compared to healthy controls [55]. Anakinra is a recombinant version of the IL-1 receptor antagonist. A small pilot study of four sIBM patients treated with anakinra did not observe any improvement of muscle strength [56]. Another open label study including 15 patients (DM, PM, and sIBM) suggested that some refractory patients with myositis could be responsive to anakinra as seven patients represented response based on IMACS criteria [57]. Thus, anakinra is a promising therapy in myositis, but still requires confirmation in a larger study.

Table 2. The information of several biological treatments.

Biological agent	Composition and target	Trade name	Trial in myositis
Rituximab	Monoclonal antibody against CD20 on B cells	Rituxan/ Mabthera	Randomized, placebo trial [33]
Abatacept	A fusion protein of CTLA-4 and a constant domain of human IgG1	Orencia	Case reports [35, 36]
Alemtuzumab	Monoclonal antibody anti-CD52	Campath-1H	Case reports [38, 39]
Infliximab	Chimeric monoclonal antibody anti TNF-α	Remicade	Case reports [42, 43], pilot study [41]
Adalimumab	Fully human monoclonal antibody against TNF-α	Humira	Case report [45]
Etanercept	A fusion protein of TNF receptor with a constant portion of human IgG1	Enbrel	Case report [46], pilot studies [47, 48]
Sifalimumab	Monoclonal antibody against IFN-α	Sifalimumab	Phase 1b clinical trial [50]
Tocilizumab	IL-6 receptor antagonist	Roactemra /Actemra	Ongoing Preparation for double-blind, controlled trial (Clinical tirals.gov)
Anakinra	IL-1 receptor antagonist	Kineret	Open pilot studies [56, 57]

1.4.3 Exercise

Except for the above discussed pharmacological therapies, physical exercise has turned out to be an effective therapy in combination with immunosuppressive treatment, for patients with DM and PM. For sIBM, there is less data available on the efficacy of exercise. Eleven patients with DM and PM exercised according to a 12-week resistive home exercise program in combination with conventional immunosuppressive treatment, with improved muscle function and quality of life without causing more inflammation [26]. A multicenter randomized controlled study displayed reduced disease activity and improved muscle performance in patients with DM and PM undergoing a 12-week endurance exercise compared to patients without exercise intervention [58]. An open study including 7 patients with sIBM showed improved muscle improvement and function with minor elevated serum CK level after a 16-week home-based exercise program [59]. The underlying molecular mechanism of the observed beneficial effects of exercise in patients with myositis is not known. DM and PM patients who participated in a 12-week endurance training showed elevated aerobic capacity and muscle mitochondrial enzyme activities [60], as well as associations between maximal oxygen uptake (V_{O2} max) and physical function [58]. Furthermore, the overexpression of genes associated with capillary growth and mitochondrial genesis and increased number of capillaries

in muscle biopsies were also observed in DM and PM patients, suggesting that increased aerobic metabolism pathway might be one possible mechanism [61].

1.5 IMMUNE MECHANISMS

Even though more knowledge of the pathological mechanisms of myositis has developed over the past decades, the detailed molecular mechanisms are still not clear. The shared clinical and histopathological features in muscle tissue suggest shared pathogenesis of some subsets of myositis patients, however, the specific characters of each subset also imply heterogeneity of the mechanisms between different subgroups of myositis. The involvement of immune-associated genes, presence of inflammatory cells and cytokines, and autoantibodies in myositis suggests the important roles of immune system in the pathogenesis of myositis.

1.5.1 Genetic risk factors

The human leukocyte antigen (HLA), named major histocompatibility complex (MHC) in vertebrates, is thought to be a major genetic factor for myositis [62]. A genome-wide association study (GWAS) of 1710 myositis patients found that multiple alleles composed of HLA 8.1 ancestral haplotype (8.1AH) could virtually cover all the genetic risk factors of phenotypes studied in white population, however, different alleles showed different associations to different subsets of myositis. *HLA-DRB1*03:01* alleles associated with JDM and DM whereas *HLA-B*08:01* with PM and myositis patients with positivity of anti-Jo-1 antibodies [13]. Another multicenter study confirmed the strongest association of 8.1AH region but found that *HLA-DRB1*03:01* was more associated with PM and *HLA-B*08:01* was more associated with DM [63]. Serotype of myositis specific autoantibodies seems to be stronger associated with certain HLA-DR genotypes than the traditional subgroups DM, PM, and IBM. Thus another study showed that *HLA-DRB1*11:01* was related to an increased risk of anti-HMGCR antibody-positive necrotizing autoimmune myositis in Caucasian and African Americans [64]. *PTPN22* was another strong genetic factor besides HLA, especially associated with PM [63]. Other loci, *UBE2L3, CD28, TRAF6*, and *STAT4* have also shown significant associations with myositis [63]. The results of a high-resolution HLA-DRB1 genotyping of sIBM patients in an Australian cohort showed that different regions in HLA-DRB1 were associated with sIBM, in which *HLA-DRB1*03:01* allele and the *HLA-DRB1*03:01/*01:01* diplotype were the highest associated loci, indicating that HLA-DRB1 was a genetic factor in sIBM [65]. The associations with HLA-DR loci support the hypothesis of involvement of the adaptive immune system in patients with myositis as the major role of the HLA-DR molecule is to present antigen to $CD4^+$ T cells.

1.5.2 Environmental risk factors

1.5.2.1 UV-light and Vitamin D

An interesting observation is the increased prevalence of some autoimmune diseases with latitude, suggesting that ultraviolet (UV) radiation may be involved in the pathogenesis of autoimmune diseases [66]. UV light is a strong environmental contributor to DM and the subset of DM with anti-Mi-2 antibodies [67], which is mainly observed in females [68]. After short time exposure of UV light, an association was found between UV exposure and anti-p155/140 antibodies in JDM patients [69].

Vitamin D (cholecalciferol) is mainly synthesized in the skin with exposure of UV light or it could be obtained by food [70]. Cholecalciferol binds with the vitamin D binding protein (DBP) and moves into liver, where cholecalciferol is converted into 25(OH)D_3 by enzymes [70]. Then 25(OH)D_3 enters into circulation and is transferred into the kidney, where is converted into 1,25$(OH)_2D_3$. 1,25$(OH)_2D_3$ is the active type of vitamin D [70].

To avoid the disturbance of vitamin D from food, the level of 25(OH)D_3 in serum is mainly used as a measurement to investigate the associations with autoimmune diseases [70]. Low serum levels of 25(OH)D_3 was found in patients with autoimmune diseases like RA and systemic lupus erythematosus (SLE). These low serum levels correlated to high disease activity [70].

LL-37 is an anti-microbial peptide with the function of defending against bacteria. LL-37 could also modulate pro- and anti-immune response including activation of type I IFN system [71]. The expression of LL-37 could be regulated by different factors like vitamin D [72], which is also an important inducer of the gene expression of LL-37 [73]. Different relationships between serum levels of 25(OH)D_3 and LL-37 were reported in different diseases [74-76]. Serum levels of 25(OH)D_3 in myositis patients with short time of disease duration was lower compared to healthy controls, with a higher expression of LL-37 compared to healthy controls [77]. A deeper investigation between the relationship of 25(OH)D_3, LL-37, and the type I IFN system would be interesting and might help understand the pathophysiology of myositis and might add information on LL-37 as a potential trigger mechanism for the type I IFN system known to be activated in some patients with myositis.

1.5.2.2 Infections

Infections are interesting environmental triggers of autoimmune diseases according to epidemiology reports and experiments in mouse model. However, a direct association between specific infections and autoimmune diseases has not been found to date [78]. Several hypothesized mechanisms have been proposed that speculate how infections may induce autoimmunity, such as (1) “molecular mimicry”, in which epitopes of a pathogen are similar to that of self-antigens; (2) “bystander activation of autoreactive cells”, in which autoreactive cells with low affinity to self-antigens may be further activated by infections [79]. The involvement of these mechanisms with the occurrence of autoimmune diseases still require further investigations.

Virus is the major infectious agent implicated in the onset of autoimmune diseases. Some viruses have been detected in patients with autoimmune diseases like multiple sclerosis and type I diabetes, but again, the evidence of a direct link to the autoimmune disease is still lacking [80]. It has been reported of some possible associations between some viral infections and specific types of myositis, like Human Immunodeficiency Virus (HIV) in DM, Human T-cell lymphotropic Virus Type 1 (HTLV-1) in sIBM, Epstein-Barr virus (EBV) and Human Cytomegalovirus (HCMV) in DM and PM, Hepatitis C virus (HCV) and Hepatitis B virus (HBV) in PM [81-85]. However, no direct association between infections with viruses and myositis etiology has been properly demonstrated.

1.5.3 Innate immunity

The innate immune system serves as the first line of defense to combat the invasion from outside pathogens, which reacts quickly and efficiently. One of the major differences between

innate immunity and adaptive immunity is the unspecific recognition of the pathogens. Most bacteria and viruses share a structure called pathogen associated molecular patterns (PAMPs), which are essential to the survival of the pathogens. PAMPs are not expressed in eukaryotes. The pathogen recognition receptors (PRRs) expressed by immune cells bind to PAMPs and initiate the activation of immune cells to kill the invading microbes. PRRs could also bind to damage associated molecular patterns (DAMPs) secreted by necrotic cells, leading to immune response and also repair processes [86].

PRRs can be found both on the surface of cells, organelles, and in the cytoplasm of different immune cells. The different types of PRRs own different functions. Here, Toll-like receptors (TLRs) is taken as an example. TLRs are expressed on the cell surface and also in the endosome. When TLRs bind to the PAMPs on the extracellular surface of microbes, two of most important downstream transcriptional factors: nuclear factor κB (NF-κB) and interferon regulator factors (IRFs) are activated. NF-κB stimulates the production of various cytokines like TNF-α, IL-1, and IRFs could induce the generation of type I IFN. These cytokines could help kill microbes and maintain the host defense [86].

The main components of innate immunity are mast cells, neutrophils, monocytes/macrophages, dendritic cells, NK cells, and complement proteins. Mast cells are present in resident tissue with abundant cytoplasm granules with histamine inside. Once mast cells recognize a pathogen, histamine is released, which will cause vasodilation and enhance vascular permeability promoting the recruitment of immune cells to the infected tissue. Neutrophils are the first cell type to respond to pathogens, but they can only survive for some hours in the tissue. Monocytes are less abundant in circulation and become macrophages when entering infected tissue. Neutrophils and monocytes/macrophages are two phagocytes which engulf and destroy pathogens and are recruited into infected sites when activated. Activated dendritic cells can release different cytokines and are important to connect innate immunity and adaptive immunity. NK cell is a type of cytotoxic lymphocytes which have the ability to recognize viral-infected cells or stressed cells in the absence of antibodies or MHC molecules, allowing for a much faster immune reaction compared to T cells. Once activated by pathogens, NK cells work to control viral infections by secreting IFN-γ and TNF-α, which will promote the activation of macrophages leading to secretion of type I IFN, IL-12, and IL-15. These cytokines will in turn enhance the activation and function of NK cells. Complement proteins are mainly circulating proteolytic enzymes, which are activated by three different pathways: classical pathway, alternative pathway, and lectin pathway. Classical pathway is activated by the binding of antibodies so it is more involved in adaptive immunity. The activation of complement system could help phagocytes recognize and kill microbes via opsonization, recruit more cells to the resident site, and form MAC on the membrane of microbes leading to the lysis and apoptosis of microbes [86].

When a pathogen like bacteria invades our body and enters into the tissue, the resident mast cells recognize the PAMPs on the surface of the invading pathogen and then release histamine, causing dilation and elevated permeability of the vessel in the tissue. The resident dendritic cells and macrophages also recognize PAMPs and secret cytokines and chemokines, which also increase the vascular permeability and attract more immune cells into the infected tissue. Then neutrophils and monocytes in circulation cross through the vessel wall and enter the tissue. Neutrophils and macrophages could engulf and kill pathogens. The complement proteins also help enhance inflammation and phagocytosis [86]. The presence of different components of innate immune response like macrophages, complement proteins, and type I

IFN in muscle biopsies of patients with myositis suggest a critical role of innate immunity in patients with myositis.

1.5.3.1 Eosinophils, macrophages, and complement proteins – relationship with myositis

Different components involved in innate immunity were found present in the inflammatory environment of myositis. Eosinophils were mainly observed in endomysial, intra- and perivascular regions of muscle biopsies from DM, PM, and sIBM patients, and in higher numbers than those observed in healthy controls, suggesting that eosinophils might play a role in the pathogenesis of myositis [87]. Macrophages are present in muscle biopsies of patients of all subtypes of myositis but the expression pattern and localization may vary between different subsets of patients with myositis [88]. Furthermore, the differentiation pattern may vary between subsets of patients [88]. A subgroup of macrophages with expression of 25F9, a late-activation marker, was co-localized with inducible nitric oxide synthetase (iNOS) and tumor growth factor (TGF)-β in muscle biopsies of DM and PM patients, indicating the contribution of late activated macrophages to the pathogenesis of DM and PM [89]. The observation of deposits of C5b-C9 and MAC in vessel walls suggest the involvement of complement system in the pathogenesis of DM.

1.5.3.2 IFN – relationship with myositis

Type I IFN is an important cytokine involved in the innate immune mechanism that has in particular been demonstrated in patients with DM, but also in patients with some autoantibodies targeting RNA-binding proteins like anti-Jo-1 antibodies [90, 91]. Type I IFN induced genes are up-regulated in peripheral blood samples of patients with DM and PM, and the overexpression of these genes showed positive correlation to the disease activity of DM and PM patients [49, 92, 93]. Several type I IFN regulated proteins overexpressed in serum correlated significantly to levels of muscle enzymes of DM patients [93]. Type I IFN signature has been observed in muscle and skin biopsies of DM patients, and also the expression of type I IFN secreted protein, myxovirus resistance protein A (MxA), and the main I IFN producer, pDC expressing BDCA-2 [90, 94].

Autoantibodies, normally connected to the adaptive immune system, may be a possible inducer of type I IFN in both DM and PM [95], in line with the discoveries in patients with SLE [96]. However, DM patients without autoantibodies also showed increased MxA expression in muscle tissue, indicating the existence of type I IFN induction molecules other than autoantibodies [95]. TLR-7 and TLR-9 identified in inflammatory cells were found to co-localize with BDCA-2 positive cells, which have been observed in muscle tissue of patients with DM/PM, suggesting their involvement in the production of type I IFN [97].

Expression of TLR-3 and retinoic acid inducible gene I (RIG-I), both inducers of type I IFN, was observed mainly in the atrophic fibers in the perifascicular areas of muscle biopsies of DM patients [97]. Type I IFN induced protein interferon-stimulated gene 15 (ISG15) was significantly upregulated in muscle biopsies of patients with DM and mainly found in the perifascicular atrophic muscle fibers [98], which may suggest the contribution of type I IFN to the atrophy of muscle fibers of DM patients. Type I IFN not only mediates the innate immunity, but also triggers a cascade of adaptive immunity response by recruiting and regulating different lymphocytes and cytokines [99]. Type I IFN attracts $CXCR3^+$ lymphocytes and this has been identified in skin biopsies of DM patients [94]. Type I IFN also promotes the activity of T cells,

B cells, macrophages, and the expression of MHC class I [99]. The upregulation of MHC class I of muscle fibers is a characteristic histopathological feature of patients with myositis [100].

1.5.4 Adaptive immunity

Adaptive immunity is the second line of the host defense, which is composed of highly specialized, systemic cells and that processes and eliminates pathogens or prevents their growth. Adaptive immunity creates immunological memory after an initial response to a specific pathogen and leads to an enhanced response to subsequent encounters with that specific pathogen. Unlike the innate immune system, the adaptive immune system is highly specific to a particular pathogen and can also provide long-lasting protection.

1.5.4.1 Cell-mediated immunity

Naïve $CD8^+$ T cells and naïve $CD4^+$ T cells are activated by recognizing antigens presented by APCs through MHC class I and MHC class II molecules, respectively, in peripheral lymphoid organs. The full activation of both $CD4^+$ T cells and $CD8^+$ T cells requires two signals in the downstream signaling pathway. Signal 1 is the recognition between T cell receptor (TCR) on the surface of T cells and peptide presented by MHC class I or II molecules. Signal 2 is the binding of CD28 expressed on the surface of T cells and CD80/CD86 (B7) expressed on the surface of APCs. CD28 plays an important role in decreasing the risk of T cell auto-immunity against host antigens. Without signal 2, T cells enter into a state without functional response named as anergy. The full activation of T cells could trigger the induction of downstream signaling pathways, which stimulates several transcription factors to regulate the proliferation and differentiation T cells and the production of different cytokines. $CD4^+$ T cells differentiate into effector T cells (T helper cells, T_H) with different subsets: T_H1, T_H2, T_H17, T regulatory (Treg), and T follicular helper (T_{FH}) cells. T_H1 cells secrete IFN-γ, and activate phagocytes to clear up the ingested antigens, and induce the opsonization of complement proteins. T_H2 cells activate eosinophils which secret IgE to combat mainly helminths by generating IL-4 and IL-5. T_H17 cells produce IL-17 which mainly help defend extracellular bacteria and fungi. Tregs inhibit the immune response and secret IL-10 and TGF-β. T_{FH} cells help the activation of B cells in the germinal center of follicles of peripheral lymphoid node. $CD8^+$ T cells differentiate into cytotoxic T cells which could kill the infected cells [101-103].

1.5.4.2 Immunological tolerance

Theoretically, as the somatic recombination of genes of T and B cell receptors is random, there must be existing T and B cell receptors which recognize self-proteins or molecules (also called self-antigens). The cells interacting with self-antigens are called autoreactive cells. This process would lead to a cascade of autoimmune responses and attack of our own tissues or organs. However, this is not the case biologically. Our immune system has an ability to tolerate self-antigens. The tolerance of self-antigens involves central tolerance and peripheral tolerance, depending on whether the tolerance occurs in central lymphoid organs (bone marrow and thymus) or in peripheral lymphoid organs (spleen and lymph nodes) [104].

Precursor T cells migrate from bone marrow to thymus and start further development. During the development, some T cells with high affinities undergo apoptosis (negative selection). But some other T cells with high affinities turn into Treg cells and go to the peripheral lymphoid organs to exert an immunosuppressive function. In peripheral lymphoid organs, the interaction of T cells with self-antigens lacks a strong co-stimulation, leading to apoptosis due to the lack

of ability to induce anti-apoptotic proteins or to anergy condition without functional response. Tregs inhibit the activation of autoreactive T cells by disturbing the costimulatory pathway and secreting inhibitory cytokines [104].

In central tolerance, B cells with weak interaction with self-antigens become functionally unresponsive. Upon very strong interaction, B cells either perform receptor editing or enter apoptosis. If the receptor editing fails, the B cells will go into apoptosis. In peripheral tolerance of B cells, the lack of help from T cells leads B cells into anergy or apoptosis.

1.5.4.3 T cells – relationship with myositis

T cells are present in muscle biopsies of all three major subgroups of myositis, but the subset and location of T cells differ between the subgroups [16]. In patients with DM, $CD4^+$ T cells, are mainly distributed around capillaries in perivascular and perimysial area [16]. While in patients with PM and sIBM, the infiltrating inflammatory cells are located mainly in the endomysial area surrounding non-necrotic muscle fibers, and the predominating cells in the infiltrates are cytotoxic $CD8^+$ T cells and $CD4^+$ T cells [16]. The two patterns of infiltrating T cells indicate that T cells may affect different subsets of myositis via different pathways, one targeting the micro-vessels in muscle tissue and the second targeting the muscle fibers, but the immune specificity of the T cells is not known. The involvement of specific HLA-DR genotypes gives further support for a role of T cells in these disorders (*HLA-DRB1*03:01* and *DQA1*05:01*), as a major role of the HLA molecules is to present antigens to T cells [105].

Thus, one discussed hypothesis is that the MHC class I expressing muscle fibers can serve as APCs to initiate the activation of cytotoxic $CD8^+$ T cells. The cytotoxic $CD8^+$ T cells could then lead to damage of non-necrotic muscle fibers via perforin pathways. In myositis, MHC class I molecules are up-regulated in muscle fibers, whereas MHC class I molecules are not expressed in healthy differentiated muscle fibers [6]. Although CD80 (B7-1) and CD86 (B7-2) have not been detected on muscle fibers in myositis, BB-1, which is also a member of B7 family of costimulatory molecules, has been detected in muscle fibers of patients with myositis [106]. Thus the MHC-class I/BB-1 positive muscle fibers may interact with cytotoxic $CD8^+$ T cells with CD28 on their surface [6, 106]. This cascade has, however, not yet been fully elucidated.

$CD4^+$ T cells share similar steps to be activated as $CD8^+$ T cells, except MHC class II molecules are presenting the antigens instead of MHC class I molecules. After activation, $CD4^+$ T cells could differentiate into T effector cells according to the microenvironment, such as T_H1, T_H2, T_H17, and Treg cells. The four types of T effector cells have all been identified in patients with myositis, both in circulation and locally in the skeletal muscle [107-109], as suggested by the observation of locally expressed cytokines produced by different T cell phenotypes.

The elevated expression of IFN-γ and IL-12 in the blood and muscle tissue of patients with myositis indicates the involvement T_H1 cells in this condition [107, 110]. Growing evidence has shown that T_H17 cells mediated pathway may also be involved and thus a potential target for therapy [109, 111]. Presence of elevated expression of IL-17 mRNA in muscle biopsies has been reported in the DM, PM, and IMB compared to controls [112, 113]. IL-22, a T_H17 related cytokine, has been found overexpressed on protein level compared to healthy controls in muscle biopsies of DM/PM patients, and significantly correlated with disease activity of DM/PM [114]. T_H1 and T_H17 cell lines could indirectly mediate the regulation of muscle fibers

through different types of cytokines, and these cytokines could then regulate other inflammatory cells, thus, leading to a sequential autoimmune response [109, 115, 116].

T_H2 related cytokines such as IL-4 and IL-13 have also been reported to be expressed in muscle tissue of myositis patients, especially in PM and sIBM patients [117]. The role of these cytokines in myositis is unclear. One possibility is that T_H2-secreted cytokines could induce generation of M2 macrophages to repair the tissue. Another possibility is that T_H2 cells could induce maturation and differentiation of B cells into autoantibody producing plasma cells [117].

FOXP3$^+$ Treg is an important part of immunological tolerance by inhibiting the immune response. FOXP3$^+$ Treg cells have been identified in muscle specimens of patients with DM, PM, and sIBM, and the expression of these cells correlates to the level of infiltration in muscle biopsies [118]. The number of FOXP3$^+$ T cells was higher in muscle biopsies of patients with JDM compared to non-autoimmune disease controls, and the suppressive capacity of these Tregs was assumed defective [119]. No difference of the frequency of Tregs in circulation of JDM patients and healthy controls was observed, while in adult patients with DM the number of Tregs was significantly reduced compared to healthy controls [119, 120]. The data imply that different pathogenic mechanisms may predominate in children and adults with DM. FOXP3$^+$ T regulatory cells could not only inhibit the function of CD4$^+$ T cells and CD8$^+$ T cells, but also suppress the maturation and antigen-presenting function of DCs [118, 121]. Thus, Tregs may play a pivotal role in human myositis. This is also supported by the data showing that specific muscle antigen like myosin could develop a spontaneous myositis in FOXP3 mutant mice [122]. Furthermore, one study showed that injection of polyclonal Tregs could decrease the severity of myositis in a mouse model of autoimmune myositis [123]. The Treg cells is a key regulator in the autoimmune disease, which definitely makes Treg an interesting target for immune therapy in patients with myositis.

Further phenotyping of CD4$^+$ and CD8$^+$ T cells have revealed a predominance of CD28^{null} T cells of both CD4 and CD8 phenotypes in muscle tissue and peripheral blood of myositis patients [9, 124]. CD28^{null} T cells constitute a subset of T cells with cytotoxic properties lacking the expression of the costimulatory molecule CD28. This subset of T cells is regarded as a marker of senescence of the immune system and is usually present in elderly healthy individuals and in patients with chronic inflammatory conditions [125-127]. CD28^{null} T cells express some receptors, which are absent in conventional CD28$^+$ T cells, such as OX40/4-1BB costimulatory receptors, activating NK cell receptors (NKG2D), and CX3CR1 (fractalkine receptor) [128]. These receptors may help the proliferation of CD28^{null} T cells compensating the lack of CD28 and exert the cytotoxic function of CD28^{null} T cells.

These pro-inflammatory and terminally differentiated CD28^{null} T cells are resistant to apoptosis and they are long-time living. These features may contribute to treatment resistance in myositis patients [9]. Compared to CD28$^+$ T cells, CD28^{null} T cells mainly secret IFN-γ and TNF-α [9, 128], and these molecule could promote the expression MHC class I and MHC class II on muscle fibers of DM and PM patients, which could give rise to the sequential immune response as mentioned earlier. Another distinct feature of CD4$^+$CD28^{null} T cells is the secretion of perforin and granzyme, a mechanism via which CD4$^+$CD28^{null} T cells gain cytotoxic function that may directly damage the muscle fibers of myositis patients [9, 128]. In fact, CD4$^+$CD28^{null} T cells and CD8$^+$CD28^{null} T cells are frequently found in muscle specimens of DM and PM

patients, and functional *in vitro* studies have demonstrated a myocytotoxic capacity of these T cells [9].

Several cytokines are important for the $CD28^{null}$ T cell activity. For example, IL-15 is an important cytokine, which could not only promote the generation and proliferation, but also enhance the cytotoxic properties of $CD28^{null}$ T cells [129, 130]. This has been proven in different disease conditions [131-133]. In contrast, IL-12 may induce the expression of CD28 and could restore the costimulatory activity of $CD4^{+}CD28^{null}$ T cells [134]. Without CD28 expression, $CD28^{null}$ T cells cannot initiate proliferation through the classical stimulation pathway CD28/B7. CD58/CD2 was reported as a primarily replaced costimulatory pathway in human $CD8^{+}CD28^{null}$ T cells *in vitro* [135]. After treatment with glucocorticoids a higher frequency of $CD28^{null}$ T cells was found in muscle tissue of patients with myositis, compared to the reduced frequency of Tregs [136].

1.5.4.4 Humoral immunity

Naïve B cells in follicles of lymph node are activated by invading pathogens or antigens presented by follicular dendritic cells, and then migrate into the T cell zones from lymphoid follicles. After the processing of the pathogens or antigens, activated B cells present the processed peptides to helper T cells in the parafollicle areas, which have been stimulated by the same antigens presented by dendritic cells. The interaction between T helper cells and B cells may induce the proliferation and differentiation of some B cells, which will only express limited isotypes of antibodies (mainly IgD and IgM) and become short-lived plasma cells. Some other activated B cells and T helper cells will migrate back to lymphoid follicles, where B cells are further activated by T helper cells (specifically named T_{FH} cells). Further activated B cells migrate into the germinal center, where they undergo somatic hyper-mutation and heavy chain class switching and proliferate and differentiate into plasma cells and memory B cells. Hyper-mutation cause mutations within the genes to increase affinity, diversity, and specificity of antibodies, and heavy chain class switching could change the isotypes of the antibodies due to different antigens involved [137, 138].

There are four different isotypes of antibodies circulating in blood secreted by plasma cells. IgG is the main antibody in circulation, with the function of opsonizing antigens to help phagocytosis, neutralizing microbes and toxins, activating classical complement pathway, and crossing placenta and gut. IgM is the first produced antibody when exposed to invading pathogens and is present as a pentamer structure in blood, helping the activation of classical complement system. IgA is secreted as a monomer or a dimer structure, mainly distributed in mucosal sites to defend against invading pathogens. IgE could bridge antigens and mast cells and simulate mast cells to secrete histamine, which is important in mediating hypersensitivity reactions. IgE are involved in the defense against parasites and worms. IgD is expressed on the membrane of B cells instead of plasma cells, which is a marker of mature B cells [138].

1.5.4.5 B cells and autoantibodies – relationship with myositis

B cells and plasma cells can be detected in muscle tissue of patients with DM, PM, and sIBM [139]. The successful application of rituximab, a B cell-blocking therapy, supports the contribution of B cells in the immune mechanism of myositis [28]. Furthermore, the presence of circulating autoantibodies also supports the involvement of B cells in the pathogenesis of myositis, at least in the subgroups with autoantibodies. Autoantibodies in patients with myositis

are separated into two categories with the generally clinical consent [140]: MSAs, which are specific for myositis patients, and MAAs, also present in other connective tissue diseases.

The MSAs include the classical autoantibodies targeting the following autoantigens [140]: aminoacyl tRNA synthetases (ARS), nucleosome remodeling deacetylase complex (NuRD), signal recognition particle (SRP), as well as several lately found autoantigens: transcription intermediary factor 1γ/α (TIF1γ/α, p155/140), melanoma differentiation-associated protein 5 (MDA5), nuclear matrix protein 2 (NXP-2), small ubiquitin-like modifier-1 (SUMO-1) activating enzyme (SAE), and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR). These autoantibodies support diagnosis of myositis, help predict the clinical manifestations, and also seem to be able to predict the prognosis of the disease although cohort studies with long term follow up are scarce and more studies are needed [12, 141]. A list of MSAs is presented in Table 3.

Table 3. Myositis-specific autoantibodies and typical extramuscular clinical symptoms.

Autoantibody	Target antigen	Associated symptoms	Associated subset of myositis
Anti-ARS	Aminoacyl t-RNA synthetases	ILD [11], arthritis, Raynaud´s phenomenon, mechanic´s hands	ASS [142]
Anti-Mi-2	Nucleosome remodeling deacetylase complex	Skin rash [12]	DM [12]
Anti-SRP	Single recognition particle	Dysphagia, high CK [143]	IMNM [143]
Anti-TIFIγ (p155)	Transcription intermediary factor 1γ	Skin rash, cancer [144]	DM [144] JDM
Anti-MDA-5	Melanoma differentiation associated protein 5	ILD, Rapidly progressive ILD, skin rash [145, 146]	DM, CADM [145, 146]
Anti-MJ/NXP2	Nuclear matrix protein 2	Heliotrope rash, calcinosis, cancer [147, 148]	DM [147, 148], JDM
Anti-SAE	Small ubiquitin-like modifier activating enzyme	Skin rash, dysphagia [149, 150]	DM [149, 150]
Anti-HMGCR	3-hydroxy-3-methylglutaryl-coenzyme (CoA) reductase	Muscle deficit, high CK [151]	IMNM [151]

Anti-FHL1	Four-and-a-half LIM domain 1	Severe myopathy, often treatment resistant	DM, PM, and sIBM
Anti-cN-1A	Cytosolic 5′-nucleotidase 1A	Muscle weakness and atrophy	sIBM

ILD: interstitial lung disease; CK: creatine kinase; ASS: anti-synthetase syndrome, IMNM: immune mediated necrotizing myopathy; CADM: clinically amyopathic dermatomyositis

1.5.4.5.1 Anti-ARS antibodies

Anti-ARS antibodies target a family of proteins termed as cytoplasmic aminoacyl tRNA synthetases. To date, autoantibodies against 8 of these ARS have been described in myositis: anti-hystidyl (Jo-1), anti-threonyl (PL-7), anti-alanyl (PL-12), anti-glycyl (EJ), anti-isoleucyl (OJ), anti-asparaginyl (KS), anti-phenylalanyl (ZO), and anti-tyrosyl (YRS/HA) antibodies. Anti-Jo-1 antibodies targeting histidyl-transfer RNA synthetase (HisRS) are more frequent than other anti-ARS antibodies, being present in up to 20-30% of patients with myositis [140, 152].

Anti-synthetase syndrome is a clinically specific subset of myositis, with symptoms of ILD, myositis, fever, polyarthritis, Raynaud’s phenomenon, and mechanic’s hands, and is associated by presence of the anti-ARS antibodies [142]. Of the symptoms generally present, ILD is common and is a leading cause of death among these patients [11]. Of note, anti-Jo-1 antibodies showed an association with ILD in myositis patients and also correlated to the disease activity of myositis patients [141], showing that anti-Jo-1 antibody is a promising biomarker to predict the presence of ILD and also the disease activity of these patients. Sera with anti-Jo-1 antibodies could induce type I IFN signaling pathway, and also stimulate the overexpression of intercellular adhesion molecule-1 (ICAM-1) in human lung endothelial cells, indicating their involvement in the pathogenesis of myositis disorders [95, 153].

1.5.4.5.2 Anti-Mi-2 antibodies

Anti-Mi-2 antibodies target a particular region of a complex protein, nucleosome remodeling-deacetylase (NuRD). The main function of NuRD is to modify the generation of chromatin [154]. Anti-Mi-2 antibodies are associated with typical skin rash of DM and usually are associated with good prognosis [12, 154].

1.5.4.5.3 Anti-SRP antibodies

The SRP antigen is a conserved cytoplasmic complex with six ribonucleoproteins and one 7S RNA, and the function of SRP is to recognize secretory proteins and regulate their translocation across the rough endoplasmic reticulum (ER) [155]. Anti-SRP antibodies are mainly detected in patients with IMNM, with the histological features of necrotizing fibers and few infiltrates in muscle biopsies [143]. Patients with anti-SRP antibodies often have pronounced muscle weakness and they respond poorly to conventional immunosuppressive treatment.

1.5.4.5.4 Other autoantibodies

Besides the above described autoantibodies, several new autoantibodies specific for myositis have also been identified. Anti-TIFIγ (p155) antibodies are associated with DM patients, especially DM patients with cancer, as well as with JDM patients [144]. MDA5 is a receptor that recognizes viral double-stranded RNA (dsRNA) in cytoplasm [156]. CADM is a subset of myositis with the typical cutaneous manifestations of DM patients, but no muscle symptoms involvement [5]. Anti-MDA5 antibody is a predictive biomarker of CADM, which is also associated with a rapidly progressive ILD [145, 146]. Anti-MJ/NXP2 antibodies mainly exist in DM patients and in patients with JDM, and are rarely found in PM patients. These autoantibodies are associated with clinical features of calcinosis and cancer, however, the clinical manifestations are not consistent across patients from different ethnical source [147, 148]. Anti-TIFIγ and anti-NXP-2 antibodies are both associated with DM and cancer, but not all patients with these antibodies develop cancer and not all patients with DM and cancer have any of these autoantibodies [144, 157]. The antigen SAE is a group of enzymes associated with post-translational processing known as protein sumoylation. The anti-SAE antibodies are primarily present in DM patients with frequent skin lesions, systemic symptoms, and dysphagia [149, 150]. Anti-HMGCR antibodies are mainly detected in patients with immune mediated necrotizing myopathy [151]. Anti-four-and-a-half LIM domain 1 (FHL1) antibody is a muscle specific autoantibody, identified in patients with DM, PM, and sIBM. Myositis patients with anti-FHL1 antibodies often have a severe muscle pathology and are treatment resistant [158]. Another muscle-specific autoantibody against cytosolic 5′-nucleotidase 1A (cN-1A) is specific for sIBM, which may be a potentially diagnostic marker of sIBM [159].

2 AIMS OF THE STUDY

The overall objective of this study was to increase the knowledge about the immune mechanisms involved in myositis from innate and adaptive immune angels. The specific aims of each project are as follows:

Project 1. To evaluate the effects of abatacept on disease activity and the target organ, skeletal muscle, of adult patients with dermatomyositis (DM) and polymyositis (PM) (ARTEMIS trial).

Project 2. To evaluate *in vivo* effects of abatacept on phenotypes of T and B cells in the circulation and in muscle tissue in available samples from the ARTEMIS trial using mass cytometry (CyTOF).

Project 3. To explore a potential role of LL-37 in relation to the type I interferon system in patients with dermatomyositis (DM) and polymyositis (PM).

Project 4. To identify biomarkers in paired muscle biopsies and blood samples, taken before and after conventional immunosuppressive therapy, which could predict therapeutic response in myositis patients.

3 PATIENTS

3.1 PATIENTS IN PROJECT 1

Twenty adult patients (13 females and 7 males) with a median age of 51.5 years (range: 45.3-58.8 years) from Karolinska University Hospital, Stockholm, Sweden; King's College Hospital NHS, London, UK; and the Institute of Rheumatology Prague, Prague, Czech Republic were included in this study. The patients were diagnosed with DM (n=9) and PM (n=11) according to Bohan and Peter criteria [18, 19]. The patients had active disease after 3 months of conventional treatment with a combination of glucocorticoids and immunosuppressive agents. Repeated muscle biopsies were available from six patients from Sweden.

Muscle biopsies were investigated by immunohistochemistry staining for different markers (CD3, CD4, CD8, Ki67, CD68, CD163, CD19, CD19+CD20 (Clone no: L27), CD20cy (Clone no: L26), CD244, CTLA-4, DC-LAMP-PE, FOXP3, CD31, TNF-α, and IL-15).

3.2 PATIENTS IN PROJECT 2

Project 2 is a sub-study of project 1. Therefore treatment, inclusion criteria, and exclusion criteria are the same as in project 1.

Twelve patients with repeated peripheral blood mononuclear cells (PBMCs) taken before and after 6-months of abatacept treatment were included in Project 2. The patients (8 females and 4 males, 6 DM and 6 PM) were form Karolinska University Hospital, Stockholm, Sweden and Institute of Rheumatology Prague, Prague, Czech Republic. Five of the twelve patients had paired muscle biopsies. Conclusive summary of patients in Project 1 and Project 2 are presented in Figure 2.

PBMCs were investigated focusing on T and B cell phenotypes by CyTOF with a panel of 29 antibodies, respectively. Muscle biopsies from Project 1 were investigated by immunohistochemistry including staining for CD3, CD4, CD8, FOXP3, CD244, CD19, CD19+CD20 (Clone no: L27), and CD20cy (Clone no: L26). Expressions of CD3, CD4, and CD8 from Project 1 were re-evaluated in Project 2. In Project 1, CD3, CD4, and CD8 expressions were quantified by the percentage of positively stained areas of the whole section area, whereas in project 2, calculation of the number of positively stained cells per total tissue area (mm^2) was used.

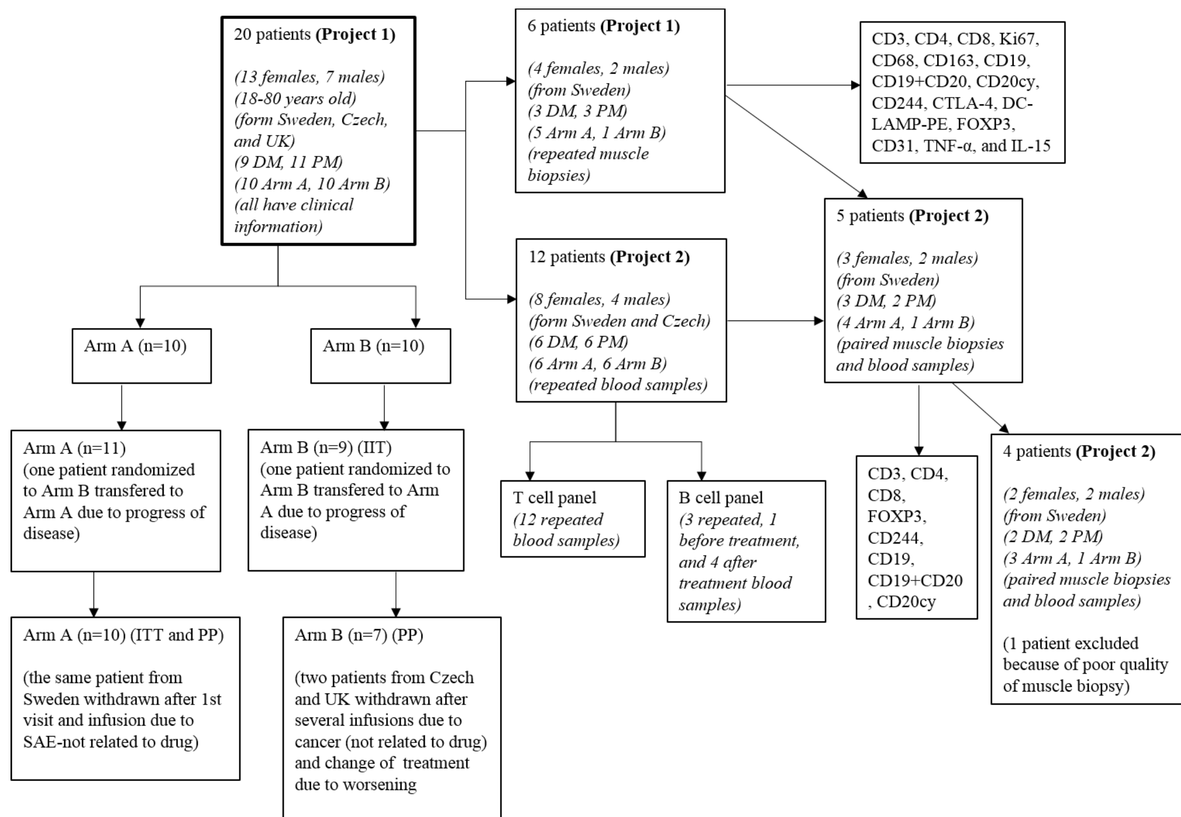

Figure 2. Diagram of patients distributed in Project 1 and Project 2. ITT: intention-to-treat population, meaning all patients randomized and given at least one baseline and following efficacy evaluation after treatment. PP: per protocol population, meaning all patients in the ITT population who also continued until week 24 without any violations according to the trial design. SAE: serious adverse event.

3.3 PATIENTS IN PROJECT 3

Patients in Project 3 include patients with myositis and SLE, and healthy controls.

Twelve patients (6 females, 6 males) with a mean age of 57 years (range: 40-77 years) were diagnosed with DM (n=6, 4 definite and 2 probable) and PM (n=6, 4 definite and 2 probable) by Bohan and Peter criteria [18, 19]. Eight of the twelve patients with short disease duration (symptoms ≤ 6 months, median 3 months, range: 1-6 months) were not treated except one patient who was given treatment 4 months before the muscle biopsy was taken. The other four patients with long disease duration (median 120 months, range: 60-216 months) were treatment resistant. Six SLE patients and eleven healthy subjects were enrolled as positive and negative controls, respectively. The median age of the six female SLE patients was 62 years (range: 37-77 years). Muscle biopsies were taken from five healthy controls with a median age of 58 years (range: 36-69 years). Skin biopsies were collected from another six healthy controls with a median age of 43 years (range: 32-51 years). The gender of the healthy controls was not known.

Muscle biopsies and skin biopsies from patients and controls were analyzed by immunohistochemistry staining for LL-37, CD66b, MxA, BDCA-2, CD68, CD163, and CD31.

Serum samples were evaluated for 25(OH)D_3 by chemiluminescence immunoassay technique. The patients and healthy controls and corresponding samples are presented in Figure 3.

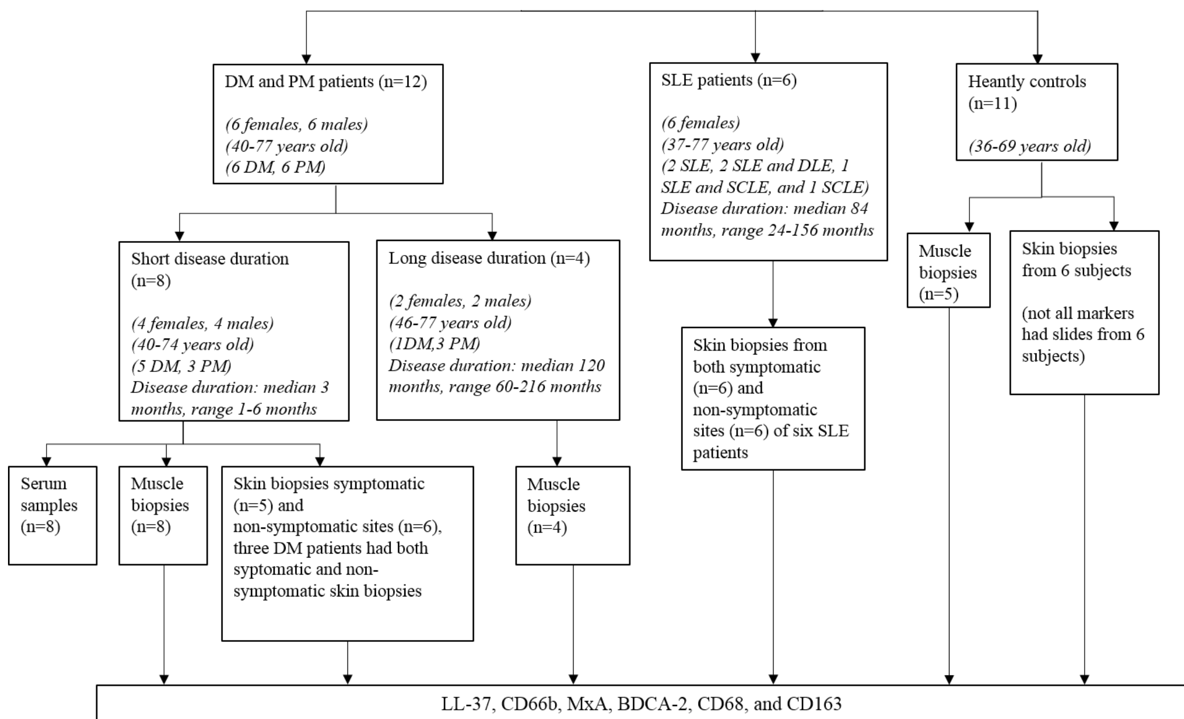

Figure 3. Diagram of the patients, muscle biopsies, and skin biopsies involved in Project 3. SLE: systemic lupus erythematosus; DLE: discoid lupus erythematosus; SCLE: subacute cutaneous lupus erythematosus.

3.4 PATIENTS IN PROJECT 4

Thirteen patients (8 females and 5 males) with myositis were enrolled in this project with the following diagnosis: DM (definite n=4), PM (definite n=4, probable n=3), IBM (definite n=2) based on the 2017 EULAR/ACR classification criteria [22, 23]. The median age at time of diagnosis was 65 years (range: 45-84 years). Clinical information was collected at time of diagnosis (pre-treatment), at 1- and 3-year follow-up. Selection of treatment was based on decision of the treating physician and consisted of a combination of glucocorticoids and immunosuppressive agents. Muscle biopsies and PBMCs were collected at time of diagnosis and within 1-year of follow-up (Figure 4).

Muscle biopsies were evaluated by immunohistochemistry including staining for markers of CD3, CD68, CD163, IL-15, MHC class I, and MHCn. Blood samples were analyzed by flow-cytometry for monocytes and T cells.

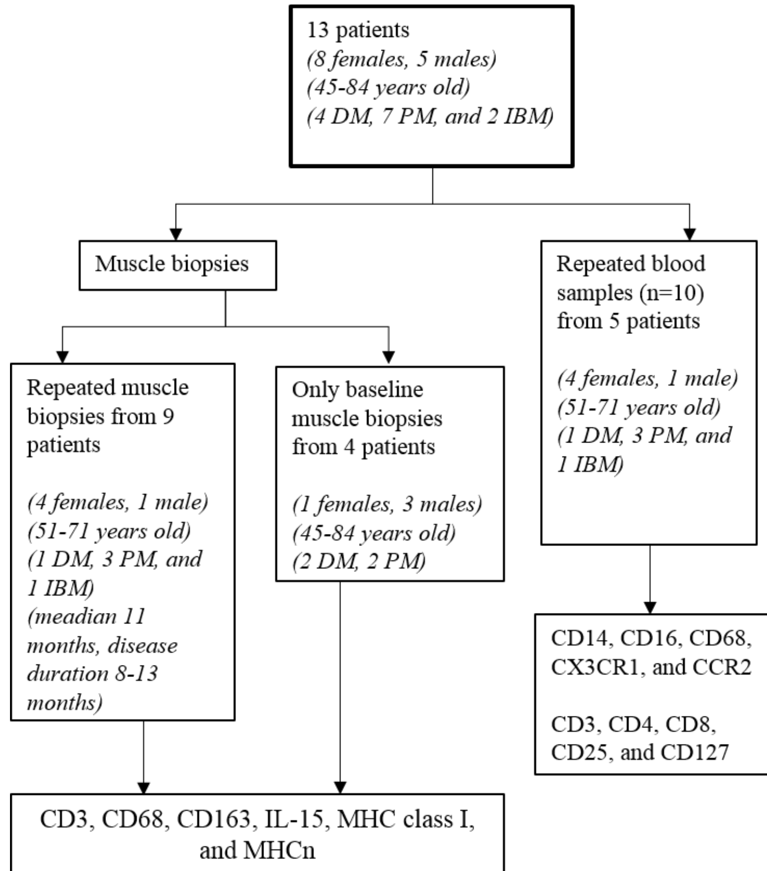

Figure 4. Diagram of the patients, muscle biopsies, and blood samples involved in Project 4. MHCn: myosin heavy chain neonatal.

4 METHODS

The following methods were used for the 4 projects.

4.1 MUSCLE BIOPSIES

4.1.1 Muscle biopsy technique

The morphological evaluation of muscle biopsies is an important tool to diagnose myositis. Immunohistochemistry staining of different molecular makers in muscle biopsies could not only provide more information for diagnosis but could also serve as an important tool to explore the underlying pathological mechanisms.

The general method for muscle biopsies used in Sweden is the "semi-open" muscle biopsy technique [160]. After local anesthesia, an alligator forceps (Weil-Blakesley´s conchotome) was used to take muscle biopsies by an experienced rheumatologist from various muscles like *vastus lateral* or *tibialis anterior* muscle. It is possible to take multiple muscle biopsies in the same site. This method is easily performed and gives enough material for clinical diagnosis and research purpose without causing much uncomfortable feelings or large scars. When repeated muscle biopsies are taken, changes in morphology and molecular expression of muscle biopsies can be investigated at different time-points in the disease course.

4.1.2 Handling of muscle biopsies

A good quality of muscle biopsies is the foundation for the morphological evaluation and immunohistochemistry staining. Muscle biopsies are very sensitive and therefore they need to be frozen properly to prevent artifacts and morphology destruction.

Different freezing methods were used for different research purposes. Muscle biopsies for PCR were frozen in isopentane pre-chilled by liquid nitrogen within seconds when taken from patients. While the muscle biopsies for immunohistochemistry were put on a moist sterile tissue in a plastic box on ice for 15 minutes before putting into pre-chilled isopentane. The frozen biopsies were then put in sterile pre-chilled tubes, containing a small piece of sterile ice in order to protect the samples from freeze drying during storage. The tubes were kept on dry ice until stored for longer time in -80 °C.

4.1.3 Sectioning of muscle biopsies

The muscle biopsies were kept on dry ice when taken from -80 °C. The muscle biopsies were put on a pre-cooled metal specimen holder in the cross-section direction and then mounted by OCT Cryomount medium (45830, HistoLAb, Gothenburg, Sweden). Then both the holder and the muscle biopsy were quickly dipped into liquid nitrogen until the OCT became frozen. Mounted muscle biopsies were sectioned into 7-um-thick specimens by using the Cryostat (CRYOSTAR NX70, Thermo Scientific, US). The cut muscle specimens were put on chrome-gelatine coated slides (Thermo Scientific, US), and air dried at room temperature for 30 minutes. To track the histology of continuous sections, Mayer´s HTX and eosin were used to stain the first and last section.

The slides were then fixed according to the features of different molecular markers to be stained. For intracellular antigen staining, slides were fixed by 4 °C 2% formaldehyde for 20 minutes and then washed twice in 4 °C 1xPBS for 5 minutes respectively before storage in -80

°C. For cell surface antigens, the slides were fixed in 4 °C 50% acetone for 3 minutes and then 4 °C 100% acetone for 30 seconds before storage in -80 °C.

4.2 SKIN BIOPSIES

Skin biopsies were taken by a punch method [161] under local anesthesia by an experienced dermatologist or rheumatologist. The handling, freezing, and sectioning of skin biopsies were similar to muscle biopsies.

4.3 IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE STAINING

Immunohistochemistry staining is a technique to investigate molecular expressions at protein level in target organs such as muscle and skin and provides information of cellular localization of the investigated molecule. Saponin is a cholesterol-like detergent, which permeabilizes cell surface membrane allowing the access of antibodies into cytoplasm. Thus, saponin is essential for intracellular staining. As the permeabilization is reversible, saponin has to be present in all steps during the whole staining process.

In our lab, avidin-biotin-peroxidase complex (ABC) method is used for immunohistochemistry staining. Avidin has four sites that binds biotin with high affinity. When the primary antibody recognizes antigens in the tissue it will bind to the biotinylated secondary antibody. Open sites of avidin then binds to biotin on the secondary antibody which is labelled with horseradish peroxidase (HRP). The substrate (DAB) is catalyzed by HRP and develop color.

To block endogenous peroxidase activity 1% H_2O_2 was often used before adding the primary antibody. Serum which the secondary antibody was produced in was used to block the non-specific binding of antibodies to tissue or to Fc receptors. After application of the secondary antibody, the sections were treated with ABC Elite kit (PK-6100, Vector Laboratories, Burlingame, CA, USA). Then peroxidase substrate kit containing 3, 3'-diaminobenzidine (DAB) (SK-4100, Vector Laboratories, Burlingame, CA, USA) was added to the sections for color reactions. Mayor's hematoxylin was used to stain the nuclei of cells. Finally, the slides were mounted with glycerol and investigated under optical microscope.

Before performing the staining of patients' materials, choosing proper primary antibodies, titration of primary antibodies, and test of optimal protocol are essential for a successful staining. Higher concentration of serum, different origins of serum blocking, and biotin-avidin blocking steps are recommended based on the basic staining protocol considering the nonspecific staining as background. Negative controls and positive controls are essential to confirm the specific staining of the target antigens.

Immunofluorescence staining was used in two of my studies to confirm the co-localization of two different molecular markers. The staining procedures are similar as for immunohistochemistry staining except that a fluorophore is used as a color signal instead of DAB. Of note, the two primary antibodies should be from different species to avoid any cross reaction. The staining should be performed in darkness after application of fluorophore. 4', 6-diamidino-2-phenylindole (DAPI) (Roche, Stockholm, Sweden) for cell nuclei.

4.4 PEPTIDE BLOCKING

A mixture of blocking peptide and primary antibody (in appropriate proportions) was prepared and kept in 4 ℃ one day before the staining procedure. The day of the staining, saponin was added to a final concentration of 0.1% (the same concentration as for the other immunohistochemistry staining). The staining procedures were then the same as for the immunohistochemistry staining.

4.5 QUANTIFICATION

We used a Reichert Polyvar II microscope (Reichert-Jung, Vienna, Austria) and a digital Leica camera DFC450 C (Leica, Cambridge, UK) to investigate the staining, visualize the morphology, take pictures, and manually count cells. A Leica DMRXA2 (Leica, Cambridge, UK) and Leica digital camera DFC450 C (Leica, Cambridge, UK) were used to take pictures of fluorescence staining and to perform computerized image analysis.

The Leica TCS SP5 Confocal (Leica Microsystems, Cambridge, UK) was used to take pictures of fluorescence staining.

Although the immunohistochemistry staining is a descriptive method, quantification can still be performed by using computerized image analysis system. This method could partially reduce the subjective bias of human eyes, but the computer cannot adjust the color threshold of positivity according to the different staining intensity in different sections, which has to be set manually to make the evaluation more consistent in different samples. The sections can also be scored by manual counting of number of positively stained cells in relation to the total tissue area calculated by computerized image analysis and presented as a grading score.

The estimation of expression of a stained molecule by image analysis can be performed in different ways according to the different expression patterns of molecular markers stained. If the molecular markers were positive in large areas of most samples, the percentage of positively stained areas of the whole section area was used. While if only scattered positive cells are detected, calculation of positively stained cells per total tissue area (mm^2) was a better alternative.

4.6 ISOLATION OF PBMCS

The heparinized blood samples were diluted in PBS buffer. Ficoll-Paque medium were carefully added to the bottom of the blood tube. The tubes were centrifuged at room temperature at 1660 rpm for 20 minutes. The PBMCs (located between the top plasma layer and the ficoll medium) were transferred to a new tube. The PBMCs were then washed with PBS and centrifuged at 1660 rpm for 7 minutes. The supernatant was then removed. The washing procedure was repeated three times. The PBMCs were counted and freezing medium (90% fetal bovine serum (FBS) in combination with 10% of dimethyl sulfoxide (DMSO)) were then added before freezing. The PBMCs were kept at -80 ℃ until use.

4.7 CYTOF

Mass cytometry, also named Time of Flight Mass Cytometry (CyTOF), is a combination of flow cytometry and mass spectrometry in which cells are stained with antibodies labeled with heavy metal ion rather than fluorochrome, allowing for up to 40-simultaneous antibodies to be

quantified in individual cells at a rate of about 500-1000 cells/second without the overlap of emission spectra [162].

CyTOF gives large amounts of data which could be a challenge to analyze. T-Distributed Stochastic Neighbor Embedding (t-SNE) map, spanning-tree progression analysis of density-normalized events (SPADE), cluster identification, characterization, and regression (Citrus) are commonly methods to analyze the CyTOF results. T-SNE map was exported by PhenoGraph [163], displaying multi-dimensional data on a two dimensional dot plot, and the location of each dot is the projection of the relationship of each cell in the high-dimension space [164]. SPADE is a tool to reduce the multidimensional data into two dimensions on a minimum spanning tree (MST) formed by cell clusters based on the similarities of surface phenotypes [165]. Citrus is used to identify clusters that predict the differences between different end points by using a hierarchical algorithm and a prediction model [166]. One disadvantage of CyTOF is that the cells are destroyed during the running, so it is important to reserve the same samples for necessary downstream experiments. As CyTOF is a new method, it is necessary to confirm the results of CyTOF by using other generally accepted methods, like flow cytometry.

4.8 FACS

Fluorescence activated cell sorting (FACS) of live cells could separate a group of cells recognized by antibodies labeled by fluorescence into sub-groups. Frozen PBMCs were thawed, filtered by Falcon 40 µm cell strainer, and counted by Scepter™ automated cell counter (Millipore, Germany). Single cell suspensions (one million cells per sample) were plated in 96-well V-bottom plate and stained with the antibodies. LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) was applied to exclude dead cells. Gallios flow cytometer (Beckman Coulter, Brea, CA) and Kaluza v1.1 software (Beckman Coulter) were used to analyze the samples.

4.9 STATISTICS

Statistical analyses were performed by GraphPad Prism 6 (San Diego, Calfornia, USA). Fisher's exact test was used to test for significance of association between the two kinds of variables. Mann-Whitney U test was used for the comparisons between two independent groups with nonparametric distribution data. Student's t-test was used to compare two groups of parametrically distributed data. Nonparametric Wilcoxon matched-pairs signed rank test was applied to compare two paired groups. Friedman test was used to calculate the differences among three groups with paired samples, followed by a Dunn's post-test to correct for multiple comparisons. Kruskal-Wallis test was used to calculate the differences of three groups with unpaired samples. Nonparametric spearman`s Rank correlation was applied to calculate the correlation between two variables. Pearson correlation was used to calculate the correlation between two variables normally distributed. $P<0.05$ was considered significantly different.

5 RESULTS AND DISCUSSION

Myositis is a group of heterogeneous and rare autoimmune disease with the target tissue of skeletal muscle. It is not curable and affects the daily life of patients. The treatment of myositis is mainly glucocorticoids in combination with immunosuppressive agents. If the patients do not respond, within 3-6 months the physicians generally change to alternative treatment. The incomplete response and common side effects of conventional immunosuppressive treatment requires new therapies. The molecular mechanisms of myositis are still not known, which is the primary limitation to the discovery of new and effective treatments.

5.1 ABATACEPT IN THE TREATMENT OF ADULT DERMATOMYOSITIS AND POLYMYOSITIS (ARTEMIS)

(Project 1 and Project 2)

5.1.1 Delayed-start design

As this was a new treatment in patients with myositis, an open label trial was planned. However, to strengthen the power of the trial, we used a randomized delayed-start design [167] (Figure 5). The patients were randomized into Arm A (active group, n=11) and Arm B (delayed group, n=9). Patients in the active group were given abatacept treatment from the first visit, while patients in the delayed-start group were treated with abatacept after 3 months delay. All the patients were given 6 months of active abatacept treatment.

After 3 months from the start of the trial (patients in Arm A with abatacept treatment for 3 months, whereas patients in Arm B with no abatacept treatment) there were more responders (n=5) in Arm A compared to Arm B (n=1) (Table 4).

Three months delay was chosen because it was hypothesized to be sufficient to observe effects of abatacept treatment on disease symptoms, furthermore, a longer delay would be unethical for the non-treated patients. Even though both groups were treated by abatacept, without a placebo group, we could still compare patients treated with abatacept for 3 months to patients without abatacept treatment. As more patients were improved after 3 months of abatacept treatment than patients without abatacept treatment this may suggest that the response was due to the beneficial effects of the short time treatment of abatacept or individual differences, rather than by modification of the disease itself.

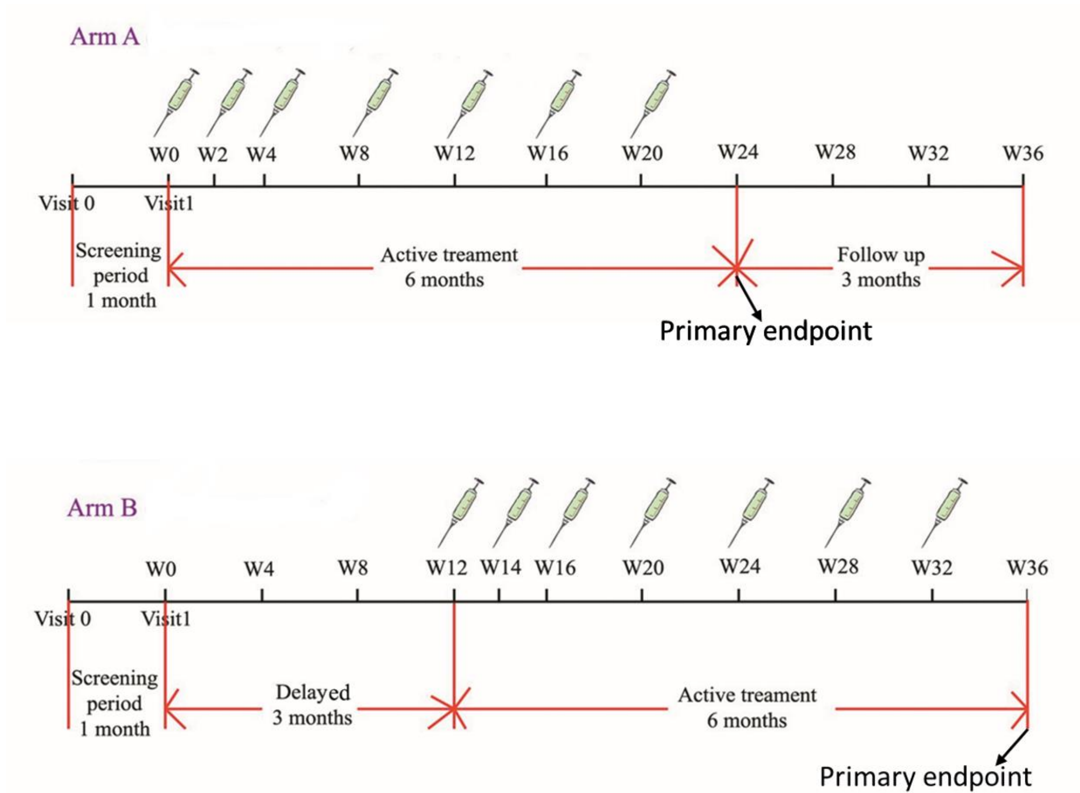

Figure 5. The design of the trial to investigate the effect of abatacept on DM and PM. The patients were randomized into two groups: Arm A and Arm B. The patients in Arm A were given abatacept treatment from the first visit, while patients in Arm B were treated after 3 months delay. All the patients were given 6 months of active abatacept treatment. W: week. Screening period is to ensure no malignancy and for wash out period of prohibited therapies.

Table 4. Response to abatacept treatment in different time points.

Response after 3 months from start		
n=19	Responders (n=6)	Non-responders (n=13)
Arm A (n=10)	5	5
Arm B (n=9)	1	8
Response after 6 months from start		
	Responders (n=7)	Non-responders (n=12)
Arm A (n=10)	6	4
Arm B (n=9)	1	8
Response after 6 months of active treatment		
	Responders (n=8)	Non-responders (n=11)
Arm A (n=10)	6	4
Arm B (n=9)	2	7

5.1.2 Clinical effect of abatacept treatment on patients with DM and PM

After 6 months from the start of the trial (patients in Arm A with abatacept treatment for 6 months, while patients in Arm B with abatacept treatment for 3 months), one more patient became responder than after 3 months in Arm A (n=6 vs n=5), whereas no difference was registered in the number of responders in Arm B (n=1). Within arm B there was one more responder after 6 months of abatacept treatment (n=2) in comparison to after 3 months of abatacept treatment (n=1) (Table 4). In general, there were more responders in Arm A than Arm B after both three months (n=5 vs n=1) and six months of active abatacept treatment (n=6 vs n=2) (Table 4).

Most patients responded to abatacept after 3 months, while a small group of patients showed response only after 6 months of abatacept treatment in both Arm A and Arm B. In line, in a 6-month trial to investigate the effect of abatacept in RA patients, more improvement of disease activity was also observed after 6 months than 3 months of treatment, however, without reaching a significant difference [168]. It seems likely that 3 months was sufficient for the beneficial effect of active abatacept treatment for the majority of patients, but 6 months of active abatacept treatment was required for a minority of patients to improve. More responders were defined in Arm A than Arm B after both three and six months of active abatacept treatment. The reasons are not known. However, the difference observed between Arm A and Arm B is the 3 months delay and the median age at baseline of Arm A was higher than Arm B ($P<0.05$), which may be the reasons leading to more responders in Arm A than Arm B after 6 months of active abatacept treatment.

In total (both Arm A and Arm B), nineteen patients were included in the analysis of the results. According to the definition of improvement by IMACS criteria [24], 8 patients were responsive to the treatment and 11 patients were not. During the treatment of DM and PM patients, eight of thirty-six adverse events reported were associated with the administration of abatacept, with four mild and four moderate, whereas no severe side events were registered. The most common adverse event was infections.

Most DM and PM patients do not completely respond to conventional therapy of gluococorticoid and immunosuppressive agents, furthermore, unwanted side effects often follow. Abatacept has shown efficacy and well tolerance in patients with rheumatoid arthritis and psoriatic arthritis [32, 169]. After 6 months of abatacept treatment, almost half of the enrolled patients who previously had not responded to conventional immunosuppressive treatment showed response to the treatment according to the IMACS definition of improvement [24] and no severe adverse events were related to abatacept treatment.

5.1.3 Effect of abatacept treatment on muscle performance of patients with DM and PM

The muscle strength presented by MMT-8 significantly improved ($P<0.05$) and the muscle disease activity also significantly decreased after 6 months of active abatacept treatment ($P<0.05$) for patients in both Arm A and Arm B. The MMT-8 of six patients with repeated muscle biopsies also showed a numerical increase (median (IQR) 72.00 (60.25-74.00) -73.50 (66.00-78.50)) after abatacept treatment. There was also a numerical improvement in all the variables of functional index (FI)-2 representing the muscle endurance of patients with myositis, especially right shoulder flexion ($P<0.05$) and heel lift ($P<0.05$) with significant improvement.

Muscle weakness is a characteristic clinical manifestation for most DM and PM patients, causing negative effect on the daily activities and the quality of life. After 6 months of abatacept treatment, the muscle performance of DM and PM patients improved significantly. Some other biological therapies have shown efficacy with lower disease activity in DM and PM patients in clinical trials, but no biological treatment has shown significant improvement of muscle performance to date. This makes abatacept a promising biological drug to directly relieve muscle weakness of treatment resistant DM and PM patients.

5.1.4 Effect of abatacept treatment on expression of molecular markers in muscle biopsies

The number of FOXP3$^+$ Tregs in muscle tissue significantly increased after 6 months of abatacept treatment although the muscle biopsies were only available for six patients. However, no significant differences were noted for any other marker which we stained for. This may be due to the different co-stimulation requirements between regulatory T cells and effector T cells, as abatacept is a modulator of CD28/B7 co-stimulation [170].

FOXP3$^+$ Treg cells mediate the tolerance of immune system by suppressing immune reaction. FOXP3$^+$ Tregs have been observed close to the effector cells in muscle tissue of DM and PM patients [109]. The number of FOXP3$^+$ Tregs was significantly higher in muscle of JDM patients comparing to controls, but the capacity to suppress inflammation of these Tregs in muscle was assumed defective due to the high number of FOXP3$^+$ Tregs with high level of inflammation. In addition, in the *in vitro* co-culture system of blood derived-Tregs and T effector cells, the suppressive ability of Tregs from active JDM patients was also lower than the cells from JDM patients in remission [110]. However, in RA patients, the suppressive ability of Tregs in synovium treated with abatacept was not affected [171]. The number of FOXP3$^+$ Tregs in muscle tissue in our study was increased after treatment of abatacept, however, the suppressive functions of Tregs were not tested. The effect of abatacept on the functions of Tregs differ between different resident tissues and different autoimmune conditions. Thus, whether Tregs have an effect in the mechanism of action of abatacept in muscle tissue of patients with DM and PM still needs to be explored. We also searched for clinical or laboratory variables that could predict treatment response, but without success.

5.1.5 Effect of abatacept treatment on phenotypes of T cells in blood samples

We used CyTOF, a mass cytometry technique, to investigate phenotypic differences of peripheral T and B cells of DM and PM patients with repeated blood samples taken before and after 6 months of active abatacept treatment, but no significant differences were found on group level.

CD28^{null} T cells and FOXP3$^+$ T regulatory cells are two distinct subsets of T cells present in both muscle and blood samples of DM and PM patients. The cytotoxic activity of CD28^{null} T cells and the suppressive capacity of FOXP3$^+$ T regulatory cells may play a role in the pathogenesis of DM and PM. In a previous study, the frequency of CD28^{null} T cells was higher while the frequency of FOXP3$^+$ T regulatory cells was lower in blood samples of DM and PM patients compared to healthy controls by using flow cytometry [172]. We did not find any changes on the frequency of these two groups of cells in the circulation after 6 months of treatment with abatacept, measured by CyTOF.

Twenty-nine antibodies focusing on markers of T cells were tested by using CyTOF. Multiple combinations of these markers provide possibilities to identify conventional T cell subgroups and also some novel subsets not reported before (Figure 6), indicating the power of CyTOF as a discovery tool. The analysis of large amounts of data is a big challenge to the users. Several specific analytical methods with different functions are recommended. T-SNE map and SPADE were applied to get the overview of the multidimensional data of CyTOF, and Citrus was used to elucidate cell groups predicting differences in different time points. By using several methods to analyze CyTOF data, we did not observe any treatment-induced changes on group level. The reasons may be the clinical heterogeneity of patients enrolled, the steady state of PBMCs without stimulation, and/or the small number of patients involved.

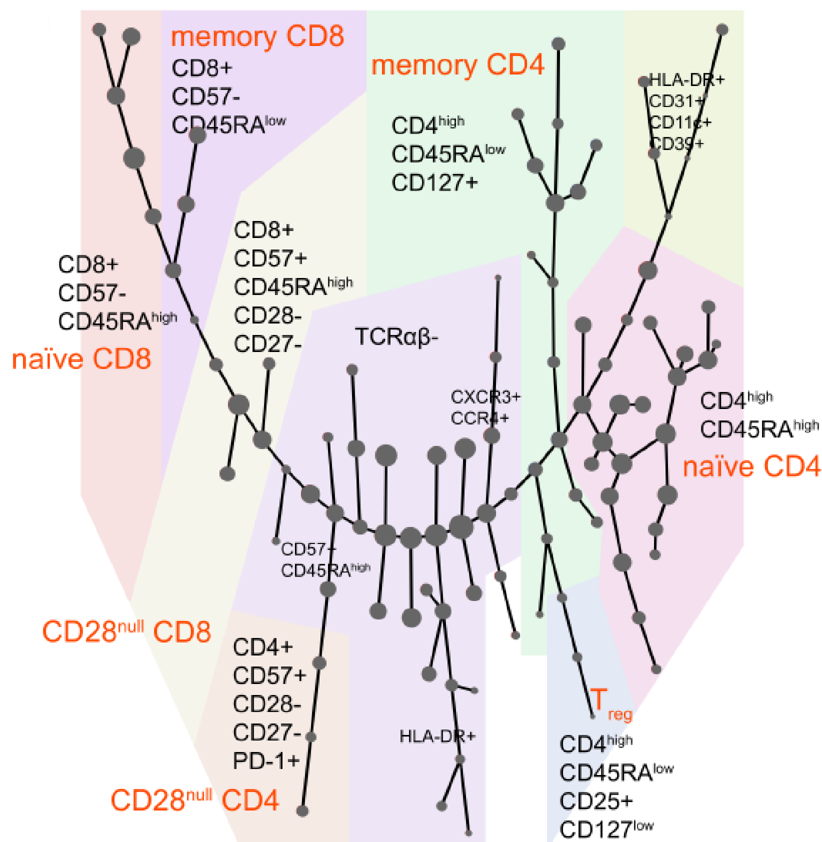

Figure 6. The clustering tree from SPADE (CyTOF analytical tool), where edges represent similarity and nodes are clusters, with area proportional to the average cell frequency among samples. The colors in the background show manually defined groups of SPADE clusters.

5.1.6 The correlation of cell phenotypes expressed in muscle and blood samples compared to clinical outcome

$FOXP3^+$ T regulatory cells not only suppress the inflammation in muscle tissue, but also exert the recovery of muscle fibers. In two different mouse models, Tregs inhibited the injury of muscle fibers [173] and promoted the proliferation of satellite cells leading to the regeneration of muscle fibers [174]. In our study (Paper I), the muscle strength of DM and PM patients improved after the 6 months of abatacept treatment. Although we did not find any correlation

between the number of $FOXP3^+$ Tregs in muscle tissue and MMT-8 of DM and PM patients before and after 6 months of abatacept treatment, $FOXP3^+$ T regulatory cells in muscle tissue may still be a potential regulator of the improvement of muscle strength.

A positive correlation between the ratio of frequency of $CD4^+$ and $CD8^+$ T cells (CD4/CD8) in peripheral blood before active abatacept treatment (r=0.74, P=0.0038) with FI-2 score improvement was observed. A positive correlation between CD4/CD8 ratio and FI-2 improvement was also found after treatment (r=0.69, P=0.013). FI-2 indicates muscle endurance of DM and PM patients [175]. The ratio of $CD4^+/CD8^+$ T cells in peripheral blood samples in RA patients after 4 weeks of abatacept treatment was a predictor of the efficacy (DAS28-CRP) of abatacept treatment [176]. This suggests that the ratio of CD4/CD8 in circulation might also be a potential parameter to measure the beneficial effects of abatacept on muscle endurance of DM and PM patients.

5.2 THE HOST DEFENSE PEPTIDE LL-37 A POSSIBLE INDUCER OF THE TYPE I INTERFERON SYSTEM IN PATIENTS WITH POLYMYOSITIS AND DERMATOMYOSITIS

(Project 3)

5.2.1 Expression pattern of LL-37, CD66b, BDCA2, and MxA in muscle and skin tissue of DM and PM patients

LL-37, CD66b, BDCA2, and MxA were present in corresponding areas of consecutive slides of muscle and skin biopsies from DM and PM patients. The expression of LL-37, CD66b, BDCA2, and MxA in muscle biopsies was similar in patients with short and long disease duration (Figure 7A-D). LL-37 also co-localized with $CD66b^+$ neutrophils in both muscle and skin biopsies of DM and PM patients, identified by using double fluorescence staining.

These results support our hypothesis that $CD66b^+$ neutrophils express LL-37, which may stimulate $BDCA2^+$ pDCs to produce type I IFN (MxA) in muscle and skin tissue of DM and PM patients, regardless of short time or long time of disease duration. But these are immunohistochemistry staining results, *in vitro* cell stimulation experiments are needed to prove this hypothesis.

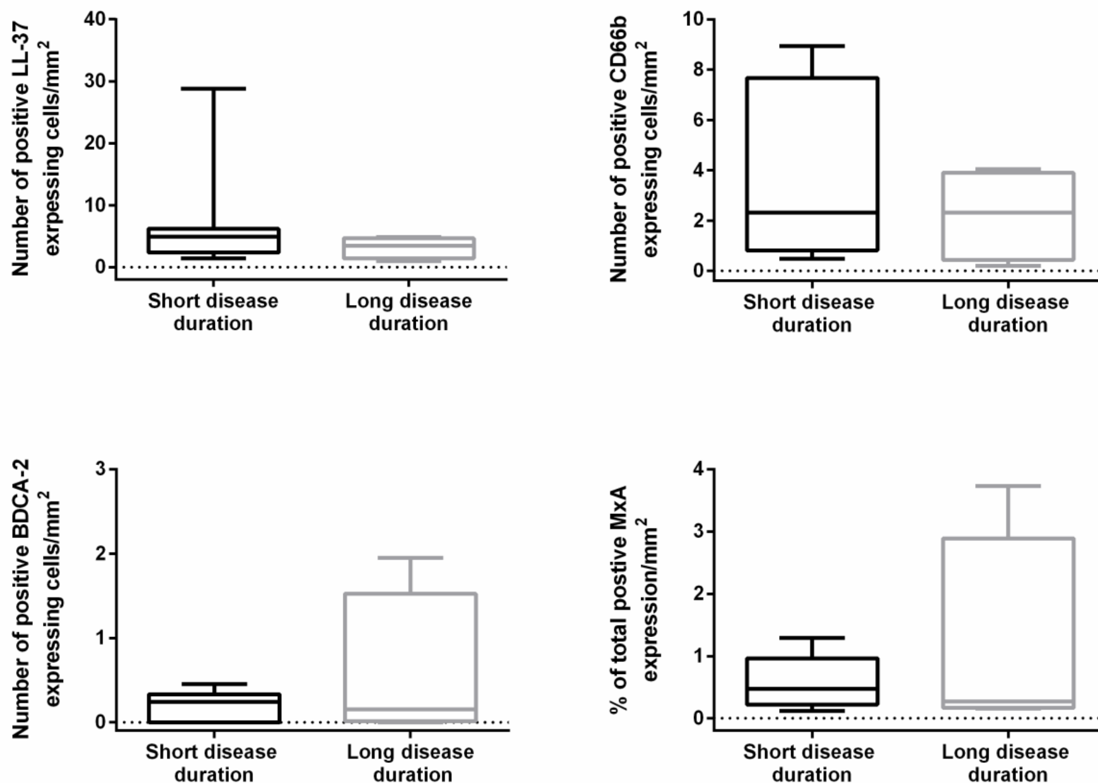

Figure 7. The comparison of expression of (A) LL-37, (B, E) CD66b, and (C) BDCA-2 presented by the number of positively stained cells per mm^2 and (D) MxA presented as percentage of positively stained areas out of the whole tissue section in muscle biopsies between patients with short disease duration and long disease duration.

5.2.2 Expression of CD66b and MxA in myositis patients with and without autoantibodies targeting RNA-binding proteins

Autoantibodies targeting RNA-binding proteins were present in one DM and four PM patients but negative in seven patients (5 DM, 2 PM). The number of CD66b$^+$ cells (neutrophils) in muscle biopsy of patients with autoantibodies targeting RNA-binding proteins were significantly lower compared to seronegative patients ($P<0.05$, Figure 8). The number of CD66b$^+$ cells showed a positive correlation to the expression of MxA (type I IFN secreted protein) in patients without autoantibodies targeting RNA-binding proteins ($r=0.79$, $P<0.05$).

Type I IFN signature was found increased in muscle tissue and blood samples of DM and PM patients [49]. Autoantibodies targeting RNA-binding proteins were associated with expression of type I IFN in patients with SLE [177], which was also observed in myositis patients with autoantibodies [91], and experimental data suggested that immune complex containing antibodies targeting RNA-binding proteins could induce type I IFN production from pDCs. However, an increased expression of MxA, indicating type I IFN activity in muscle tissue, was

also seen in DM patients without autoantibodies, suggesting the presence of alternative inducers of type I IFN in myositis patients [95]. The higher number of $CD66b^+$ neutrophils and the association between neutrophils and MxA in patients negative of autoantibodies targeting RNA-binding proteins could indicate that LL-37 is a potential alternative pathway to induce the elevated type I IFN in myositis patients without these autoantibodies. Another observation from this study was the presence of neutrophils in muscle and skin samples. Neutrophils have rarely been reported in muscle biopsies before, whether this can be explained by different staining protocols or that they have not been looked for in earlier studies is not known. Whether neutrophils have a role in the pathogenesis of patients with myositis could not be answered by our study.

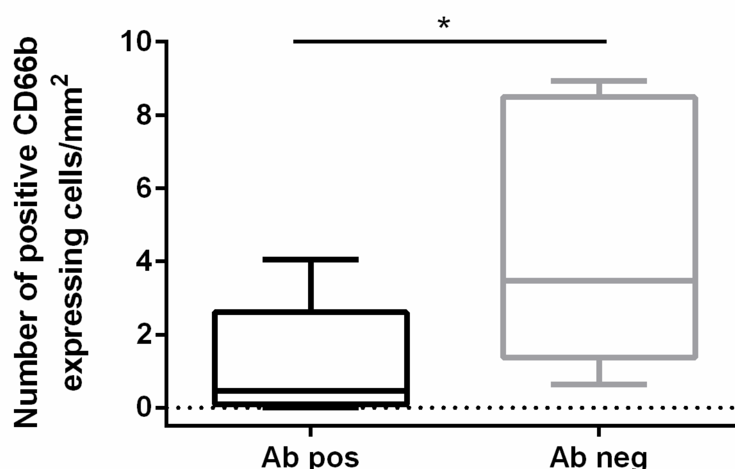

Figure 8. The expression of CD66b in muscle tissue of patients with autoantibodies targeting RNA-binding proteins compared to patients negative for autoantibodies targeting RNA-binding proteins. *P<0.05.

5.3 MUSCLE BIOPSY FEATURES IN REPEATED BIOPSIES AND RESPONSE TO TREATMENT IN PATIENTS WITH IDIOPATHIC INFLAMMATORY MYOPATHY – A PILOT STUDY

(Project 4)

As there is a variety of clinical outcome in patients with myositis to glucocorticoids and immunosuppressive agents, it is important to identify predictors of treatment response of the patients. Skeletal muscle is the major target tissue in myositis. Thus, we investigated the expression of different molecules present in muscle tissue of myositis patients to identify possible markers to predict response to conventional immunosuppressive treatment.

5.3.1 Expression of CD68, CD163, and IL-15 in muscle biopsies of responders and non-responders pre- and post- treatment

In the pre-treatment biopsies, the expression of CD68 of responders was numerically higher than in non-responders (median (IQR), 0.50 (0.16-1.58)-0.39 (0.05-0.66)), but there was no statistically significant difference of the expression of CD68 between responders and non-

responders. The expression of CD68 in the repeated muscle biopsies in responders was significantly lower than in non-responders after approximately 1-year treatment, although follow-up biopsies in responders were only available from three responders ($P<0.05$, median (IQR), 0.13 (0.04-0.13)-0.31 (0.19-0.37)). The expression of CD68 showed a within-group trend towards decrease after treatment in responders (median (IQR), 0.87 (0.21-3.71)-0.13 (0.04-0.13)), but there were no trends in non-responders. The same pattern was observed for the expression of CD163 and IL-15. In the pre-treatment biopsies, there was a moderate negative correlation between CD68 expression and MMT-8 ($r = -0.5641$, $P=0.0454$).

$CD68^+$ and $CD163^+$ macrophages are commonly observed in muscle biopsies of most myositis patients. IL-15 secreted by macrophages is an important cytokine activating T cells. The reduction of macrophages and IL-15 expression in muscle tissue of patients with myositis after treatment is consistent with previously observed results [178]. The expression of CD68 in pre-treatment muscle biopsies correlated with muscle strength presented by MMT-8. A previous study showed that the expression of IL-15 associated with the improvement of muscle endurance measured by FI-2 [178]. But we did not observe this association in our study, maybe due to the low number of patients. Conclusively, CD68 expression in muscle tissue, particularly a reduction after treatment, seems to be a potential marker to predict clinical long-term outcome of conventional immunosuppressive treatment, especially muscle performance. Notably, the T cells did not decrease after treatment in the repeated muscle biopsy. This might due to the low number of patients or suggest a need for more T cell targeting therapies in patients with T cells in infiltrates in muscle tissue.

5.3.2 Frequency of monocytes in blood samples of responders and non-responders pre- and post- treatment

Repeated blood samples were available from five patients, 3 responders and 2 non-responders. Classical monocytes, intermediate monocytes, and non-classical monocytes were present in the blood samples of the five patients. The expression of the intermediate subset of monocytes showed a trend to increase in responders whereas an opposite trend was observed in non-responders after treatment. Before treatment the fraction of intermediate subset was lower in responders than in non-responders, but the opposite was observed after treatment.

Intermediate monocytes are associated with inflammatory processes in healthy controls and in individuals with autoimmune diseases [179, 180]. A negative correlation between frequency of intermediate monocytes and reduction of disease activity after methotrexate treatment was found in patients with RA suggesting that the frequency of intermediate monocytes is predictive to methotrexate therapy [181]. We only tested the intermediate monocytes in five patients without statistically significant results. As macrophages are commonly found in muscle biopsies of myositis patients, and considering the inflammation-related regulatory function exerted by intermediate monocytes, it would be interesting to investigate the frequency of intermediate monocytes in relation to treatment response in a larger cohort of patients with myositis.

6 CONCLUSIONS AND PERSPECTIVES

The immunological mechanisms of myositis have been generally accepted as an interaction between the innate immunity and the adaptive immunity. In my research, Project 1 and Project 2 mainly focused on adaptive immunity and Project 3 was mainly related to innate immunity. In project 4, we investigated if some of the markers involved in both the innate and adaptive immunity could predict the clinical outcome of myositis patients. In our projects, we found that abatacpet is a potential new treatment for myositis with improved muscle performance of patients, LL-37 might induce the upregulation of type I IFN, and the effects of conventional immunosuppressive treatment on $CD68^+$ macrophages in muscle biopsies might be predictive to the clinical long-term outcome in patients with myositis.

A few randomized and controlled trials performed during recent years have reported some efficacy of biological treatments on myositis. However, the therapeutic effects are still not satisfactory. New therapies are still needed. In **Project 1 and 2**, we investigated the effects of a selectively co-stimulation modulator-abatacept (CTLA-4Ig) on clinical parameters, molecular expressions in muscle tissue, and phenotypes of T cells in blood samples of DM and PM patients. The clinical evaluation showed beneficial effects of abatacept treatment in almost half of the previously treatment resistant patients with myositis and accepted tolerance. The results provide support for a role of T cells in a subset of patients with DM and PM.

As the modulator of co-stimulation of T cells, abatacept is a T cell activation blocking agent. We also aimed to get a hint of the important roles of T cells in the pathogenesis of myositis by investigating the molecular expression of T cells in muscle tissue as well as the phenotypes of T cells in circulation. The number of $FOXP3^+$ Tregs in muscle tissue increased significantly in a subgroup of patients together with improved muscle strength evaluated by MMT-8 after abatacept treatment for 6 months. Therefore, in this subgroup of patients treatment with abatacept looks promising as it appears to improve the muscle strength. In addition, we conclude that $FOXP3^+$ Tregs seemed to be sensitive to the modulation of abatacept treatment, hence, highlighting the importance of $FOXP3^+$ Tregs in the pathogenesis of DM and PM. In our study, repeated muscle biopsies were only available for six patients. Thus, the increased number of Tregs observed in muscle biopsies of abatacept-treated patients needs to be confirmed in a larger cohort with a higher number of repeated muscle biopsies. The observation from the repeated muscle biopsies together with the clinical improvement of muscle strength supports the hypothesis that abatacept could be a potential biological treatment for a subgroup of patients with myositis. However, a larger, randomized, controlled and placebo trial is important to confirm the efficacy and safety of abatacept treatment in myositis and the trial is ongoing. Furthermore, in a larger trial, biomarkers that could predict response to abatacept treatment among patients with myositis may be identified.

In **Project 2**, we did not see any statistically significant changes of phenotypes of T cells in circulation after compared to before abatacept treatment by using CyTOF. However, we found a positive correlation between the ratio of $CD4^+$/$CD8^+$ T cells in circulation, both at baseline and after treatment, with a measure of muscle performance, FI-2 score improvement, suggesting that CD4/CD8 ratio in circulation may be a predictor of the improvement of muscle endurance of myositis patients with abatacept treatment. Again, more patients are required in a larger trial to confirm these results. The Citrus (CyTOF analytical tool) results showed that $FOXP3^+$ and $CD28^{null}$ T cell subsets were sensitive to the abatacept treatment in the circulation, however, due to high false discovery rate (FDR) the changes did not reach any significant

difference. For future trials, the markers of $FOXP3^+$ and $CD28^{null}$ T cell subsets should be paid more attention. Another parallel conventional method, like flow cytometry, is also recommended to be taken into consideration to confirm the results from the CyTOF analyses.

Both innate and adaptive immunity are involved in the immune mechanism of myositis. Type I IFN is an important member of innate immunity and may also be an important cytokine in the pathogenesis of myositis. In **Project 3**, we provide evidence to support our hypothesis that LL-37 expressed by neutrophils may be an inducer of Type I IFN by stimulating pDCs. But further investigation is required to confirm these results. *In vitro* experiments with the co-culture of these cells taken from patients are recommended.

In **Project 4**, we tested several molecules to identify markers that could predict the clinical outcome of conventional treatment. The staining of inflammatory cells with immunohistochemistry in repeated muscle biopsies taken within the first year of treatment together with evaluation of disease activity and muscle strength may be a way to predict prognosis for recovery of muscle performance. CD68 may be a promising predictive marker, but this needs to be confirmed in a larger study.

Myositis is a rare group of autoimmune diseases leading to difficulties to recruit large number of patients for randomized and controlled studies. The high heterogeneity of myositis patients also brings difficulties to reach statistically significant results at group level. Therefore multicenter collaborations are important like we did in my first two projects. Still it took a long time to recruit the 20 patients in this pilot study. For future studies more homogenous subgroups e.g. selected by autoantibody profiles could be considered. A strength of our studies is the combination of detailed clinical longitudinal data with repeated muscle biopsies and blood samples. However, the muscle biopsy samples, are often small especially follow-up muscle biopsies, limiting the number of investigations to perform on each specimen which is a limitation for studies using immunohistochemistry or immunofluorescence staining. These are general limitations of all my four studies. New technologies where several molecules could be investigated in the same biopsy sample would be interesting to test in future studies, as well as investigations transcripts in single cells in tissue samples. Even though our cohorts are small, we have collected different types of tissue samples from patients and included different molecular markers to investigate the effects of biological treatment on myositis, the underlying pathogenesis of myositis, and the prognosis prediction of myositis.

In conclusion, our findings strengthen the critical roles of innate and adaptive immune response in the pathogenesis of myositis by investigations from different perspectives and in different cohorts. In addition, there are individual differences regarding molecular patterns among patients with myositis, which needs to be taken into account in future studies. Finally, our data may provide some clues on how to develop novel and effective therapies for patients with myositis.

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