Surface binding autoantibodies and their functional effects in dermatomyositis and polymyositis

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Date of the Doctoral Defense: 21st September, 2010.

Dedicated to my grand mother and my parents.....

Declaration

I declare that the present thesis is my original work and that it has not been previously presented in this or any other university for any degree. I have also abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

INDEX

Abbreviations

2D	Two Dimensional
AECA	anti endothelial cell antibodies
anti-ARS	anti-aminoacyl tRNA synthetase antibodies
anti-Ku	anti-Ku antibodies
anti-Mi-2	anti-Mi-2 antibodies
anti-PM-Scl	anti-PM-Scl antibodies
anti-SAE	anti-small ubiquitin-like modifier activating enzyme
anti-SRP	anti-Signal-Recognition particle antibodies
anti-U1-RNP	anti-U1-ribonucleoprotein antibodies
anti-U3-RNP	anti-U3-ribonucleoprotein antibodies
AP	Alkaline Phosphatase;
DM	Dermatomyositis
E.Coli	Escherichia coli
EC	Endothelial cell
ELISA	Enzyme Linked Immunosorbent Assay
FITC	Fluorescinisothiocyanate
HCMEC	Human chorionic micro-vascular endothelial cells
HDMEC	human dermal micro-vascular endothelial cells
HUVEC	Human umbilical vascular endothelial cells
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IIMs	Idiopathic inflammatory myopathies
LEMS	Lambert-Eaton Myasthenic syndrome
LMP2 / 7	Low molecular mass polypeptide 2 / 7
MAAs	myositis-associated autoantibodies
MCTD	Mixed connective tissue diseases
MHC class I	Major histocompatibility complex class I

MSAs	myositis-specific autoantibodies
mTOR	mammalian target of rapamycin
OIM	Other inflammatory myopathies
PBGD	Porphobilinogen deaminase
PM	Polymyositis
SEREX	serological analysis of recombinant cDNA expression library
SERPA	serological proteome analysis
SKMC	Human primary skeletal muscle cells
SLE	Systemic Lupus Erythematosus
TAP1 / 2	Transporter associated with antigen processing 1 / 2
TE671	Human rhabdomyosarcoma cells
WG	Wegener's granulomatosis

1. Introduction

1.1. Immune system

The immune system is the body's natural guardian against disease. It has evolved as a way for us to defend ourselves from invading pathogenic organisms like bacteria, parasites and viruses. It equals in complexity the intricacies of the brain and nervous system, displays several remarkable characteristics which include distinction between "self" and "non-self" and the ability to remember previous experiences and react accordingly. Based on the type of response whether it is unspecific or specific, immune system can be divided into innate and adaptive immune system respectively (Goldsby et al. 2000; Janeway et al. 2001). These two systems are integrated and often interact with each other (Hoebe et al. 2004).

Our first line of defence against any invasion from outside is the innate immune system, which also includes a series of unspecific barriers that try to stop the invading organisms. Our body surfaces are defended by epithelium, which provides a physical barrier between the internal milieu and the external world that, contain pathogens. Infections occur only when pathogen can colonize or pass through these barriers. The low pH in the intestine and the presence of degrading enzymes also inhibit invading pathogens. If a microorganism crosses the epithelial barrier and begins to replicate in the tissues of host, in most cases it is immediately recognized by the mononuclear phagocytes, or macrophages, that reside in these tissues (Fig. 1). While many cell types are capable of endocytosis, only specialized cells like blood monocytes, tissue macrophages and neutrophils are capable of phagocytosis. The next line of barrier also includes the complement system, which consists of serum proteins that exist in from of inactive proenzymes, which gets activated in response to different specific and non-specific immunologic mechanisms. Once activated, they lead to direct damage to the cell membrane of the pathogenic microorganisms or make them more prone to phagocytic uptake by binding to the pathogens (opsonisation).

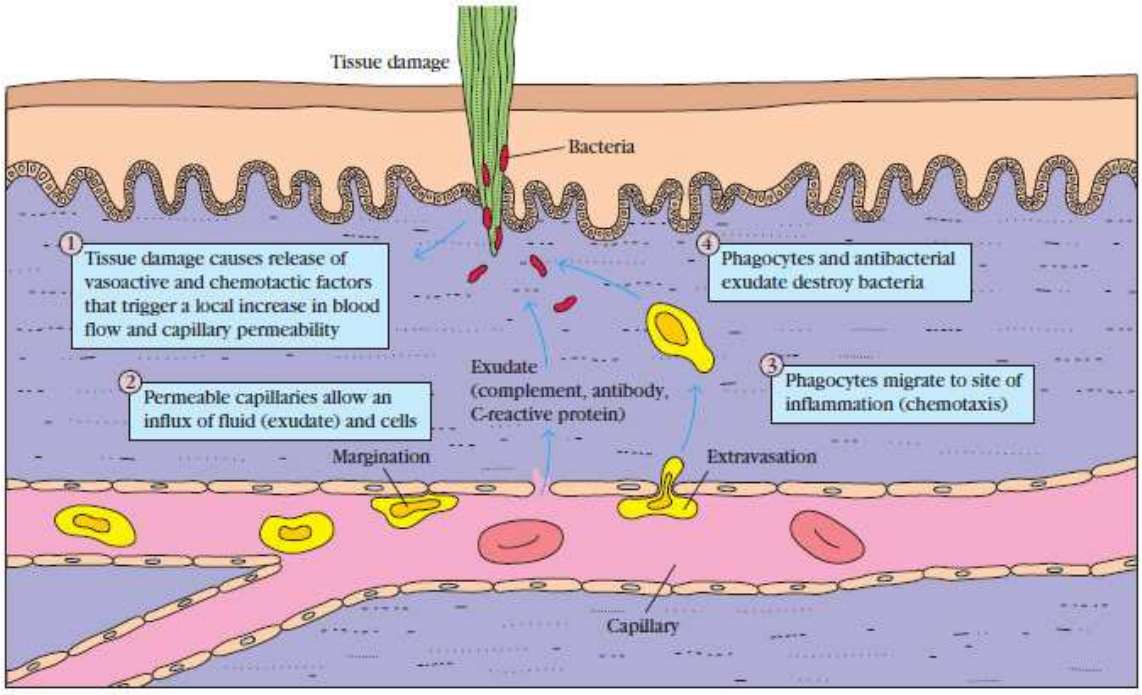

Figure 1. Major events in the inflammatory response. A bacterial infection causes tissue damage with release of various vasoactive and chemotactic factors. These factors induce increased blood flow to the area, increased capillary permeability, and an influx of white blood cells, including phagocytes and lymphocytes, from the blood into the tissues. The serum proteins contained in the exudate have antibacterial properties, and the phagocytes begin to engulf the bacteria. (Goldsby et al. 2000).

As is evident from the very name of it, “adaptive immune system”, it has the ability to adapt to the changes in the threat from the pathogenic microorganisms. Four attributes that characterize the adaptive immune system are: specificity, diversity, memory and self / non-self recognition. The antigenic specificity of the immune system permits it to distinguish subtle differences among the antigens. The immune system is capable of generating tremendous diversity in generating its recognition molecules, allowing it to recognize billions of unique structure on foreign antigens (Fig. 2). Some of the cell types of the adaptive immune system are long lived and these cells contribute to the immunologic memory that makes it possible for the immune system to remember foreign molecules that it has experienced before. The next time the same foreign

molecule is recognized again, the immune system can mount a specific response much faster. Finally, the immune system normally responds only to foreign antigens, indicating that it is capable of self/nonself recognition. It is intuitive to think that the only important thing for the immune system is to defend the body from pathogenic microorganisms, but not to attack the own body is of the same importance, as the outcome of an inappropriate response to self molecules can be fatal.

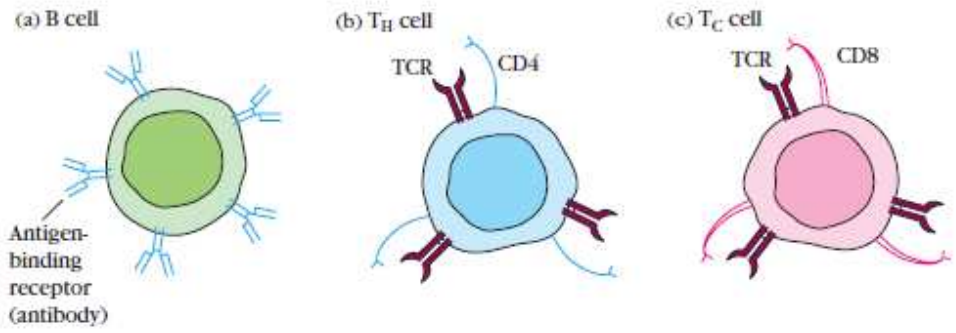

Figure 2. Distinctive membrane molecules on lymphocytes. (a) B cells have about 105 molecules of membrane-bound antibody per cell. All the antibody molecules on a given B cell have the same antigenic specificity and can interact directly with antigen. (b) T cells bearing CD4 (CD4+ cells) recognize only extracellular antigen bound to class II MHC molecules. (c) T cells bearing CD8 (CD8+ cells) recognize only intracellular antigen associated with class I MHC molecules. In general, CD4+ cells act as helper cells and CD8+ cells act as cytotoxic cells. Both types of T cells express about 105 identical molecules of the antigenbinding T-cell receptor (TCR) per cell, all with the same antigenic specificity.(Goldsby et al. 2000).

An effective immune response involves two major groups of cells: lymphocytes and antigen presenting cells. The lymphocytes can be of many different types and they are all produced by haematopoiesis in the bone marrow. The major groups of lymphocytes are T cells and B cells, and both these cell types are antigen specific in their mature state. Lymphocytes leave the bone marrow, circulate in the blood and lymphatic system, and reside in various lymphoid organs. Because they produce and display the antigen binding cell-surface receptors, lymophocytes mediate the defining immunologic attributes of specificity, diversity, memory and self / non-self recognition.

B cells function as professional antigen presenting cells and they produce antibodies as well as different cytokines. The antigen specific receptor of the T cells is called a T cell receptor while the antigen specific receptors of B cells are membrane bound antibodies. After binding of specific antigen to receptor antibodies, the antigen gets internalized and processed into small peptides, which will then be expressed on the surface of B cells with MHC class II. As soon as this MHC II-peptide is recognized by a T helper cell, it will activate the B cell to start proliferating and differentiating into antibody producing plasma cells or memory cells.

The antibodies produced by B cells are either secreted (plasma cells) or remain as membrane bound receptors (memory cells). These molecules are antigen specific. There are five different isotypes of antibodies with different functions. These antibodies bind to antigens and result in opsonisation or complement activation (Kuby 1997).

1.2. Autoimmunity

The concept of autoimmunity was first predicted by Nobel Laureate Paul Ehrlich and he described it as `horror autotoxicus´. We now understand that, while mechanisms of self-tolerance normally protect an individual from potentially self-reactive lymphocytes, there are failures. They result in an inappropriate response of the immune system against self-components termed autoimmunity. Autoimmune diseases result from three interacting components: genetic, environmental and regulatory (Ermann and Fathman 2001). Potential mechanisms of loss of self tolerance and potential induction of autoimmunity could be:

1 **Abnormal B, T cell signalling :** Abnormal T cell activation and cell death signalling underline the pathology of SLE (Kyttaris et al. 2005). Lupus B cells also exhibit abnormal signalling through the B-cell receptor (BCR) (Jenks and Sanz 2009), mammalian target of rapamycin (mTOR) activation and enhanced Ca^{2+} flux (Fernandez et al. 2006).

2 **Abnormal B, T cell apoptosis :** impaired clearance of apoptotic cardiocytes, in infants born to mothers with SLE or Sjogren's syndrome has been linked to anti-

SSA/Ro and –SSB/La antibodies in the pathogenesis of congenital heart block (Clancy et al. 2006).

3 **Abnormal autoantigen handling:** abnormal clearance mechanisms can allow the persistence of antigenic stimulation. Immune responses that specificall target apoptotically modified form of lupus autoantigen have been identified in lupus patients (Greidinger 2001).

4 **Abnormal autoantigen modification:** Although autoantigens targeted in systemic autoimmune diseases share little in common in terms of structure, subcellular distribution, or function in normal cells, these molecules are unified by becoming clustered and concentrated in the surface blebs of apoptotic cells. Furthermore, their structure is altered during some types of cell death to generate structures not previously generated during development and homeostasis (Rosen and Casciola-Rosen 2004).

5 Abnormal recognition of MHC class II: A striking characteristic of human autoimmune diseases is the increased frequency of certain HLA class II alleles in affected individuals. Since alleles positively associated with autoimmune diseases share amino acid residues in the hypervariable HLA regions involved in peptide binding, it is likely that disease associated class II molecules have the capacity to bind the autoantigen and present it to T cells, thereby inducing and maintaining the autoimmune disease (Adorini 1992).

6 Molecular mimicry: Although the triggering event in most autoimmune diseases is unknown, an infectious cause has long been postulated to explain the development of autoimmunity. Molecular mimicry is one mechanism by which infectious agents (or other exogenous substances) may trigger an immune response against autoantigens. According to this hypothesis a susceptible host acquires an infection with an agent that has antigens that are immunologically similar to the host antigens but differ sufficiently to induce an immune response when presented to T cells. As a result, the tolerance to autoantigens breaks down, and the pathogen-specific immune response that is generated cross-

reacts with host structures to cause tissue damage and disease. In Guillain-Barré syndrome antigenic epitopes are shared between Campylobactor glycoproteins and structures of the myelin sheath (Albert and Inman 1999; Goodyear et al. 1999) .

5 **Abnormal regulatory cells:** The crucial role of regulatory cells in self-tolerance and autoimmunity has been clearly established in numerous types of regulatory cells, the majority of which are $CD4^+$ T cells. Much focus has been placed on thymically derived $CD4^+CD25^+$ regulatory T cells, given that the depletion of this subset in murine models results in the spontaneous development of autoimmune diseases (Asano et al. 1996).

6 **Excessive polymorphism of autoantigens:** Autoimmune diseases can be either organ-specific or systemic. In an organ-specific autoimmune disease, the immune response is directed to a target antigen unique to a single organ or gland, so that the manifestations are largely limited to that organ. The cells of the target organs may be damaged directly by humoral or cell-mediated effector mechanisms. Alternatively, the antibodies may overstimulate or block the normal function of the target organ. Few examples of this type of autoimmune disease are Hashimoto's thyroiditis, Goodpasture's syndrome, Insulin-dependent diabetes mellitus, which are mediated by direct cellular damage and others are Graves' disease, Myasthenia gravis, which are mediated by stimulating or blocking autoantibodies (Besinger et al. 1983; Bach 1994; Drachman 1994). In systemic autoimmune diseases, the response is directed towards a broad range of target antigens and involves a number of organs and tissues. These diseases reflect a general defect in immune regulation that results in hyperactive T cells and B cells. Tissue damage is widespread, both from cell-mediated immune responses and from direct cellular damage caused by autoantibodies or by accumulation of immune complexes (Klinman 1989; Lin et al. 1991).

Myasthenia gravis (MG) usually presents in young adult or later adult life as muscle weakness and excessive fatigue during repetitive movements. It most often involves the

extraocular muscles of the eye with double vision and ptosis at onset, but usually progresses to generalized weakness. Cholinesterase inhibitors, by prolonging the action of ACh, tend to lead to clinical improvement. It is the prototype autoimmune disease mediated by pathogenic antibodies. These autoantibodies are directed against various nicotinic Acetylcholine receptor (AChR) on the neuromuscular junction or helper proteins of the receptor such as the muscle-specific kinase MUSK (Vincent and Newsom-Davis 1985; McConville et al. 2004). Interestingly, the anti-AChR antibodies do not block the receptor directly, but they lead to a cross-linking of receptor molecules and an internalisation of the receptor-antibody complexes (Elias et al. 1978; Appel et al. 1979).

1.3. Idiopathic inflammatory myopathies/ myositis

The idiopathic inflammatory myopathies comprise several diseases of which polymyositis (PM), dermatomyositis (DM) and sporadic inclusion body myositis (IBM), collectively called myositis are the most common. Apart from these three well-characterised syndromes, some patients can have postinfectious or unclassified myositis. The inflammatory myopathies are a heterogeneous group of subacute, chronic, or acute acquired diseases of skeletal muscle (Dalakas and Hohlfeld 2003) (Christopher-Stine and Plotz 2004). These disorders are characterized by a clinical spectrum which includes muscle, skin and lung diseases, other associations including cancer and specific autoantibodies (Bohan and Peter 1975 a; Bohan and Peter 1975 b; Love et al. 1991; Targoff 2006; Targoff et al. 2006). Their etiology is not known but genetic and environmental factors are believed to contribute to disease susceptibility and to clinical phenotypes.

1.3.1. Clinical features

The most often used diagnostic or classification criteria for PM and DM were proposed by Bohan and Peter in 1975 (Bohan and Peter 1975; Bohan and Peter 1975 a; Bohan and Peter 1975 b). Later in a proposed revision of the Bohan and Peter criteria, Magnetic resonance imaging to identify inflamed areas in muscle and autoantibodies were also included. Although all classifications identify these disorders as part of a

single disease spectrum, certain important features are used to separate subsets.

- Symmetrical proximal muscle weakness of limb-girdle muscles and anterior neck flexors progressing over weeks to months, with or without disphagia or respiratory muscle involvement.

- Elevation of serum skeletal muscle enzymes, particularly creatine phosphokinase (CK) often aldolase, serum alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and lactate dehydrogenase (LD).

- Electromyographic changes (EMG) indicating muscular impairment i.e. polyphasic short small motor neuron potentials, fibrillation, positive sharp waves, increased insertional irritability, and repetitive high frequency discharges.

- Muscle biopsy evidence of Type I and II fibre phagocytosis, regeneration with basophila, large vesicular sarcolemal nuclei and prominent nucleoli, atrophy in a perifascicular distribution, variation in fibre size and inflammatory exudates, often perivascular.

- Characteristic cutaneous manifestations of dermatomyositis (DM) including lilac discoloration of eyelids (heliotrope) with periorbital edema, a scaly, erythemathosis dermatitis over dorsum of the hands (especially the metacarpophalengeal and proximal interphalangeal joints (Gottron's sign)). This type of distribution is considered to be virtually pathognomonic of DM.

Myositis (refers to all three subsets) patients within different subsets have distinct clinical, histological and serological characteristics. Certain important features used to separate these subsets include the distinctions between childhood and adult onset, PM versus DM and the presence or absence of malignancy and other connective tissue diseases. This suggests the involvement of different mechanisms in the development of different subsets of myositis.

Most patients with PM or DM present with subacute or slowly progressive, usually symmetric, proximal muscle weakness. These patients also suffer from decreased

muscle endurance and fatigue, disabling them from daily activities.

DM is primarily characterized by muscular and cutaneous manifestations (Dalakas and Hohlfeld 2003; Briani et al. 2006; Callen and Wortmann 2006). The onset of DM may be acute or insidious and distal muscles being involved very late. The extensor muscles of the neck may be involved, causing difficulty in holding up the head. Myositis patients with severe muscle weakness can potentially end up wheelchair-bound and requiring assisted ventilation. The most common clinical signs are reduction of the muscular strength in the proximal muscles, contractures and, late in the course of the disease, muscular atrophy.

1.3.2. Extramuscular manifestations

1.3.2.1. Cutaneous manifestations: Dermatomyositis is identified by a characteristic rash accompanying or, preceding muscle weakness. The most common and peculiar skin manifestations are: (1) violaceous erythematous papules which may be observed on the extensor surface of metacarpophalangeal, proximal and distal interphalangeal joints (Gottron's sign) (Fig. 3 A), while Gottron's papules are found covering other bony prominences such as elbows, knees etc; (2) heliotrope rash, a purplish erythema, with or without oedema, in a symmetrical distribution involving periorbital skin (Fig. 3 B). Several other cutaneous features, characteristic of the disease are malar erythema, periungual teleangiectasia with or without dystrophic cuticles, vasculitic skin manifestations consisting of subcutaneous nodules, erythema, periungual infarctions, and digital ulcers, calcification in the subcutaneous tissues leading to subcutaneous painful hard nodules, and Raynaud's phenomenon which is more common in patients with idiopathic DM and in DM associated with connective tissue diseases.

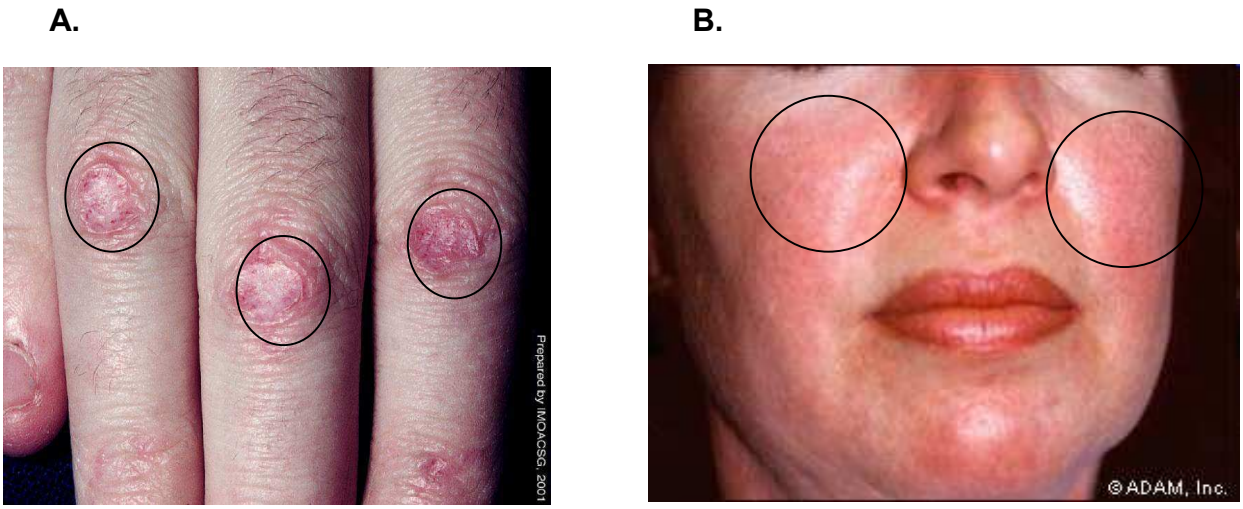

Figure 3. Rash in dermatomyositis. (A) Gottron's rash (circled); (B) Heliotrop rash (circled).

In children, DM resembles the adult disease, except for more frequent subcutaneous calcifications, vasculitic skin changes, and extramuscular manifestations.

DM can be associated with cancer or may overlap with systemic sclerosis and mixed connective-tissue disease. Patients with inflammatory myopathies have higher risk of malignancy than the normal population. In DM, it has been reported to occur in approximately 30% of cases with a higher occurrence in men and in old age (Dalakas and Hohlfeld 2003; Briani et al. 2006; Callen and Wortmann 2006).

1.3.2.2. Pulmonary disease: Pulmonary complications constitute important clinical manifestations of PM or DM. The lungs may be involved either primarily or as a complication of muscle weakness. The reported prevalence of lung involvement in PM and DM varies from 5 to 65 %, (Hepper et al. 1964; Marie et al. 1998; Douglas et al. 2001; Fathi et al. 2004) depending on whether clinical, radiological, functional or pathological criteria have been used. The most common pulmonary disorder in PM and DM is interstitial lung disease (ILD), not always related to muscle symptoms and is one of the hallmarks of anti-synthetase syndrome. ILD can appear before, at the same time or after the onset of skin or muscle symptoms. The presence of ILD in patients with

myositis affects the prognosis, with increased morbidity and mortality, and this often also has an influence on the choice of immunosuppressive treatment.

1.3.2.3. Heart involvement: The clinical cardiac manifestations most frequently reported in IIMs, myositis, are congestive heart failure, conduction abnormalities and coronary artery disease. The frequency of heart involvement in myositis varies between 6% and 75% depending on patient selection, definition of heart involvement, whether clinical manifest or subclinical cardiac involvement is considered, and methods used to detect cardiac involvement (Bohan et al. 1977; Gottdiener et al. 1978; Taylor et al. 1993). Also children with juvenile DM may develop cardiac involvement although the frequency seems to be low. ECG abnormalities observed in PM and DM patients include atrial and ventricular arrhythmias, bundle branch block**,** A-V blocks, high- grade heart block, prolongation of PR-intervals, ventricular premature beats, left atrial abnormality, abnormal Q-waves, as well as non-specific ST-T wave changes.

Though clinically significant cardiac involvement is infrequent in PM and DM, but cardiovascular manifestations constitute a major cause of death (Bohan et al. 1977 ; Hochberg et al. 1986). Based on available data it can be suggested that inflammation of the heart muscle could be important in the pathophysiologic mechanisms which could lead to myocarditis and congestive heart failure or conduction disturbances in patients with myositis.

1.3.2.4. Other organ involvement: Other organs that are frequently involved in myositis are the pharyngeal and gastrointestinal muscles. The most common GI symptom is dysphagia and disordered esophageal motility.

1.3.2.5. Malignancy: Although all the inflammatory myopathies can have a chance association with malignant disease, especially in older age-groups, the frequency of cancer is definitely increased in DM (Sigurgeirsson et al. 1992; Buchbinder et al. 2001). The most common cancers are those of the ovaries, gastrointestinal tract, lung, and breast and non-Hodgkin lymphomas (Hill et al. 2001). The increased risk is both at the time of DM diagnosis but also after more than 10 years duration of disease (Callen 2002). There have been some recent reports linking polymyositis also with malignancy

of one or the other type. These reports include: paraneoplastic polymyositis associated with; squamous cell carcinoma of the lung (Gabrilovich et al. 2006); with transitional cell carcinoma of the bladder (Bouropoulos et al. 1997); thymic carcinoma (Inoue et al. 2009) and a renal carcinoma (Wurzer et al. 1993).

1.3.2.6. Overlap syndromes: PM and DM are seen in association with various autoimmune and connective tissue diseases (Table 1). The term overlap syndrome indicates that certain clinical signs are shared by both disorders. Myositis-overlap syndromes are characterized by a heterogeneous group of clinical syndromes of which many are closely linked with specific autoantibodies. It is only DM, and not PM, that truly overlaps and that too only with systemic sclerosis and mixed connective-tissue diseases. In a recent report DM has been shown to overlap with lupus (Dayal and Isenberg 2002).

Characteristic	Dermatomyositis	Polymyositis	Inclusion-body myositis
Age at onset	All ages	>18 years	>50 years
Familial association	No	No	In some cases
Extramuscular manifestations	Yes	Yes	Yes
Associated disorders			
Connective-tissue diseases*	Only with scleroderma and mixed connective-tissue disease	Yes, with all	Yes, in up to 20% of cases
Overlap syndrome†	Only with scleroderma and mixed connective-tissue disease	No	No
Systemic autoimmune diseases	Rarely	Frequently	Infrequently
Malignant disorders	Yes, in up to 15% of cases	No	No
Viruses	Unproven	Yes‡	Yes‡
Parasites and bacteria	No	Yes§	No
Drug-induced myotoxicity‖	Rarely	Yes	No

Table 1. Conditions and factors associated with inflammatory myopathies (Dalakas and Hohlfeld 2003).

1.3.3. Epidemiology

The IIMs are rare disorders and the reports of incidence and prevalence are limited. In PM/DM several different classification systems have been proposed. Although all classifications identify these disorders as part of a single disease spectrum, certain important features are used to separate subsets. The different incidences in different population based studies, might be due to varying inclusion criteria as well as varying case retrieval strategies used in these studies, which makes comparisons difficult, but

ethnic or geographical differences can not be excluded (Benbassat et al. 1980; Oddis et al. 1990; Weitoft 1997).

1.3.3.1. Incidence by Age, Race and Sex: Although inflammatory myopathy can occur at any age, the observed pattern of incidence includes childhood and adult peak and a paucity of patients with onset in the adolescent and young adult years. The incidence sex ratio is: 2.5:1 female to male (Benbassat et al. 1980; Oddis et al. 1990; Leff et al. 1991; Symmons et al. 1995; Vegosen et al. 2007). This ratio is lower (nearly 1:1) in childhood disease and with associated malignancy, but is very high 10:1 when there is an associated connective tissue disease. PM/DM has a 3-4:1 Black to White incidence ratio.

1.3.3.2. Environmental factors: The etiology of myositis is most likely interplay between genetic susceptibility and exposure to certain environmental factors. Disease onset is more frequent in the winter and spring months, especially in childhood cases, consistent with precipitation by viral and bacterial infections. Seasonal patterns in the onset of myositis characterized by disease-specific autoantibodies such as anti-Jo1 and anti-signal recognition particle (SRP) has been reported, indicating a common environmental factor contributing to the disease (Leff et al. 1991; Vegosen et al. 2007). In one study, PM/DM patients reported excessive physical exercise, antedating illness significantly more frequently than controls, but this association has not been confirmed.

1.3.3.3. Genetic susceptibility: Myositis is associated with certain HLA-DR genotypes (Arnett et al. 1996; Shamim et al. 2000; Badrising et al. 2004; O'Hanlon et al. 2006). White children with DM and adults with PM have an increased frequency of HLA-B8/DR3, and HLA-B14 and B40 have been observed more commonly in adults with DM coexisting with another connective tissue disease. HLA is considerably more closely linked to several recently identified serum autoantibodies which have been found to define clinically homogenous patient groups. Anti-Jo1 antibody patients have a significantly increased frequency of HLA-DRw52 compared with control person, and those with anti-PM-Scl, nearly all possess HLA-DR3 or DRw52.

1.3.3.4. Viral infections: Many patients with myositis have reported that their clinical symptoms have appeared and persisted after a cold or flu episode. Infection with different viruses such as coxacievirus, adenovirus, parvovirus B19 and the retrovirus human immunodeficiency virus (HIV) has been associated with myositis (Travers et al. 1977; Dalakas et al. 1986; Harland et al. 1991; Jongen et al. 1994; Chevrel et al. 2000; Crowson et al. 2000; Douche-Aourik et al. 2003; Dalakas et al. 2007). Potential mechanisms through which an infection can lead to an autoimmune disease process include molecular mimicry, epitope spreading, bystander activation, polyclonal activation and viral superantigen activation (Wucherpfennig 2001).

1.3.4. Etiology

As the name idiopathic inflammatory myopathies, the causes of the PM or DM are unknown. However, infectious agents, drugs, toxins, genetic factors all have claimed for attention as etiologic agents in IIMs. The rarity of myositis has precluded concordance studies in twins, but reports of multicase families support a familial predisposition (Shamim et al. 2000). According to a recent study different immunogenetic profiles influence both clinical phenotype and the pattern of circulating myositis-specific autoantibodies (Chinoy et al. 2006).

A recent work provides evidence that seasonal birth distributions of Hispanic juvenile IIM patients, juvenile IIM patients with the p155 autoantibody, and juvenile patients with certain HLA alleles differ from the birth distributions of patients in other subgroups or from the birth distribution of a population of individuals not known to have an autoimmune disease. It also suggests that early environmental influences have a greater influence on childhood-onset myositis than on adult-onset myositis (Vegosen et al. 2007).

1.3.5. Pathogenesis

1.3.5.1. Immune mechanisms: The causes and pathogenesis of myositis remains unclear but immune mechanisms are strongly implicated. The autoimmune origin of these diseases is supported by their association with other autoimmune disorders, autoantibodies (Targoff 2002), and histocompatibility genes; the evidence of T-cell-

mediated myocytotoxicity or complement-mediated microangiopathy, and their response to immunotherapy. Investigations of skeletal muscle tissue suggest that both the skeletal muscle fibres and the microvasculature could serve as targets of the immune response through involvement of mononuclear inflammatory cells and their mediators (Hohlfeld and Engel 1994; Targoff 2000; Dalakas 2002 a; Dalakas and Hohlfeld 2003).

1.3.5.2. Inflammatory infiltrates: Based on major patterns of inflammatory infiltrates in skeletal muscle tissue in myositis, these diseases can be distinguished: (a) Endomysial infiltrates with primarily CD8+ T cells surrounding and invading muscle fibers and with macrophages, less common CD4+ T cells, mainly observed in patients with PM and IBM; (b) Perivascular infiltrates predominantly composed of CD4+T cells, macrophages and occasionally B cells are mainly detected in DM patients (Fig. 4 A and B) (Arahata and Engel 1984; Engel and Arahata 1984). Though these diseases share three dominant histological features that are ultimately responsible for the clinical signs of muscle weakeness: inflammation, fibrosis and loss of muscle fibers, yet they are clinically and immunopathologically distinct (Engel and Arahata 1984; Hohlfeld and Engel 1994; Dalakas 1995).

A B

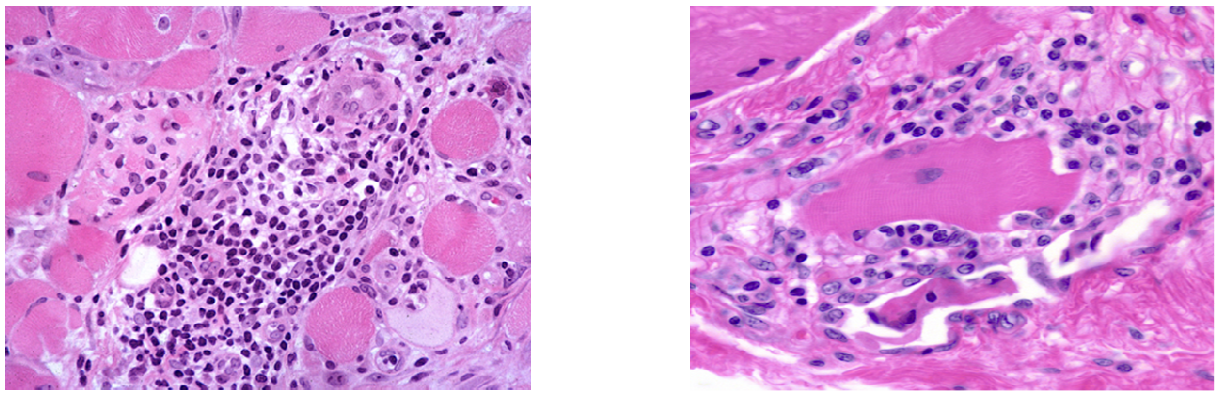

Figure 4. (A) H & E staining of a muscle from Dermatomyositis patient showing perivascular infiltration. (B) H & E staining of a muscle from Polymyositis patient showing endomysial infiltration.

1.3.5.3. Immune cells in myositis: T cells are frequently present in the muscle tissue in all subsets of myositis but with large individual variations. Electron microscopy studies of inflamed muscle tissue from polymyositis patients suggested that CD8+T cells are cytotoxic to muscle fibres (Arahata and Engel 1986). These CD8+ as well as CD4+ muscle-infiltrating T cells have been shown to be perforin positive (Goebels et al. 1996), suggesting a possible T cell-muscle cell interaction. In patients with PM and IBM, but not in DM, only certain T cells of specific TCRα and TCRβ families are recruited to the muscle from the circulation (Mantegazza et al. 1993; O'Hanlon et al. 1994a; Bender et al. 1995). Cloning and sequencing of the amplified endomysial or autoinvasive TCR-gene families have demonstrated a restricted use of the *Jβ* gene with a conserved amino acid sequence in the CDR3 region, indicating that CD8+ cells are specifically selected and clonally expanded *in situ* by muscle specific autoantigens (Salajegheh et al. 2007). A cytotoxic effect of T cells is still a subject of controversy since no muscle-specific antigens have been identified and since an expression of the co-stimulatory molecules CD80/86, normally required for functional interaction, has not been detected in inflamed muscle fibres (Yamada et al. 2001).

Though inflammatory cell infiltrates in muscle tissue, often decrease after conventional immunosuppressive treatment, yet in some cases the inflammatory cells may persist (Bunch et al. 1980; Lundberg and Chung 2000; Korotkova et al. 2008).

1.3.5.4. Immunological molecules: In the skeletal muscle tissue of healthy individuals only the presence of occasional mononuclear cells or expression of immunological molecules can be observed (Malm et al. 2000; Dorph et al. 2006). Inflammatory cells, adhesion molecules, pro-inflammatory cytokines and chemokines detected by immunohistochemistry are usually over-expressed in muscle tissue of myositis patients compared to in healthy individuals, leading to the belief of their involvement in the pathogenesis of myositis. Complement activation leading to the formation and deposition of membranolytic attack complex on the endomysial microvasculature, resulting in capillary necrosis, inflammation, ischemia and perifscicular atrophy has been seen in DM (Hohlfeld and Engel 1994; Dalakas 2002 b; Dalakas and Hohlfeld 2003) while in polymyositis an antigen-directed and MHC class I restricted CD8 T cel

mediated cytotoxicity has been implicated. The above is supported by following reports: the cytotoxicity of endomysial T cells to autologous myotubes (Hohlfeld and Engel 1994); the clonal expansion of autoinvasive T cells and the restricted usage of T cell receptor gene families (Mantegazza et al. 1993; O'Hanlon et al. 1994b; Bender et al. 1995; Amemiya et al. 2000; Benveniste et al. 2001; Nishio et al. 2001); the upregulation of co-stimulatory molecules (Behrens et al. 1998; Murata and Dalakas 1999); and the release of perforin granules by autoinvasive CD8 cells to lyse muscle fibres (Goebels et al. 1996). Upregulated chemokines, cytokines and adhesion molecules enhance the transmigration of T cells from the circulation to the muscle (Choi and Dalakas 2000; Figarella-Branger et al. 2003). What triggers complement activation in DM or T cell activation in PM still remains unclear.

1.3.5.5. Humoral immunity: Presence of B cells in DM and to a lesser extent in PM and IBM patients support the involvement of humoral adaptive immunity in these diseases (Arahata and Engel 1984; Greenberg et al. 2005; Bradshaw et al. 2007). It appears that the disease is driven, at least partly, by a loss of self tolerance with the production of autoantibodies. Up to 80% of patients with PM or DM, but less commonly in patients with IBM, have autoantibodies (Levine 2005; Noss et al. 2006). Evidently, plasma cells detected in muscle tissues of PM and IBM patients had undergone oligoclonal expansion, and affinity maturation as well as isotype switching had occurred in individual cells, suggesting that antigen drives a B cell antigen-specific response in muscle tissue of myositis patients (Bradshaw et al. 2007).

The most common autoantibodies are antinuclear autoantibodies. Some of the autoantibodies are often found in other inflammatory connective tissue diseases (for eg. Anti-PMScl, anti-SSA, and anti-SSB) and are called myositis-associated autoantibodies. Other autoantibodies, called myositis specific autoantibodies, are more specific for myositis, although they might not be found exclusively in myositis but occasionally in other patients. Recent investigations have demonstrated that about 20% to 30% of myositis patients have autoantibodies seen mainly in patients with myositis. Anti-Jo-1 (antihistidyl-tRNA synthetase) is the most prevalent MSA (myositis specific antibodies) and the most common of the eight anti-aminoacyl-tRNA synthetase autoantibodies

(anti-ARSs) currently described (Nishikai and Reichlin 1980; Mimori et al. 2007; Gunawardena et al. 2009a). These autoantibodies target cytoplasmic enzymes that catalyze the binding of specific amino acids to their cognate tRNA and define the anti-synthetase syndrome (ASS) (Targoff and Reichlin 1985). Autoantibodies to Mi-2 are detected in patients with hallmark dermatomyositis features. Mi-2 is a nuclear helicase protein that forms part of the nucleosome remodelling deacetylase (NuRD) complex playing a role in gene transcription (Wang and Zhang 2001). In recent years, a number of novel MSAs have also been described. Sato et al. (Sato et al. 2005) identified an autoantibody in Japanese patients with clinically amyopathic dermatomyositis (CADM) and rapidly progressive lung disease. This autoantibody (known as anti-CADM140) targets a cytoplasmic 140-kDa protein melanoma-differentiation associated gene 5 (MDA5). This autoantigen is involved in the innate immune defence against viral infections through the detection of viral dsDNA (Takeuchi and Akira 2008). In addition, anti-p155/140 autoantibodies have been reported in adult and juvenile dermatomyositis (Targoff et al. 2006; Kaji et al. 2007; Chinoy et al. 2007 a). Targoff et al. identified p155/140 as transcriptional intermediary factor 1-gamma (TIF1-g), a nuclear protein involved in cellular differentiation. The most striking feature of anti-p155/140 autoantibodies is the association with malignancy in adults. Gunawardena et al. have described autoantibodies to a p140 kDa target in juvenile dermatomyositis (Gunawardena et al. 2009b). The p140 kDa target has recently been identified as nuclear matrix protein NXP-2, involved in nuclear transcription. Finally, Betteridge et al. have described anti-SAE (small ubiquitin-like modifier activating enzyme) autoantibodies in adult dermatomyositis patients (Betteridge et al. 2007; Betteridge et al. 2009 a). The target autoantigen, small ubiquitin like modifier-activating enzyme is a protein involved in posttranslational modification that is located in both the nucleus and cytoplasm of cells (Dohmen 2004).

1.3.5.6. Major histocompatibility complex (MHC) class I antigen: The Major Histocompatibility Complex (MHC) is a set of molecules displayed on cell surfaces that are responsible for lymphocyte recognition and "antigen presentation". The MHC molecules control the immune response through recognition of "self" and "non-self". The Class I and Class II MHC molecules belong to a group of molecules known as the

Immunoglobulin Supergene Family, which includes immunoglobulins, T-cell receptors, CD4, CD8, and others. Class I MHC molecules bind peptides and present them to CD8+T cells. In general these peptides are derived from enoodgenous intracellular proteins that are digested in the cytosol. The peptides are then transported from the cytosol into the cisternae of the endoplasmic reticulum, where they interact with class I MHC molecules. This process is know as the cytosolic or endogenous processing pathway (Fig. 5).

Muscle fibres of most myositis patients, in contrast to those of healthy individuals, express MHC class I antigen. The over-expression of MHC class I molecules is an early event in many autoimmune diseases, since it is a prerequisite for the cytolytic action of cytotoxic T lymphocytes. MHC class I molecules by themselves can have a deleterious effect on cell types that do not constitutively express these molecules (Nagaraju 2005). Normal human skeletal myoblasts constitutively express low levels of MHC class I molecules under cell culture conditions (Hohlfeld and Engel 1991; Nagaraju et al. 1998). Muscle fibres of healthy individuals do not express detectable levels of MHC class I antigens, although these fibres have been shown to express MHC class I in several autoimmune muscle diseases (Appleyard et al. 1985; Emslie-Smith et al. 1989). A transgenic mouse model that constitutively over-expresses MHC class I in skeletal muscle fibres develops clinical, biochemical, histological and immunological features similar to those of human myositis (Nagaraju et al. 2000). Some investigators advocate characterize DM patients histologically by MHC class I antigen expression predominantly located to perifascicular muscle.

The explanation for the MHC class I expression on muscle fibres in human myositis is unknown. An endoplasmic reticulum stress response is suggested to be a link between MHC class I up-regulation and muscle damage and dysfunction in PM and DM. Over-expression of MHC class I induced an ER-stress response in mouse model, by up-regulating nuclear factor (NF)-kB, which is strongly activated in myositis and can suppress myoblast differentiation and induce pro-inflammatory cytokines causing muscle damage.

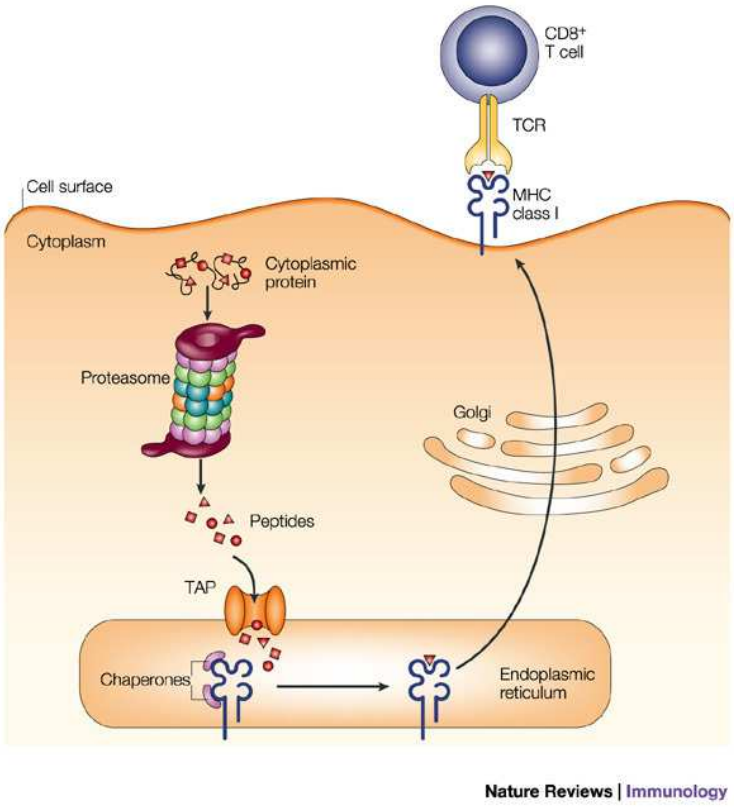

Figure 5. Cytosolic and nuclear proteins are degraded by the proteasome into peptides. The transporter for antigen processing (TAP) then translocates peptides into the lumen of the endoplasmic reticulum (ER) while consuming ATP. MHC class I heterodimers wait in the ER for the third subunit, a peptide. Peptide binding is required for correct folding of MHC class I molecules and release from the ER and transport to the plasma membrane, where the peptide is presented to the immune system. TCR, T-cell receptor (Kuby 1997).

1.3.5.7. Enhanced autoantigen expression: Casciola-Rosen et al demonstrated enhanced expression of both Mi-2 and Jo-1 in myositis muscle compared with normal muscle, and this was primarily observed in regenerating muscle rather than in mature myotubes (Casciola-Rosen et al. 2005). They also showed that myositis autoantigen expression is also markedly increased in several cancers known to be associated with autoimmune myositis, but not in their related normal tissues, showing the similarity between tomor cells and undifferentiated myoblasts. Work from Casciola-Rosen et al.

and Zampieri et al supports the hypothesis that the presence of candidate myositis autoantigens during reparative myogenesis and in cancer-associated myositis, an autoimmune response directed against cancer, cross reacting to regenerating muscle cells can drive induction and propagation of the autoimmune response (Casciola-Rosen et al. 2005; Zampieri et al. 2008).

1.3.6. Treatment

The general recommended treatment of PM and DM is based on immunosuppressive agents, starting with high dosage of glucocorticosteroids often in combination with methotrexate, azathioprine (Oddis 1994; Adams and Plotz 1995; Mastaglia et al. 1997). Glucocorticosteroids can reduce the inflammatory infiltrates in muscle tissue and thereby decrease the expression of some pro-inflammatory molecules and adhesion molecules (Lundberg and Chung 2000).

In a placebo-controlled trial high-dose IVIg was beneficial in treatment resistant DM patients, both regarding muscle function and muscle histopathology (Dalakas et al. 1993). Although the clinical effects were accompanied by decreased ICAM-1 and MHC class I expression in repeat muscle biopsies, the number of patients that were subject to repeat biopsy was small. In case of PM patients treated with IVIg, only the clinical reports are available to date (Cherin et al. 1990; Cherin et al. 1991; Cherin et al. 2002; Danieli et al. 2002). So before plunging to start treating myositis patients with IVIg, further investigation of the underlying molecular mechanisms is necessary.

There are few studies reporting varying effects of treatment with tumor necrosis factor (TNF) blockers in patients with PM or DM. TNF blockade has been inconsistent and even worsened disease in some reported cases (Hengstman et al. 2003; Hengstman et al. 2004), again underscoring the need to understand the molecular mechanisms involved in these diseases.

2. Aim

Since inflammatory histopathology could be found in the muscles of IIM patients and steroids or other immunosuppressantrs are helpful in DM/PM, an autoimmune etiology was proposed in these diseases. Aim of the study was to identify and characterize the muscle specific autoantigens in IIMs, using different autoantibody detection methods and protein biochemistry approach, such as 2-DE and mass spectrometry. The project aimed at answering following questions:

First, do the patients have muscle-specific or endothelial cell specific autoantibodies? If yes, which are the underlying autoantigens?

Second, do the autoantibodies from myositis patients have any functional effects?

Thirdly, do the statins, reported to stimulate expression of MHC class I on muscle cells leading to the development of myopathy, affect expression of MHC class I , TAP and LMP (involved in class I antigen presentation) *in vitro* on muscle cells?

3. Patients

Serum was obtained from 41 patients with inflammatory muscle diseases (mean age 50.3 ± 17.8 years; 24 female / 17 male) after informed consent and approval of the local ethical committee. 11 patients had polymyositis and 15 patients had dermatomyositis according to the criteria of Bohan and Peter (Bohan and Peter 1975; Bohan and Peter 1975 a; Bohan and Peter 1975 b). The other myositis patients (n=15) had unclassified or postinfectious myositis. The epidemiological data of the groups are given in table 2. The mean duration of myositis at the time the serum sample was obtained was 1.1 ± 0.7 years. Sera of 26 healthy people (HC) served as controls (mean age 51.5 ± 18.2 years, 15f / 11m).

Diagnosis	n	Mean age ± StDev	Female / Male
Dermatomyositis	**15**	**42.4 ± 19.0**	**12f / 3m**
Polymyositis	**11**	**63.1 ± 12.1**	**6f / 5m**
Unclassified myositis	**15**	**49.2 ± 14.9**	**6f / 9m**

Table 2. Patient data of myositis patients.

4. Materials

4.1. Chemicals and Solutions

Acetic Acid 100%	Merck
Agarose multipurpose	Bioline GmbH
Ammoniumpersulfat APS	Roth
Antioxidant	Invitrogen
5-bromo 4-chloro 3-indolyl phosphate / nitro-blue tetrazolium chloride (BCIP/NBT)-Blue Liquid Substrate	Sigma Aldrich
BDH crystalline Trypsin (bovine pancreas)	Sigma Aldrich
Bovine Serum Albumine, Fraction V (BSA)	Sigma Aldrich
Bromphenolblue	Neolab
β-Mercaptoethanol	Fluka
CelLytic™ M Cell Lysis Reagent	Sigma
Deoxiribunuclease 1, Type 4 bovine pancreas	Sigma Aldrich
Destilled Water „Aqua ad injectabilia“	Braun
D(+)Glucose	Gibco
Dimethylsulfoxide (DMSO)	Roth
Ethylendiamintetracetic acid (EDTA) 0,5M	Roth
Ethanol 100 %	Sigma Aldrich
Ethanol for Molecularbiology 100%	Merck
Ethidiumbromid	Merck
Ficoll-PaqueTM Plus	Amersham
5-Fluoro-2´-deoxyuridine	Merck
Glycerol	Roth
Glycin	Merck
H-EBSS	Gibco
Hepes	Sigma
Interferon γ (IFN-γ)	Provitro
IgG-Standard	Sigma
Isopropanol	Merck
Potassiumchlorid (KCl)	Merck
Potassiumdihydrogenphosphat (KH_2PO_4)	Merck
4x SDS sample Buffer (for WB)	Invitrogen
Methanol	Merck
Magnesiumsulphate ($MgSO_4$)	Sigma
Sodiumchloride (NaCl)	Roth
Sodiumhydrogencarbonate ($NaHCO_3$)	Merck
Disodiumhydrogenphosphate (Na_2HPO_4)	Merck
Sodiumdihydrogenphoaphate (NaH_2PO_4)	Merck
Sodiumazide (NaN_3)	Merck
Non-fat dry milk, blotting grade	Bio-Rad

NuPage SDS MOPS Running Buffer	Invitrogen
PageRuler™ Prestained Protein Ladder	Fermentas
Paraformaldehyde (PFA)	Sigma Aldrich
Phosphate Buffered Saline (PBS) 10x (for cellcultur!)	Gibco
Poly-L-lysin-hydrobromide	Sigma
Ponceau S	Sigma / Roth
Protease Inhibitor Cocktail	Sigma
Protein G-Sepharose	GE Healthcare Bio-Science AB
Quick Load 1kb DNA Ladder	Biolabs
RNAse free Water	
Rotiphorese Gel 30 = 30 % Acrylamide-Mix	Applichem
Sample reducing Agent (10x)	Roth
Saponin	Invitrogen
Sodiumdodecylsulfate (SDS)	Sigma
Tris-Acetat-EDTA Buffer (TAE) 10x	Neolab
Trichlor-Acetic acid (20%)	Roth
Trishydroxymethylaminomethan (Tris)	Roth
Tris-HCl	Sigma
Trypanblue	USB
Trypsin (2,5g/l)	Roth
5% Trypsin-EDTA (10x)	Gibco
Trypsin inhibitor Type I-S: from Soybean	Gibco
Trypsin Type XII-S	Sigma Aldrich
Tween20	Sigma Aldrich

4.2. Consumables

Cellstar® 6 Well Cell Culture Plate	Greiner bio-one
Cellstar® Plastikpipettes (5 ml, 10 ml)	Greiner bio-one
Cellstar® U-shape with Lid, TC-Plate, 96 well, sterile	Greiner Bio-one
Cellstar® 75 cm^2 Cell cultur flasks	Greiner Bio-one
Cryobox 136x136x130 mm	Ratiolab GmbH
Cryo Tube™ vials (1,8 ml; 4,5 ml)	Nunc
Disposable scalpel, sterile	Feather safity razorco
2D-well Gradient gel NuPage 4-12% (1,0 mm thick)	Invitrogen
Disposable cuvettes	Ratiolab
FACS-Tubes 0,5 ml 38x6,5 mm PS	Sarstedt
Falcon 5ml Polystyrene Round-Bottom Tube	Becton Dickinson
Falcon® Plastic pipettes 25 ml	Becton Dickinson Labware
Falcon tubes (15 und 50 ml)	Becton Dickinson
Gel documentation Thermal Image System FTI-500	Fuji Film
Tissue culture dishes steril 35,0 / 10 mm	Greiner bio-one
Glaswares (different sorts)	Fisherbrand; IDL; Schott&Gen;

	Simax
Kodan® Tinktur Forte (alcoholic skin disinfectant)	Schülke & Mayr
LightCycler® Capillaries [20 µl] (for Real time-PCR)	Roche
Minisart single use filter (0,2 µm, 0,45 µm)	Biotech
Neubauer improved	Brand
Nitra-Tex® powder free	Ansell
Nitrocellulose membrane	Biometra
NobaGlove® –Latex powder free	NOBA GmbH
Nunclon™ surface 96-Well plates with flat bottom	Nunc™
Parafilm	American National Can
Glas Pasteur pipettes 150 mm	Brand
Pipette tipps (10µl, 100µl, 1000µl)	Sarstedt
PP-PCR-Tubes 0,2ml thin walled	Greiner bio-one
Grid inserts for Cryobox	Ratiolab GmbH
Reaction tubes 1,5 ml	Sarstedt
Safety-Multifly®–Set, sterile, pyrogenfree (Cannulae)	Sarstedt
Servapor® dialysis tubing (6mm, 25mm)	Serva Electrophoresis GmbH
S-Monovette® 7,5 ml Z (Serum-Tubes)	Sarstedt
Sterile Pipette tips with filter	Nerbe Plus
UV-spectroscopic cuvettes	Bio-Rad
Whatmann-Filterpaper 3 mm	A. Hartenstein
Cell scrapper	Greiner bio-one

4.3. Instruments

Liquid Nitrogen tank	Arpege 75
Blotapparatur	Invitrogen
ClasII Type A/B3 (Sterilbank)	Nuaire Biological Safety Cabinets
Easia shaker	Medgenix diagnostics
FACSCalibur	Becoton Dickinson
Fluorescence microscope DM RB	Leitz
Gel documentation Image Masters VDS	Pharmacia Biotech
Gel electrophoresis chamber	Peqlab
Gel trays und Gel combs	Peqlab
Heating block / Thermoshaker	Peqlab
HiTrapTM Protein G HP (1ml und 5ml Protein G columns)	Amersham Biosciences
Inverse Light microscope MBL 3100	A.Krüss Optronic
Refrigerators and Freezers	Bosch, Liebherr, Nuaire, Santo, Premium
Light Cycler 1.5	Roche Diagnostics
LightCycler centrifuge adapter	Roche Diagnostics

Magnetic mixer	IKA® Werke
Microwell	SHARP Electronics
Multiscan Ex (ELISA-Reader)	Thermo Electron Corporation
Nalgene™ Cryo 1 °C Freezing Container	Nalgene®
NOVEX Xcell surelock Blotting chamber	Invitrogen
PC-System, Printer	Hewlett Packard
pH-Meter	Schott Geräte
Pipettes (different volumes)	Gilson, Eppendorf
Pipette boy	Integra Biosciences
ProSpec (Nephelometer)	Dade Behring
Pump P-1 (Pump for IgG purification)	Pharmacia Biotech
Rotamax 120 (Shaker)	Heidolph
Swivel platform	Peqlab
SmartSpec™ Plus Spectrophotometer	Bio-Rad
Sonopuls HD 2070 (Ultra sonicator)	Bandelin Elektronik
Power pack	Peqlab
Steri-Cult 200 Incubator for cell culture	Labotec GmbH
Sterile bench	Köttermann
Thermocycler	Biometra
Table top centrifuge EBA 20	Hettich
Table top centrifuge micro 120	Hettich
Universal 32 R (centrifuge)	Hettich
Vortex Minishaker	IKA® Werke
Vortexer Vortex-Genie2	Scientific Industries
Weighing balance	Sartorius AG
Waterbath	Memmert
Centrifuge Typ 2-6	Sigma
Centrifuge Universal 32 R (cell culture)	Hettich

4.4. Molecular biology and biochemistry kits

Cytotoxicity Detection Kit (LDH)	Roche Applied Science
Quanti Fast™ SYBR Green PCR Kit	Qiagen
RevertAid™ First Strand cDNA Synthesis Kit	Fermentas

4.5. Buffers and Solutions

DNA-loading buffer (10x):	250 mg	Bromphenolblue
	33 ml	Tris (150 mM, pH 7.6)
	60 ml	Glycerol
	7 ml	H_2O

Ethidium bromide staining solution	2.5 mg	Ethidiumbromide
	1 L	1x TAE
FACS buffer	500 ml	1x PBS
	5 ml	10 % NaN_3
	5 ml	Fetal calf serum (FCS)
Glycine buffer	3.75 g	Glycine
	In 500 ml	dH_2O
		pH 9.0
IgG-Elution buffer	0.75 g	Glycine
	100 ml	dH2O
		pH 2.7
		(= 0.1 M)
Blocking buffer	0.4 g	Non fat dry milk
	10 ml	PBS Tween
1 % Paraformaldehyde (PFA)	1 g	PFA
	100 ml	1x PBS
PBS (10x)	80 g	NaCl
	2 g	KCl
	14.4 g	Na_2HPO_4
	2.4 g	KH_2PO_4
	Dissolve in 1 L	dH_2O
1x PBS	900 ml	dH_2O
	100 ml	PBS (10x)
PBS Tween	1 L	1x PBS
	500 µl	Tween 20
Permeabilisation buffer	500 ml	FACS buffer
	0.5 g	Saponin
Poly-L-Lysin	60 ml	dH_2O
	3 mg	Poly-L-Lysin
Ponceau S-solution	0.25 g	Ponceau S
	15 ml Make up	Trichloracetate (TCA)
	100 ml	with H_2O (protect from light)

Protein (SDS)-loading-Buffer 4x	25 ml	1M tris pH 6.8
	4 g	SDS
	40 ml	Glycerin
	0.8 ml	0.5 EDTA (pH between 7.5 und 8.5)
	20 ml	Methanol
	2 pinches	Bromphenol blue
	in 100 ml	with dH_2O (short term storage 4C, long term storage at -20 C)
Stacking gel buffer	9.09 g	Tris-HCl
	in 50 ml	dH_2O dissolve
		adjust pH to 6.8
SDS (10%)	100 g	SDS
	in 900 ml	dH_2O dissolve by heating at 37°C
	make up 1 L	
SDS-running buffer (10x)	10 g	SDS
	30.3 g	Tris
	144.1 g	Glycin
	in 1 L	H_2O
TAE (Tris-Acetate-EDTA)-buffer (50x)	242 g	Tris
	57.1 ml	Acetic Acid
	100 ml	0.5M EDTA
	Make upto 1 L	with dH_2O
		adjust pH to 8.5
TBS-buffer	4.5 g	NaCl
	0.71 g	Na_2HPO_4
	0.15 g	NaH_2PO_4
	5.5 g	Non fat dry milk
	2.5 ml	Tween 20
	500 ml	dH_2O
Transfer-buffer	1.513 g	Tris (25 mM)
	5.63 g	Glycin (150 mM)

	50 ml	Methanol (10 %) Adjust pH to 8.3
Resolving gel buffer	18.17 g Disslove in 100 ml	Tris-HCl dH_2O adjust pH to 8.8
Trypsin-solution	10 ml 2.5 mg	Solution 1H Trypsin
Trypsin Inhibitor solution	10 ml 1.6 ml	Solution 1H Konz. D/T-I Lsg

4.6. Media and solutions for cell culture

TE671	RPMI1640	10 % 2mM 1 %	Fetal calf serum (FCS) Glutamin (200 mM) PenStrep (Penicillin/ Streptomycin)	Gibco Hyclone Gibco Gibco
C2C12	RPMI1640	10 % 2mM 1 %	Fetal calf serum (FCS) Glutamin (200 mM) PenStrep (Penicillin/ Streptomycin)	Gibco Hyclone Gibco Gibco
SKMC	RPMI1640	10 % 2mM 1 %	Fetal calf serum (FCS) Glutamin (200 mM) PenStrep (Penicillin/ Streptomycin)	Gibco Hyclone Gibco Gibco
HEK 293	RPMI1640			
Eahy 296	DMEM	10 % 2mM 1 % 1X 1%	Fetal calf serum (FCS) Glutamin (200 mM) PenStrep (Penicillin/ Streptomycin) HAT SodiumPyruvate	
HCMEC	Microvascular endothelial			Provitro

HDMEC	cell growth medium Endothelial Cell Growth Medium MV	Promocell

4.7. Cell lines

Name	Origin	Source
TE671	Rhabdomyosarcoma	Prof. H. Wiendl, Würzburg
C2C12	Mouse myoblast	ECCAC
SKMC	Primary human skeletal muscle cells	PromoCell, Heidelberg
HEK 293	Fibroblast cells	
HCMEC	Human chorionic microvascular endothelial cells	Prof. K.T. Preissner, Giessen
Eahy926	Endothelial cells	Prof. K.T.Preissner, Giessen
HDMEC	Human dermal microvascular endothelial cells	PromoCell, Heidelberg

4.8. Antibodies

Ab-Name	against	raised in	Conjugation	Company
GAPDH	human	mouse	-	Chemicon
HLA-ABC (MHC I)	human	mouse	-	Dako
IgG	human	rabbit	AP	Dako
IgG	human	rabbit	FITC	Dako
IgG	mouse	rabbit	FITC	Dako
Isotype-control		mouse	FITC	R&D Systems
Isotyp-control		mouse	-	Dako

4.9. Primers

Description	Nucleotide sequence	NCBI GenBank
PBGD 8F (fwd)	5´-TGCAACGGCGGAAGAAAAC-3´	NM_00190
PBGD 3.1 R (rev)	5´-GGCTCCGATGGTGAAGCC-3´	
MHC class I(fwd)	5’ - GCTACTACAACCAGAGCGAGG - 3’	NM_002116
MHC class I(rev)	5’ - CCTCGTTCAGGGCGATGTA - 3’	
LMP 2 (fwd)	5’- CGTTGTGATGGGTTCTGA - 3’	NM_148954
LMP 2 (rev)	5’ - GCAATAGCGTCTGTGGTG - 3’	
LMP 7 (fwd)	5’ - TGGGGACGGAGAAAGGA - 3’	NM_148954
LMP 7 (rev)	5’ - GGCTGCCGACACTGAAAT - 3’	
TAP 1 (fwd)	5’ - TGGTCTGTTGACTCCCTTACAC - 3’	NM_00593
TAP 1 (rev)	5’ - AAATACCTGTGGCTCTTGTCC - 3’	
TAP 2 (fwd)	5’ - TACAACACCCGCCATCAG - 3	NM_018833
TAP 2 (rev)	5 ’- AGGTCTCTCCGCCAATACAG - 3’	

4.10. Software

CellQuest® BD (FACSCalibur)
Excel 2002 Microsoft
Graph Pad Prism Software Version 4.02 (Statistical analysis + Grafics)
Microsoft Office PowerPoint 2003
Microsoft Office Word 2003
QC-Net (Unity Real Time) Bio Rad (Nephalometer analysis)
Roche Molecular Biochemicals Light Cycler Software Version 3.5 (LightCycler)
Windows 2002 Microsoft
WinMDI 2.9 (analysis of FACS data)

5. Methods

5.1. Cell culture

Human primary skeletal muscle cells (SKMC) (PromoCell, Heidelberg, Germany) were cultured in skeletal muscle cell growth medium with supplements (PromoCell, Heidelberg, Germany). Human rhabdomyosarcoma cell line (TE671) was purchased from ATCC (Manassas, VA, USA) and cultured in RPMI Glutamax medium supplemented with 10% FCS (fetal calf serum) and 1% penicillin-streptomycin. Mouse C2C12 cells (ECACC) were cultured in DMEM Glutamax medium with 10% FCS and 1% penicillin-streptomycin. C2C12 cells were also differentiated to form myotubes by culturing them in a medium containing 2% horse serum instead of FBS. The medium used for culture of Ea.hy 926 cells (a kind gift from Department of Biochemistry, Giessen) was DMEM containing 10 mM Hepes, 2mM L-glutamine, antibiotics (1% penicillin-streptomycin), HAT (Hypoxanthine 100µM; aminopterin 0.4 µM; and thymidine 16 µM) 1% Na-pyruvate and 10% FCS. Human dermal microvascular endothelial cells (HDMEC) (PromoCell, Heidelberg, Germany) were cultured in endothelial cell growth medium MV with supplements (Promocell, Heidelberg, Germany). HCMEC cells were grown on dishes pre-coated with collagen. The medium was endothelial cell growth medium with supplements (PromoCell, Heidelberg, Germany). All cell lines were maintained at 37°C and 5% CO_2.

5.2. Flow cytometry

5.2.1. Autoantibody detection by flow cytometry

We recently introduced flow cytometry (FACS) to detect autoantibodies in neuroimmunological diseases (Blaes et al. 2000). To avoid unspecific binding to ubiquitous antigens we pre-absorbed the sera by incubating in PBS containing 1% FCS and 0.1% NaN3 (FACS buffer) with HEK 293 cells for 24 hours at 4ºC (serum dilution 1/10) prior to incubation with the other cell cultures. The different cell lines in culture were detached by incubation in 0.025% trypsin-EDTA (Gibco) in phosphate buffered saline (PBS) for 1-3 minutes at room temperature, and were then washed in fresh PBS.

To see the binding of sera to the detached cells, cells were resuspended in FACS buffer. Briefly, cells were washed twice in FACS buffer and then incubated with patient or control sera (1:100) for 30 min at 4°C. Secondary antibody polyclonal rabbit anti-human immunoglobulin FITC –conjugated (DAKO) was used in a 1:100 dilution (30 min at 4°C in dark). Isotype matched non-binding antibodies were used as controls for specific binding. After secondary antibody staining, cells were washed twice in FACS buffer and analyzed in a Becton Dickinson FACScan flow cytometer (Beckton-Dickinson, Heidelberg, Germany). Data were analyzed using CELLQUEST® software. Mean fluorescence intensitiy (mfi) was measured for each sample. A percentage above mean mfi + 2.5 standard deviations of the controls was considered positive.

5.2.2. MHC Class I detection

MHC class I antigen expression was determined using flow cytometry (FACS) analysis. Briefly, cells were washed twice in PBS supplemented with 1% fetal calf serum and 0.1% sodium azide (FACS buffer) throughout the experiment and then incubated with mouse anti-human MHC class I antibody (1:100) for 30 min at 4°C. Secondary antibody (rabbit anti-mouse immunoglobulin FITC -conjugated) was used in a 1:100 dilution (30 min at 4°C in dark). Isotype matched non-binding antibodies were used as controls for specific binding. After secondary antibody staining, cells were washed twice in FACS buffer and analyzed in a Becton Dickinson FACScan flow cytometer. Data were analyzed using CELLQUEST® software.

5.3. RNA isolation, RT-PCR, and Real time PCR

Total cellular RNA was isolated and MHC class I, LMP2, LMP7, TAP1 and TAP2 expression were analyzed by real time PCR. Primer sequences used for PCR were designed using Primer Premier 5® software. Primer sequences are mentioned in Table 9. PBGD mRNA was used as a housekeeping gene, because its level is neither affected by IFN-γ nor by statin treatment. A mix of the following reaction components was prepared for each sample: 8 µl water, 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 10 µl QuantiFast SYBR Green PCR kit® (Qiagen, Germany) and 1 µl of reverse transcribed mRNA sample as PCR template. Capillaries were closed,

centrifuged and placed into the rotor. The following general real-time PCR protocol was used: denaturation program (10 min at 95°C), a three-segment amplification repeated 40 times (20 s at 95°C; 20 s at different annealing temperature for each primer pair; 30 s at 72°C), melting curve program (60–99°C), and finally a cooling down to 40°C.

5.4. Cytotoxicity assay

Cytotoxicity was assessed with an lactate dehydrogenase (LDH)-cytotoxicity detection kit (Roche Diagnostics), which measures LDH activity released from the cytosol of damaged cells. Cells were grown in 96-well culture plates, at 37 °C, for 24 h to near confluence. At this time they were incubated for more than 24 h in the absence or the presence of IgGs from myositis patients or healthy controls. Cells grown in 96-well plates were exposed to various concentrations of IgGs (50 to 800 µM) for 24 h. After 24 h, 100 µl of supernatant per well was harvested and transferred into a new 96-well, flat-bottom plate. LDH substrate (100µl) was added to each well and incubated for 30 min at room temperature (RT) protected from light. The absorbance of the samples was measure at 490 nm with an ELISA reader. Cytotoxicity was calculated with the formula: % cytotoxicity = (experimental value - low control) x 100/(high control - low control), where low control is assay medium plus cells and high control is assay medium (plus 2% Triton X-100) plus cells.

5.5. Calcium imaging

Relative changes in the intracellular Ca^{2+} concentration were measured using the Ca^{2+} -sensitive fluorescent dye fura-2 as described previously (Haschke, Schafer et al. 2002). The wavelength, at which fura-2 is maximally excited, shifts in dependence of the cytoplasmatic Ca^{2+} concentration. The HUVECs grown on coverslips were loaded for 60 min with 2.5 µmol/L fura-2 acetoxymethylester (fura-2/AM). Fura-2 was then washed away. In these experiments, the cells were first superfused with tyroid solution (1 mL/min), baseline was recorded, and then control or myositis IgG were added at the concentration of 30 mg/L, and then the effect was recorded for approximately 30 min. Experiments were carried out at room temperature on an inverted microscope (Olympus IX-50) equipped with an epifluorescence and an image analysis system (Till Photonics,

Martinsried, Germany). The emission above 470 nm was measured from several regions of interest, each with the size of about one cell. The cells were excited alternatively at 340 and 380 nm and the ratio of the emission signal at both excitation wavelengths was calculated. Data were sampled at 0.33 Hz. The baseline in the fluorescence ratio of fura-2 was measured during at least 5 min, Superfusing with a standard or a Ca^{2+} free Tyrode solution (depending on the experiment), before any IgG was administered. ATP (ATP, 50 µmol/l) was used for vitality test of cells.

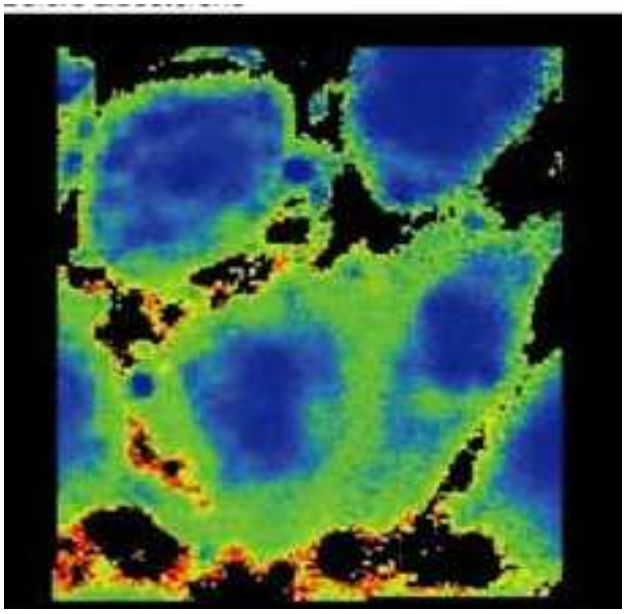

Figure 6. Fura-2 loaded endothelial cells.

5.5. Protein extraction

When cells were confluent, monolayers were washed twice with 1 % PBS and then incubated with 0.025% trypsin-EDTA (Gibco) for 1-3 minutes at room temperature to detach them. Cells were centrifuged and then resuspended in 1 % PBS. Cells were lysed using Dounce homogeniser and ultra sonication (3 times, 15 seconds for each). The cell extract was centrifuged at 14,000 rpm and the supernatant was collected. Protein quantification was performed on the supernatant using Bradford method.

5.5. One-dimensional gel electrophoresis (1-DE)

Proteins were separated by 1-DE on 4% to 12% precast Bis–Tris NuPAGE gels, using MOPS running buffer (Invitrogen, Carlsbad, CA, USA). After separation, proteins were transferred onto nitrocellulose membranes (Hybond™-c extra; GE Healthcare Life Sciences, Piscataway, NY, USA) and stained with Ponceau red (Sigma-Aldrich). Membranes were cut and strips were first saturated with PBS–5% dry milk, and then incubated with patient sera (1:100 dilution) for 1 hour followed by incubation with biotinylated conjugated mouse monoclonal anti-human IgG (Fc) (Southern Biotechnology Associates Inc., Birmingham, AL, USA) and alkaline phosphatase-conjugated streptavidin (CALTAG; Invitrogen), and finally revealed with NBT/BCIP (Roche Applied Science, Indianapolis, IN, USA). Each step was followed by three washes with PBS/Tween 0.05% buffer.

5.6. Two-dimensional gel electrophoresis (2-DE)

Adherent cells were washed with PBS and than disrupted by grinding under liquid nitrogen in a mortar. Proteins were solubilised in sample solution (8 M urea, 2 M thiourea, 2% pharmalyte buffer [v/v, pH 3-10]; 4% CHAPS; 30 mM DTT, 20 mM Tris). Samples were incubated for 2 hours at 4°C, vortexed, centrifuged (18.000 × g, 30 min) and the supernatant was subjected to isoelectric focusing (IEF) after protein determination (BCA Protein Assay, Pierce, Rockford, IL).

2-DE was performed as described (O'Farrell 1975; Gorg, Obermaier et al. 2000). Proteins were rehydrated over night of immobilized pH gradient (IPG) strips, 11 cm, pH 3-10 linear, (Amersham Biosciences, Freiburg, Germany). IEF of 250 - 460 µg protein per strip was carried out in a Multiphor chamber (Amersham Biosciences) at 20°C. Electrophoresis was carried out in SDS gels (10% or 15% acrylamide) at 25°C (600 V for 3.5 hours) in a Hoefer SE600 (Amersham Biosciences) chamber. The gels were either stained with Coomassie or blotted. Stained gels were scanned and molecular weight of the proteins was calculated using the protein ladder (Invitrogen, Karlsruhe, Germany) and pI (isoelectric point) of the proteins were determined according to the pH value of the strips. Some gels were blotted onto polyvinylidene difluoride membrane

(PVDF; Hybond-P, Amersham Biosciences) by tank blotting (Hoefer Transphor, Amersham Biosciences) using Towbin buffer with 20% ethanol for 570 Vh at 5°C. Anti-human HRP (Dako Cytomation, Hamburg, Germany) and ECL Western Blotting System (Amersham Biosciences) or autoradiography was used to visualize reactivities.

5.7. Protein identification by peptide mass fingerprinting

Myositis and HC sera were analyzed by western blot using 2-DE membranes. Positive spots were excised from Coomassie stained gels and were washed once with water and twice with 50 mM ammonium hydrogen carbonate: acetonitrile (1:1) and acetonitrile, alternately. Gel pieces were incubated in a minimal volume of a 10 ng/μL trypsin solution (sequencing grade, Roche Diagnostics, Mannheim, Germany) in 25 mM ammonium hydrogen carbonate (16 h, 37°C). Peptides were extracted with 10 μL of 1% (v/v) trifluoroacetic acid. 1 μL of the solution was mixed with 1 μL 2.5-dihidroxybenzoic acid (20 mg/ml) in 1% phosphoric acid, 50% acetonitrile on a stainless steel target and air dried (dried droplet). Mass fingerprints of tryptic digests were obtained by MALDI-TOF mass spectrometry using an UltraflexTM TOF/TOF mass spectrometer (Bruker Daltonik). Proteins were identified by database searching with peptide masses using the MASCOT search engine. Protein identification was completed by significant Z scores or by significant $p < 0.05$ probability-based Mowse Scores, respectively.

5.8. Statistical Analysis

Frequency of autoantibodies and comparison with clinical data was analysed by Fisher's exact test. Surface binding were analysed by two-tailed Student's t-test between Myositis and HC groups after validating the normal distribution of these datasets (Kolmogorov-Smirnov test). The comparison of cytotoxicity cytotoxicity level between different groups was done using one way ANOVA. All statistical analyses were performed using Prism® 4.02 Software (Graph Pad Inc.). The probability level accepted for significance was $p < 0.05$.

Differences between groups in flow cytometry experiments were analyzed by the Kruskal Wallis test followed by the Mann Whitney U test for a posteriori comparison of

means. Results are expressed as mean of triplicates ± SD. All reported *p* values are based on two-tailed statistic test, with a level of significance established at 5%. Analyses were performed using GraphPad PRISM 4.0 (San Diego, CA).

The relative expression ratio of target gene transcripts in comparison to a reference gene transcript was calculated based on the PCR efficiency and crossing point deviation of each investigated transcript, as described in an in-depth report of a mathematical model (Pfaffl 2001).

6. Results

6.1. Presence of surface binding autoantobodies

Flow cytometry was used to show the surface binding of autoantibodies to different cell lines (muscle endothelial and fibroblast cell lines). These cells were incubated with 1:100 diluted sera and IgG binding to the cells was measured by flow cytometry after the application of a FITC-labelled anti-human IgG secondary antibody. Eight of 26 patients (26.9%) tested, but none of the controls showed binding to TE671 cells (t test, $p<0.0001$) (Fig. 7). None of the patients or controls showed substantial binding to SKMC, or to undifferentiated C2C12 or myotubes-differentiated (Fig. 7). Additionally, there was no significant binding to the control cell line HEK 293 (Fig. 10). When the resulty were analyzed based on the different myositis groups (DM, PM, OIM), all three subgroups had elevated binding to TE671 cells compared to controls. However, there was no difference between the myositis subgroups in surface binding to the different muscle cell lines (Fig. 8). DM patients showed a tendency to a higher surface-binding against C2C12 mouse muscle cells. Interestingly, the patients' sera did not bind to the rat cardiomyocyte cell line HL-1 (Fig. 9).

The patients have also been tested for the binding to three different endothelial cell lines. No significant binding could be found to the HCMEC or Ea.Hy926 cells. However, 5/26 (19.2%) patients showed binding above the cut-off to HDMEC cells at a level higher than the mean ± 2.5 standard deviations of the healthy control sera (Fig. 10). Though DM patients were expected to show more binding compared to PM patients but in our experiments I could not see this difference.

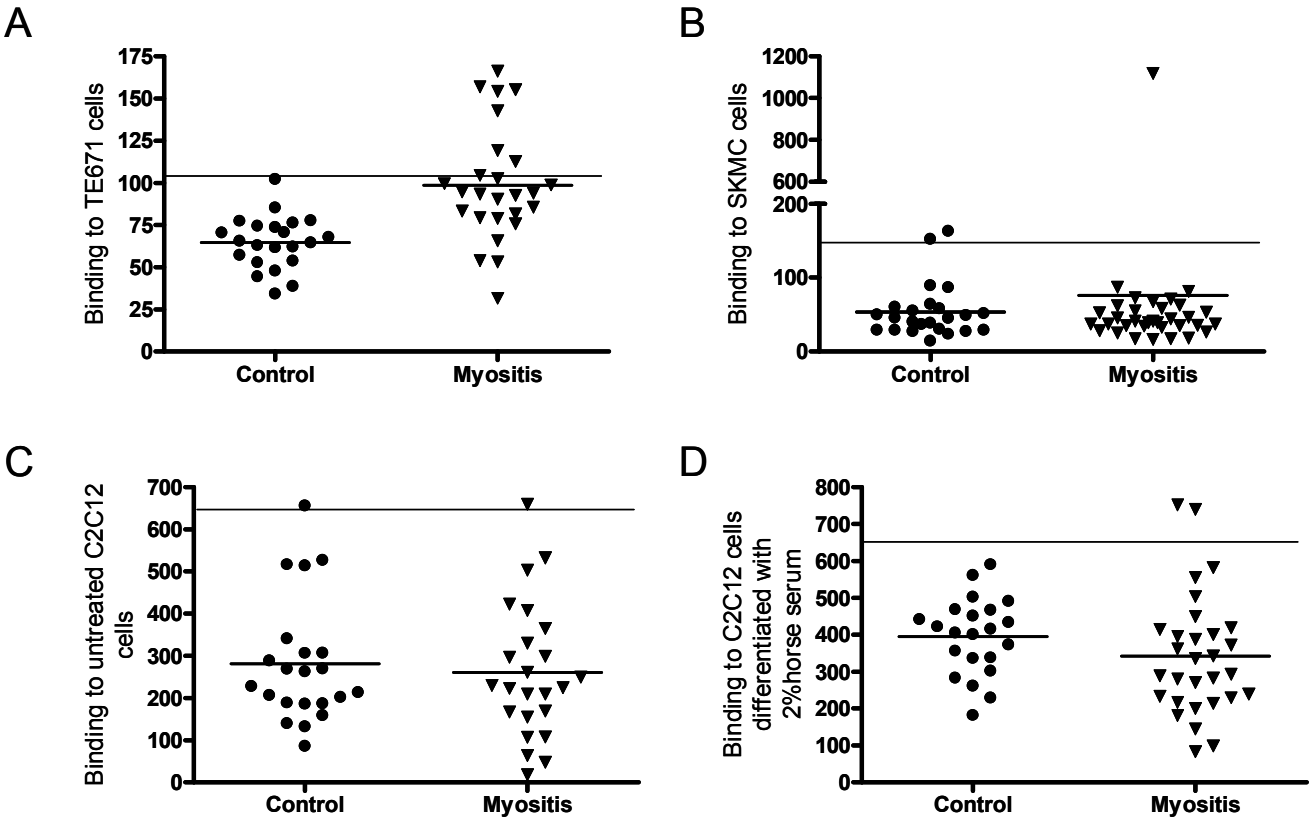

Figure 7. Surface binding of myositis sera to different muscle cell lines (expressed as mean fluorescence intensity) compared to healthy control sera. Cut off, marked as horizontal line, was determined as mean of the controls + 2.5 x std. dev. Myositis patients have more binding to TE671 cell line compared to healthy controls.

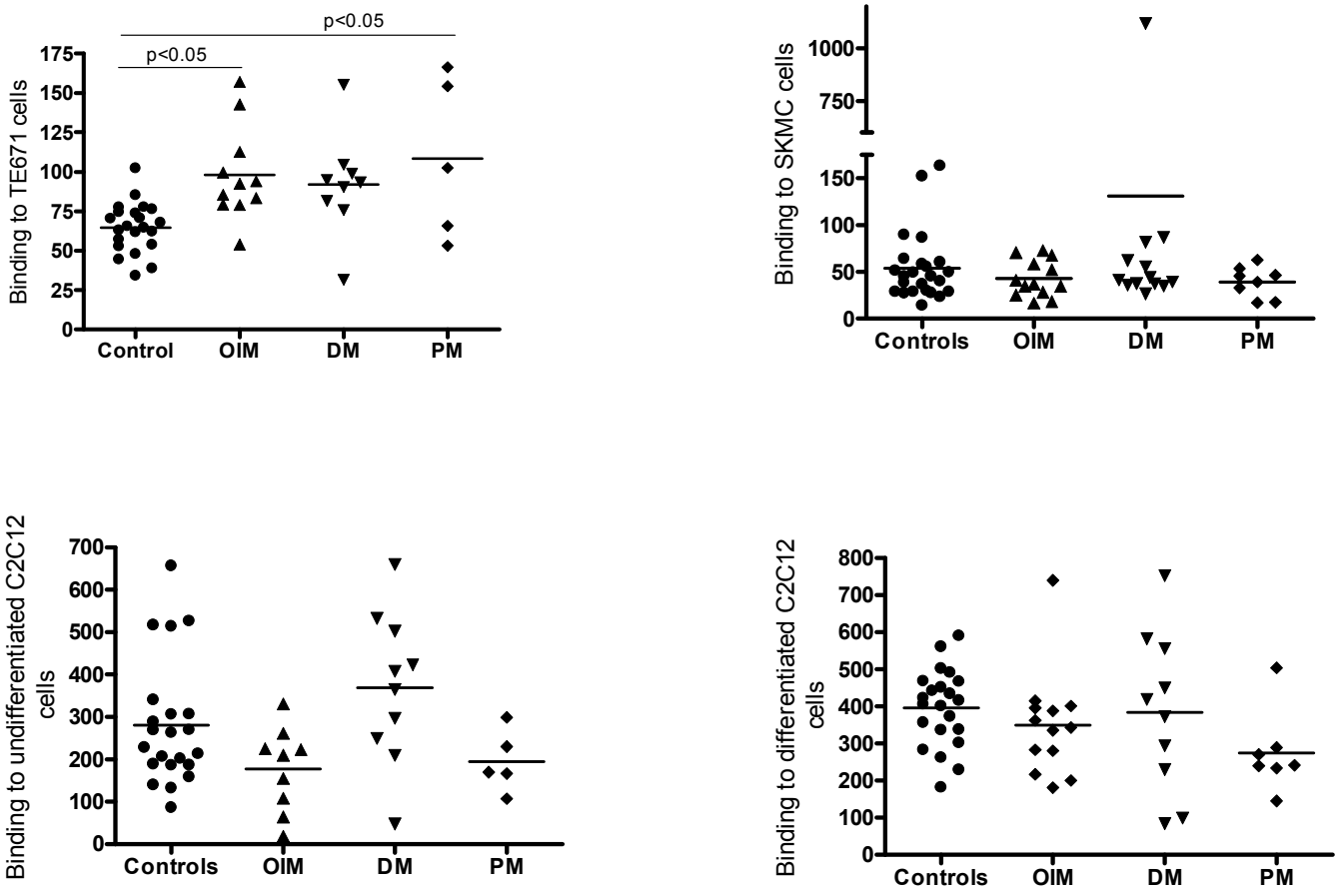

Figure 8. Surface binding of OIM, PM and DM sera to different muscle cell lines (expressed as mean fluorescence intensity) compared to healthy control sera. Cut off, marked as horizontal line, was determined as mean of the controls + 2.5 x std. dev. Myositis patients have more binding to TE671 cell line compared to healthy controls.

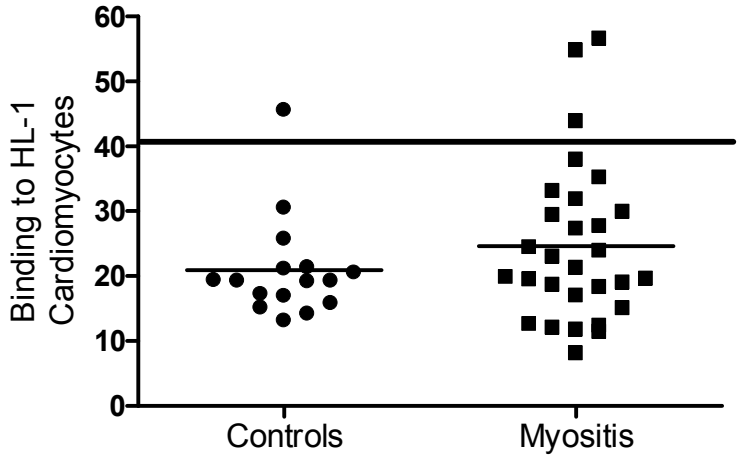

Figure 9. PM and DM patient sera have been analyzed using flow cytometry for the presence of surface binding autoantibodies to mouse primary cardimyocytes (HL-1 cells). There is no significant difference between the binding of control and patient sera.

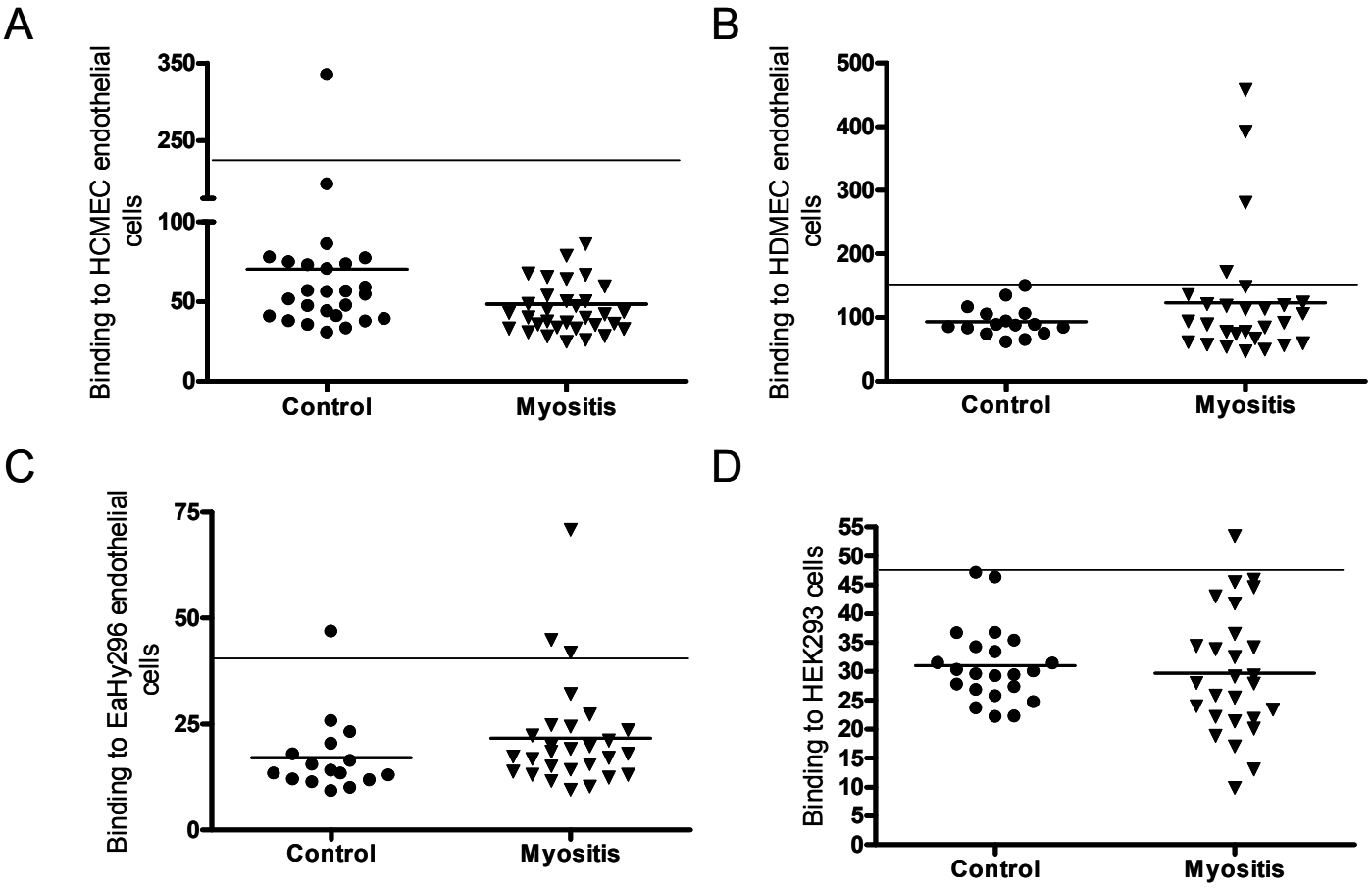

Figure 10. Surface binding of myositis sera to different endothelial cell lines (expressed as mean fluorescence intensity) compared to healthy control sera. Cut off, marked as horizontal line, was determined as mean of the controls + 2.5 x std. dev. Myositis patients have more binding to HDMEC cell line compared to healthy controls.

In a recent report statins have been implicated to trigger myositis. Therefore, muscle cell lines (TE671 and SKMC) were treated with different statins (Lovastatin, Mevastatin and Simvastatin) in combination with IFN-γ to increase the immunogenecity of the muscle cells. These cells were then used to analyze the binding of myositis sera using flow cytometry. In TE671 cells, only the treatment with mevastatin led to an increased binding of myositis sera, whereas the binding to simvastatin was even lower than to unstimulated TE671 ($p<0.001$ and $p<0.05$). Using the primary muscle cell line SKMC, Mevastatin- and Simvastatin-treated cells show significant higher binding of myositis sera (Fig. 11). The different patient groups (OIM, DM, and PM) were then analyzed. DM patients showed higher binding to simvastatin-stimulated SKMC compared to OIM and PM patients ($p<0.05$ and $p<0.01$, Fig. 12) There was no difference between different patients in binding to Mevastatin treated SKMC cells (Fig. 12).

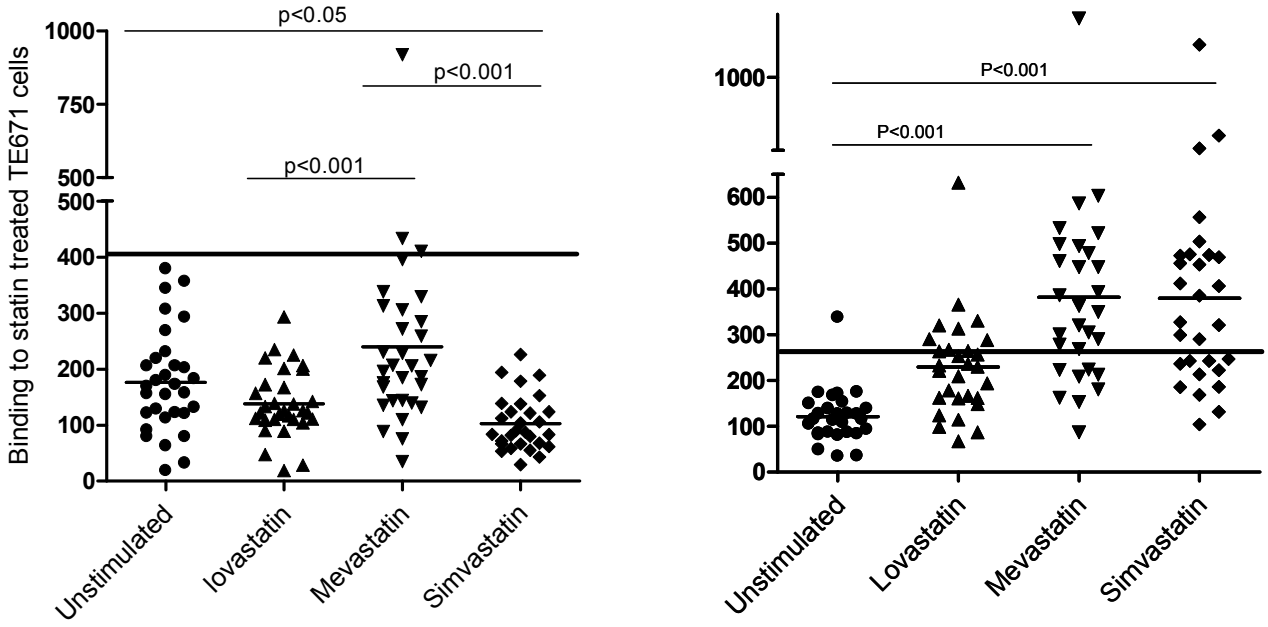

Figure 11. Surface binding of PM and DM patient sera to statin (Lovastatin, Mevastatin and Simvastatin) treated TE671 and SKMC cells. Cells were first treated with different statins for 48 Hrs and then analyzed by flow cytometry for binding of autoantibodies to these cells.

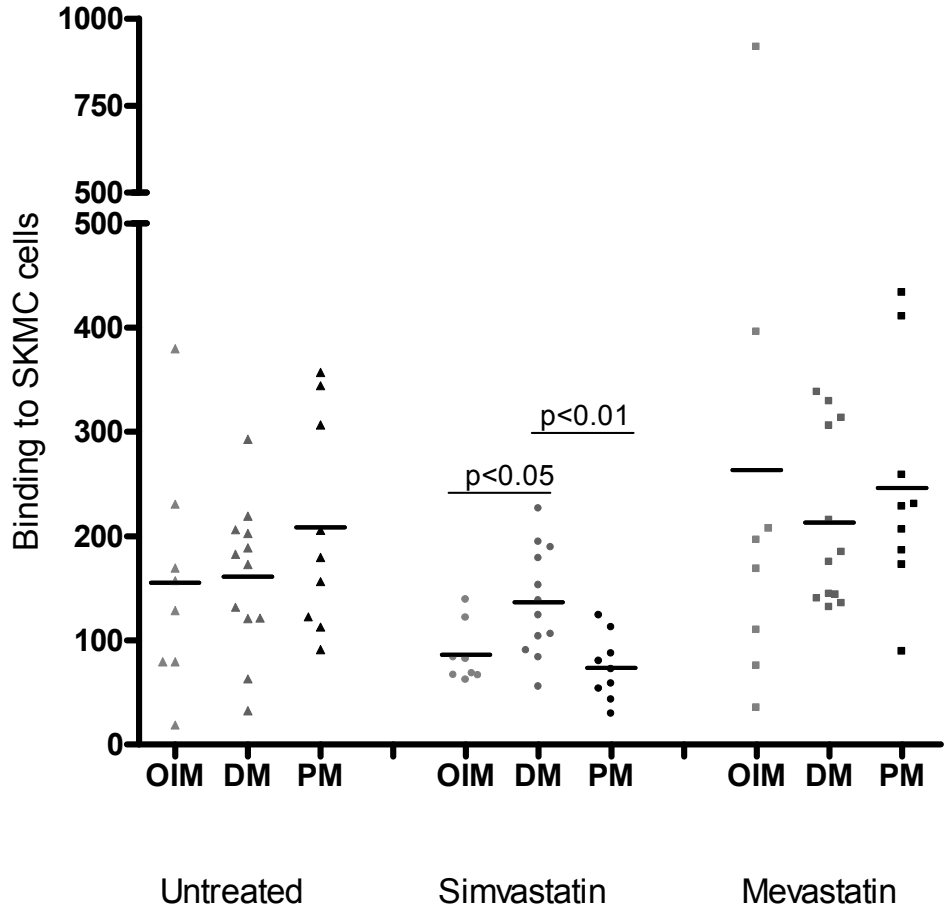

Figure 12. Surface binding of PM and DM patient sera to statin (Lovastatin, Mevastatin and Simvastatin) treated TE671 and SKMC cells. Cells were first treated with different statins for 48 Hrs and then analyzed by flow cytometry for binding of autoantibodies to these cells.

6.2. Western blotting of autoantibodies with muscle cell extract

Next the myositis sera were checked for their binding to muscle cell protein using western blotting. As is shown in Fig. 13, patient sera recognized a protein in the size range of 97-120 KDa which was absent in the fibroblast cell lysate. It again shows that PM and DM patients do have muscle antigen specific autoantibodies.

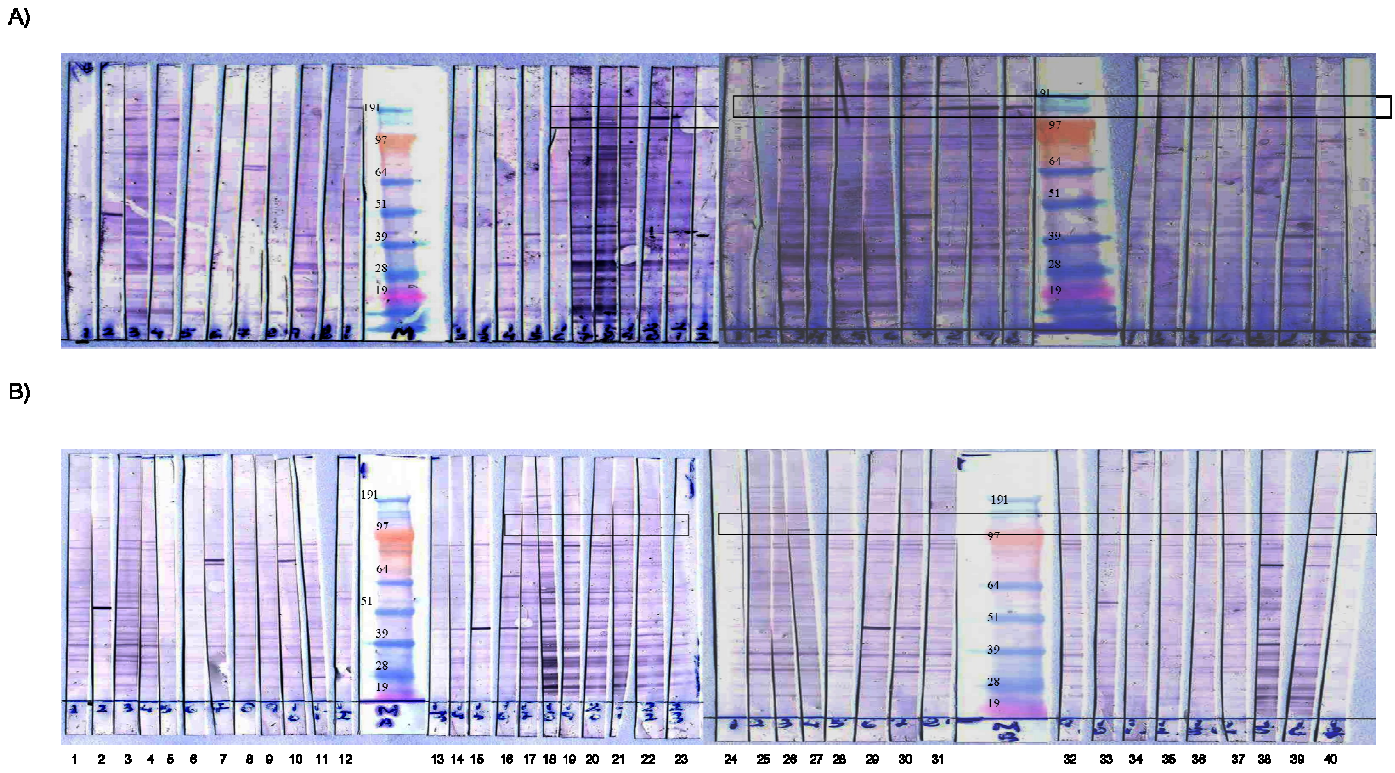

Figure 13. Immunoblot incubated with the patient's serum on TE671 cells (A) and on HEK 293 (B) as non-muscle control. Cell lysate was prepared from muscle and fibroblast cells and run onto a 4-12% gradient SDS-PAGE. After blotting of proteins to nitrocellulose membrane, the membranes were incubated with patients and healthy control sera and then the binding was visualized by using enzyme conjugated secondary antibody and NBT substrate. Line 1 to 15 represents health controls and line 16 to 40 patients. Patient sera show a specific reactivity, to proteins from muscle cell lysate, in range of 97-112 KDa ((A) inside the rectangle) which is absent in blot of fibroblast cell lysate (B).

6.3. Identification of autoantigens in muscle cells

Our experiments have shown the presence of surface binding autoantibodies in myositis patients. To see if they bind to intracellular antigens, western blotting was performed using the cell extract from muscle cells. It has been found that 10 out of 25 (40.0%) myositis patients show a specific band, against muscle cell lysate, between 95-110 KDa (Fig. 13 see the boxed area), compared to none aginst fibroblast cell lysate (5 DM, 1 PM, and 4 OIM).

In order to identify muscle proteins targeted by serum IgG from myositis patients, muscle proteins from TE671 cells were submitted to 2-DE and transferred to PVDF membranes (Fig. 14). Serum IgGs from 4 different myositis patients and a healthy control individual were incubated with the PVDF membranes transferred with muscle proteins.

Among many spots transferred to the PVDF membranes, 60 or so spots were recognized by serum IgG from patients and controls. Only the spots which showed immunoreactivity with patients' serum IgGs were submitted to MALDI-TOF mass spectrometric analysis. These spots were identified as glycyl-tRNA synthetase, aldose reductase, Proteasome subunit beta type 7 precursor and others (Table 3).

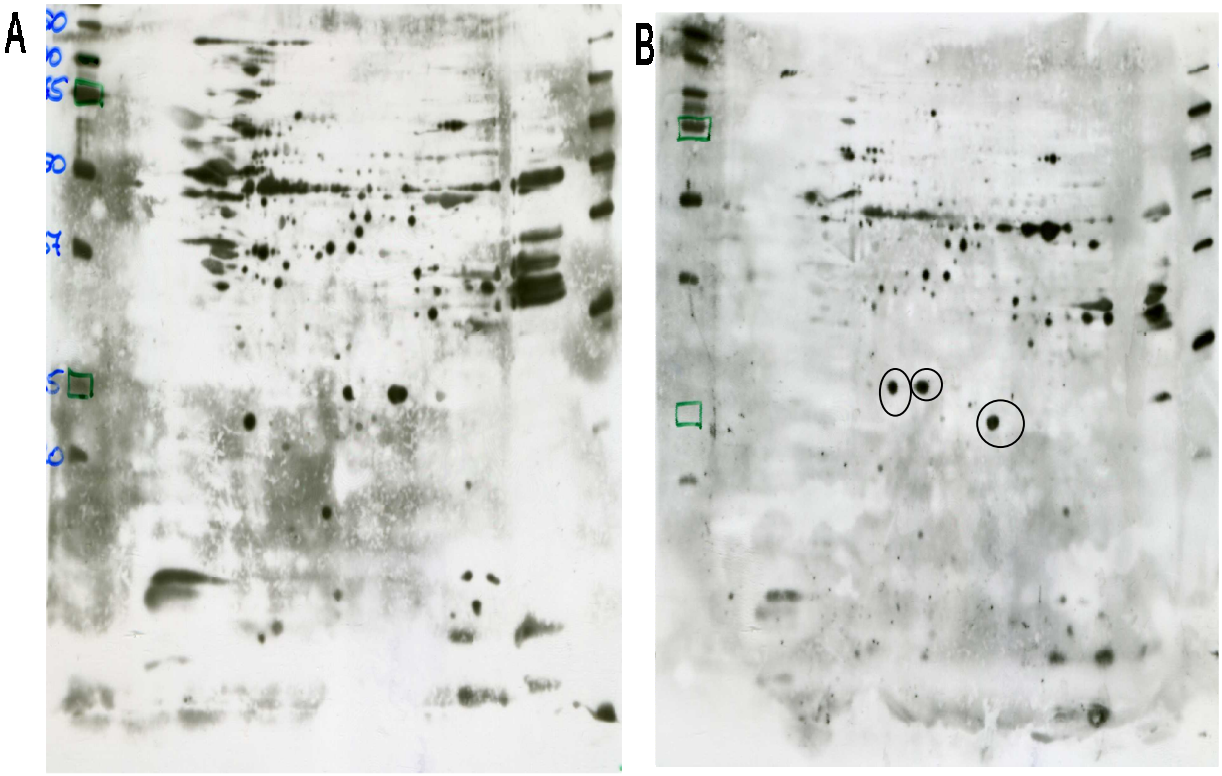

Figure 14. 2D-PAGE blots of cell lysate from TE671 cells with healthy control (A) and patient (B). Cell lysate was prepared and first applied to isoelectric focussing and then the isoelectric strips were placed onto SDS-PAGE for second dimensional electrophoresis. After the electrophoresis the protein were blotted to PVDF membranes and these membranes were then probed with either control (A) or myositis (B) sera.

Spot-Nr.	Protein	Accession number Human	M (kDa) exp.	M (kDa) theor.	pI exp.	pI theor.	Mowse score (>55)	Seq. cov. (%)	Number of ident. peptides
1	glycyl-tRNA synthetase	SYG	75	83.8	5.7	6.61	206	38	28
2	Annexin A2 + Aldose reductase	ANXA2 ALDR	35	38.8	7.0	7.57	350 162 160	54 72	20 22
3	Proteasome subunit beta type 7 precursor + Platelet-activating factor acetylhydrolase IB subunit beta	PSB7 PA1B2	28	30.3 25.7	5.5	7.57 5.57	259 143 109	59 47	18 12
4	Proteasome subunit beta type 7 precursor	PSB7	28	30.3	5.6	7.57	155	57	16
Blank	___		--		--				
BSA	BSA		--		--		144		

Table 3. Summary of proteins identified from the spots picked up from 2D gel after immunoblotting.

6.4. Cytotoxic effect of autoantibodies

To measure the cytotoxic effect of different patients' IgG on various cell lines, cells were incubated with purified IgGs in 96 well plates for 24 hours and later the cytotoxicity was measured using LDH cytotoxicity assay kit. Before incubating the cells with IgGs, first cells were plated at different densities for 24 hours in 1% FBS medium and then incubated with IgGs to find out the right cell density for further experiments. It has been deduced that 10,000 cells per well is the optimum cell density which has a linear function in the cytotoxicity assay. Ten thousands cells per well were then incubated with various concentrations of purified IgGs (50 to 800 µM), and it was found that for some cells the effective IgG concentration was 10 ug/ml and for some 50 ug/ml. The above density of cells and appropriate concentration of IgGs was then used for further

experiments. The results obtained after 24 hours incubation of IgGs with different cell lines are summarised in (Fig. 15). The cytotoxic effects of myositis IgG was visible only against the endothelial cell lines Ea.Hy and HDMEC (student's t test) $p<0.05$. Though surface binding antibodies have been found in myositis sera against TE671, which is an established model cell line for muscles, I could not see any cytotoxicity in this cell line. None of the healthy controls showed any cytotoxicity (Fig. 15).

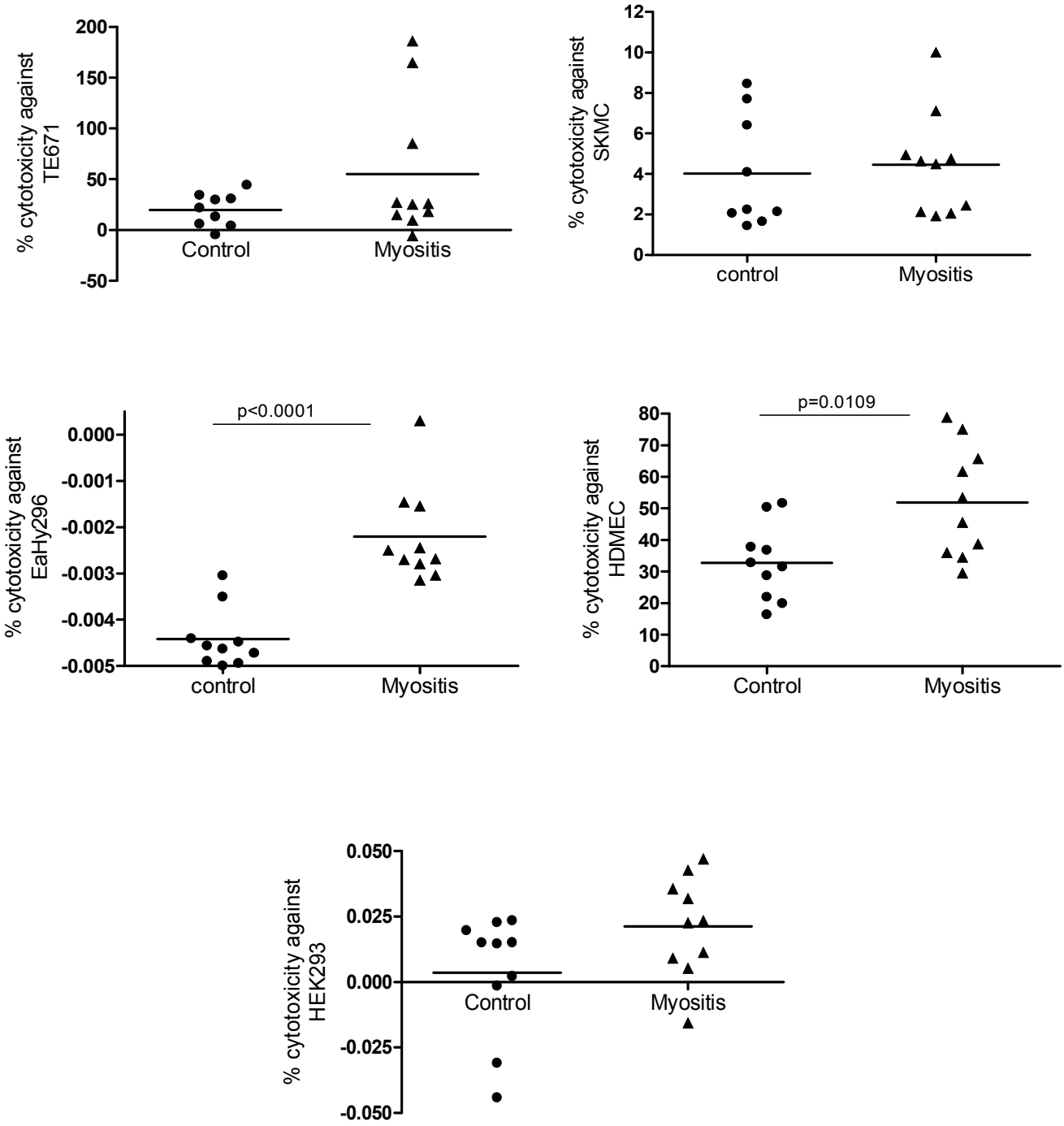

Figure 15. Purified IgGs from PM and DM patients have been tested with endothelial and muscle cell lines for their cytotoxic effects using LDH assay. Cells were incubated with IgGs for 24 hours and then supernatant was used to detect cytotoxicity using LDH cytotoxicity assay.

6.5. Effect of autoantibodies on endothelial calcium flux

As has already been shown that IgG from some of the patients show cytotoxicity against endothelial cells, it becomes necessary to check if this cytotoxic effect is due to change in Ca^{2+} influx. To investigate this effect HUVEC cells, which are an established model for Ca^{2+} measurement, were used. The cells were cultured on coverglass and then mounted on the microscope in Ca^{2+} free medium. The IgG were then added to the cells after monitoring the cells for 5 minutes without adding anything. Two myositis patients IgG (1 DM and 1 PM) and a control IgG were used for the experiment. It has been found that HUVEC cells show a significant change in calcium influx after addition of IgGs from myositis patients while healthy control IgG failed to induce any change in calcium influx (Fig. 16).

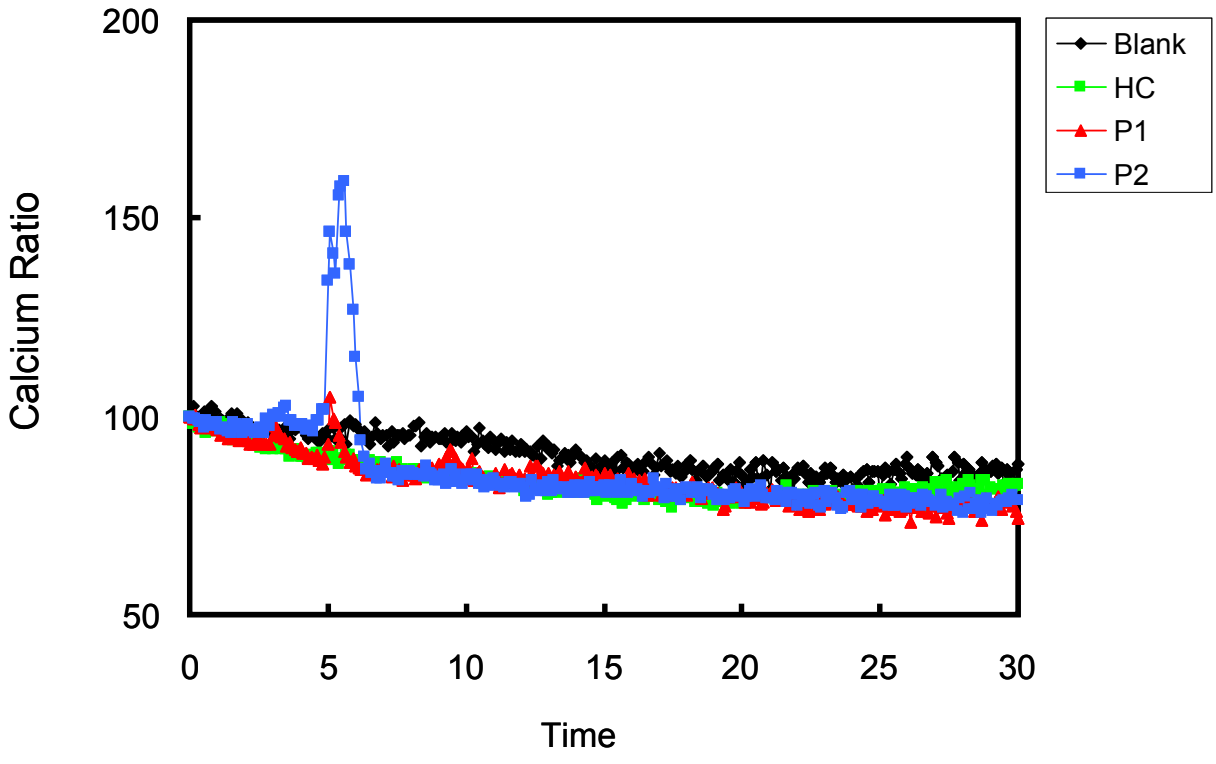

Figure 16. Purified IgGs (3g/L) were added to the primary HUVEC cells and then Ca^{2+} influx was measured using image analysis system (Till Photonics, Martinsried, Germany).

6.6. Effects of statins on muscles cells

In surface binding experiments using flow cytometry, it could be shown that statins treatment of SKMC cells do indeed affect the surface binding of myositis sera to these cells ($p<0.001$) (Fig. 11). The very obvious difference was seen in mevastatin ($p<0.01$) and simvastain ($p<0.01$) treated cells, but lovastatin treatment did not show any increase in surface binding. However, in case of TE671 cells no difference could be seen, infact simvastatin treated TE671 show less surface binding to myositis sera even compared to controls (Fig. 11)

Statins, have been prescribed extensively for their cholesterol-lowering properties and efficacy in cardiovascular diseases (Greenwood et al. 2006). Statins also have additional immunomodulatory properties that operate independently of lipid lowering. In some patients, statins can induce necrotizing or inflammatory myopathies (Needham et al. 2007). Increased MHC class I expression has been shown in muscle biopsies of statin-induced myopathy. Therefore, the effect of statins on the expression of MHC class I in muscle cells was investigated in order to evaluate the hypothesis of an altered immunogenecity of muscle cells induced by statins.

6.6.1. Statins enhance IFN-γ-induced MHC class I expression in TE671 but not in SKMC

The effect of statins on the MHC class I expression was analyzed in two different muscle cell lines – the established cell line TE671 and the primary cell line SKMC. Additionally, all three statins (Lovastatin, Mevastatin and Simvastatin) were also tested for their ability to affect IFN-γ-induced MHC class I expression in TE671 and SKMC cell lines. First, cells were treated with different concentrations of statins to see if they show any dose dependent effect on MHC class I expression, but they failed to show any dose dependency (Fig. 18). Therefore 10 µM concentrations of statins was used in further experiments, as it has been cited in other reports as well. Cells were treated with statins (10µM) and/ or with IFN-γ (100ng/ml) for 48 hours, and MHC class I expression was examined using flow cytometry.

SKMC and TE671 in the untreated condition expressed MHC class I (Fig. 19 and 20).

IFN-γ showed an induction of MHC class I expression in both cell lines (MFI 781 vs. 312 in controls in SKMC; MFI 85 vs. 28 in TE671). Surprisingly, statins alone induced a significant reduction of the baseline expression of MHC class I in primary muscle cells (SKMC); however, they had no effect in TE671 (Fig. 19 and 20).

Statins alter the IFN-γ-induced expression of MHC class I differently in SKMC and in TE671. Statins significantly reduced the IFN-γ-induced MHC class I expression in primary muscle cells (SKMC) (a decrease in MFI from 781 to 312) while they promoted a robust opposite effect in TE671 (an increase in MFI from 85 to 200) (Fig. 19 and 20).

Further, this effect has been checked at the RNA level, and it was found to complement the protein level. I could detect an increase in MHC class I gene expression after IFN-γ treatment in SKMC (17.8 fold) and TE671 (8.9 fold) (Fig. 21). The expression of MHC class I was not substantially affected by any statin treatment in any of the cell lines. This highlights the ineffectiveness of statins to enhance the MHC class I expression in vitro.

A. **B.**

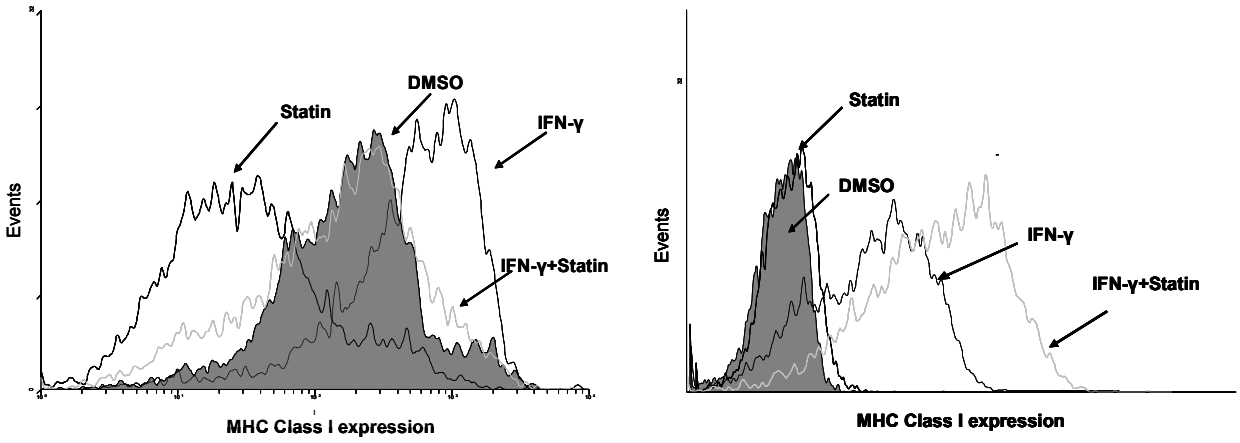

Figure 17. Effect of statins on MHC class I expression accessed by flow cytometry. (A) and (B) are representative flow cytometry analysis of MHC class I expression following different treatments of SKMC and TE671, respectively.

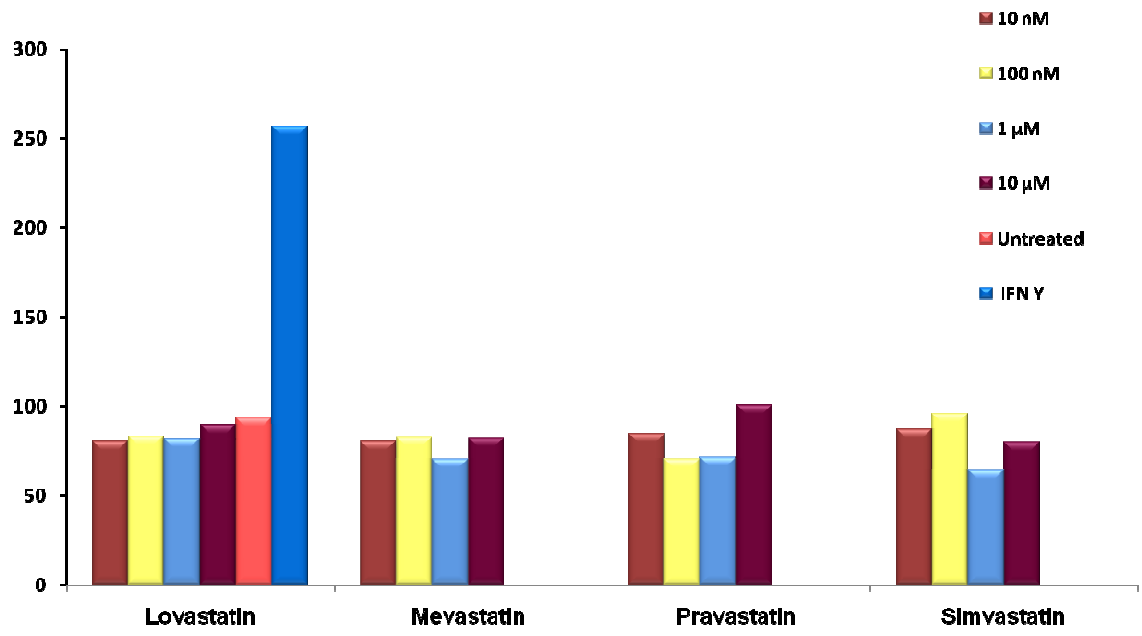

Figure 18. Effect of different concentrations of statins on MHC class I surface expression. SKMC and TE671 cells were treated with different concentrations of statins ranging from 10 nM to 10 µM.

On the other hand, a 53-fold increase in MHC class I gene expression on average was detected when SKMC cells were treated with statins along with IFN-γ (Fig. 21). This effect could nevertheless not be seen at the protein level, which might indicate possible involvement of a translational regulation of MHC class I in primary cells. A positive effect was also exhibited in the case of simvastatin and slightly in the case of lovastatin on IFN-γ-induced expression of MHC class I in the TE671 cell line, in accordance with the FACS data (Fig. 21).

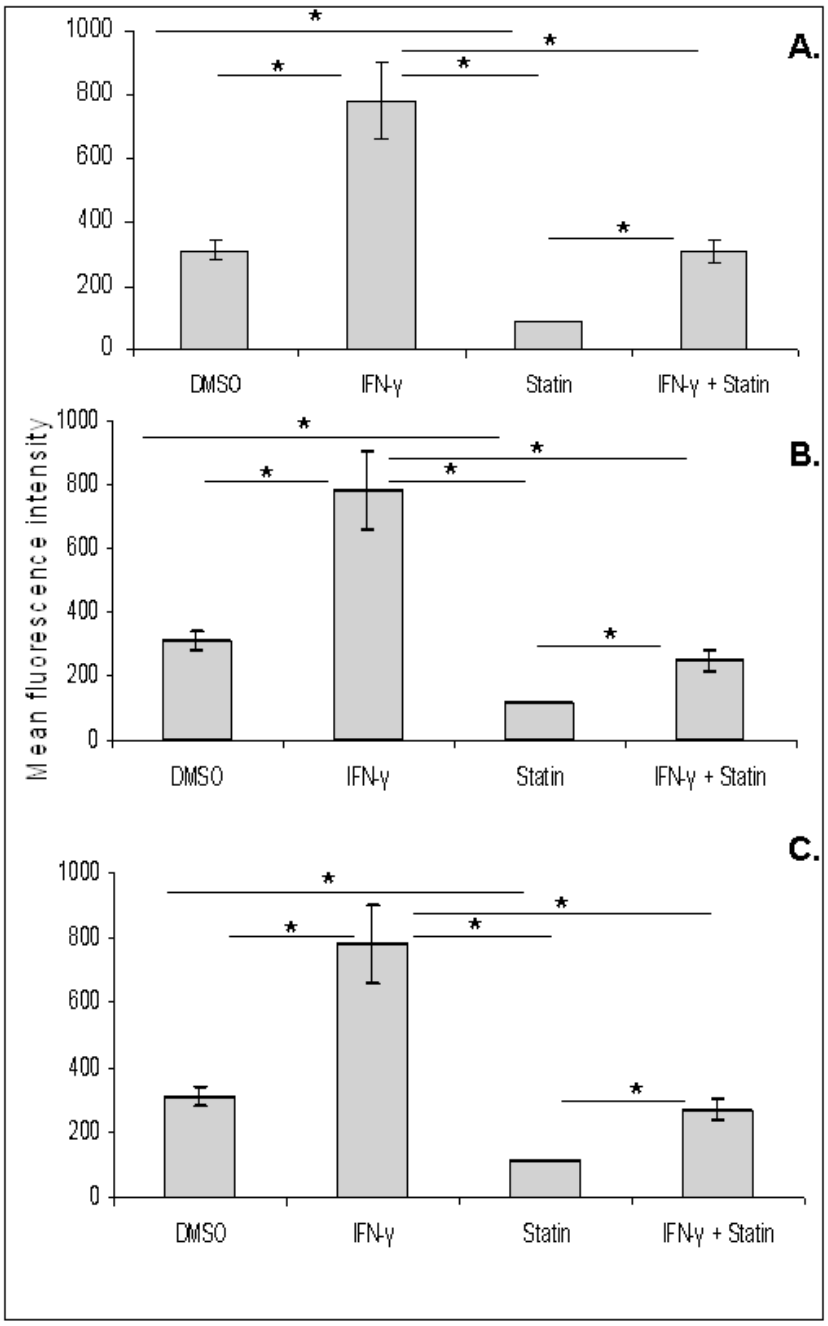

Figure 19. Effect of statins on MHC class I expression assessed by flow cytometry. (A, B, C) SKMC cells treated with IFN-γ (100ng/ml), statins (10μM) or statins along with IFN-γ. Treatment with statins reduced significantly MHC class I expression and IFN-γ inducible MHC class I expression in SKMC. (A) Treatment with Lovastatin; (B) treatment with Mevastatin; and (C) treatment with Simvastatin. Data are represented as mean of triplicates ± SD. *Values significantly different from corresponding treatment (Kruskal Wallis test followed by the Mann Whitney test, $p < 0.05$).

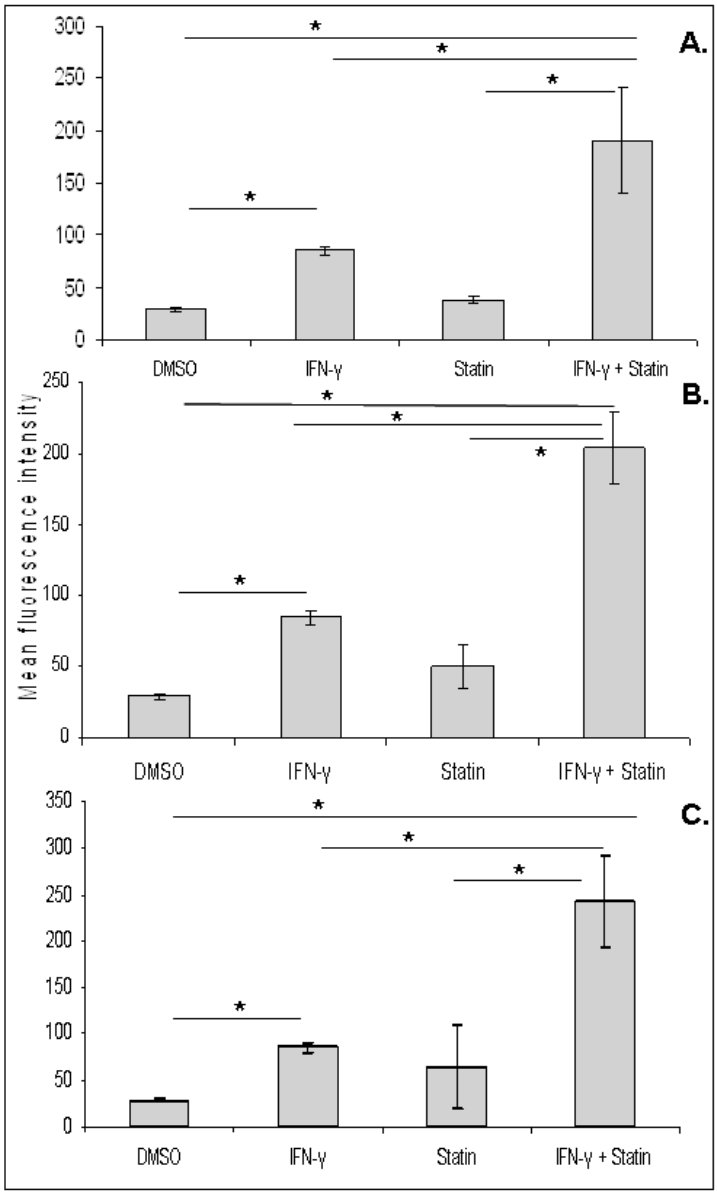

Figure 20. Effect of statins on MHC class I expression accessed by flow cytometry. (A, B, C) TE671 cell line treated with IFN-γ (100ng/ml), statins (10μM) or statins along with IFN-γ. Statins showed differential effects in two different cell lines. Treatment with statins reduced significantly MHC class I expression and IFN-γ inducible MHC class I expression in SKMC. Same treatment here showed no considerable effect on MHC class I expression although conversely enhanced IFN-γ-induced MHC class I expression in TE671. (A) Treatment with Lovastatin; (B) treatment with Mevastatin; and (C) treatment with Simvastatin. Data are represented as mean of triplicates ± SD. *Values significantly different from corresponding treatment (Kruskal Wallis test followed by the Mann Whitney test, $p < 0.05$).

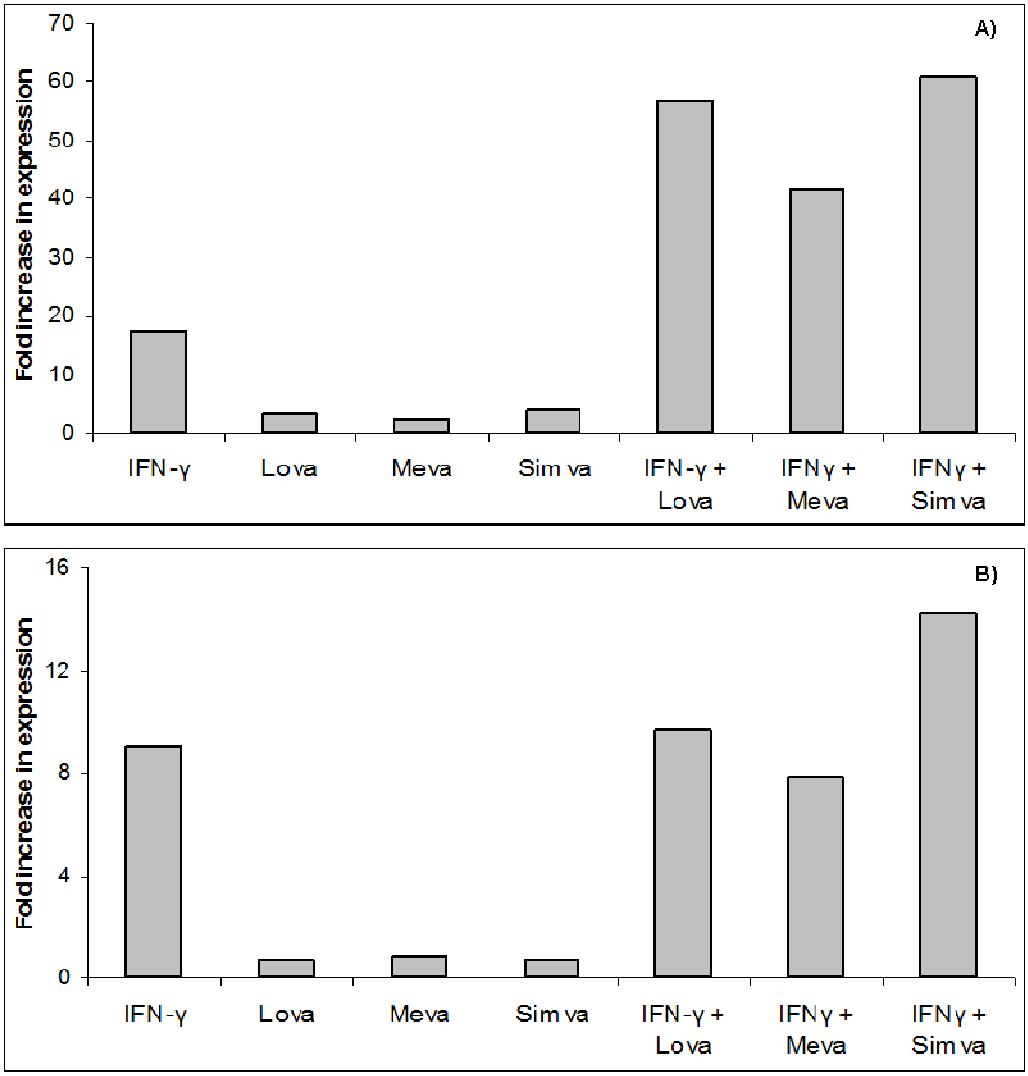

Figure 21. Effect of statins on MHC class I expression at transcriptional level accessed by real-time PCR in (A) SKMC cells and (B) TE671 cells. The graphic shows the fold induction level of MHC class I expression compared to untreated cells. These values are representative of three experiments done in triplicate.

6.6.2. TAP and LMP expression

The assembly of MHC class I molecules with peptides is orchestrated by several factors, including the transporter associated with antigen processing (TAP) (Raghavan et al. 2008). TAP1 and TAP2 gene products are responsible for the transport of the peptides from the cytosol to the endoplasmic reticulum, where peptides bind to newly sythesized MHC class I (Elliott 1997). The low- molecular-mass polypeptides 2 (LMP2) and 7 (LMP 7) increase the amount of generated peptides available for binding to MHC class I molecules (Driscoll et al. 1993; Gaczynska et al. 1994; Groettrup et al. 1995; Niedermann et al. 1995). Up-regulation of the TAP and LMP genes by immune regulators, such as interferon-gamma (IFN-γ), leads to an increase in MHC class I expression (Yang et al. 1992; Boes et al. 1994; Tanaka 1994). Determination of TAP1, TAP2, LMP2 and LMP7 expression was done at RNA level after 48 h of treatment with IFN-γ, statins, or a combination of both. IFN-γ treatment increased the expression of all four genes (TAP1, TAP2, LMP2 and LMP7) (Table4). Statins alone increased the expression level of TAP1 (5 fold) and LMP7 (2.8 fold) in SKMC cells, but they failed to show any effect in TE671. No significant change could be detected in other genes evaluated in response to statin treatment. All three statins potentiated the IFN-γ induction of TAP2, LMP2 and LMP7 in SKMC but not in TE671 (Table 4).

GENE / TREATMENT		IFN-γ	lova	meva	simva	IFN-γ + lova	IFN-γ + meva	IFN-γ + simva
SKMC	LMP2	17.83	1.29	0.94	1,10	30.21	36.03	37.09
	LMP7	9.45	2.40	1.77	2.87	11.62	16.22	16.35
	TAP1	28.03	4.81	2.53	5.06	90.82	89.21	123.37
	TAP2	8.47	0.33	0.71	0.27	16.53	10.47	18.83
TE671	LMP2	123.05	0.66	0.61	0.60	97.40	101.19	104.91
	LMP7	23.87	0.78	0.85	0.59	23.84	25.40	8.51
	TAP1	53.77	0.80	0.73	1.34	53.86	49.13	38.61
	TAP2	7.96	0.72	0.41	0.82	6.24	9.31	5.09

Table 4. Relative gene expression analysis by quantitative real time PCR of genes involved in MHC class I antigen presentation after statins and/or IFN-γ treatment in muscle cell lines.

7. Discussion

In this thesis, a combination of different autoantibody detection methods and protein biochemistry methods, such as 2D-gelelectrophoresis and mass spectrometry has been used to detect muscle- or muscle-endothelium-specific autoantigens and their functionality in inflammatory myopathies. The identification of such antigens could contribute significantly to elucidate the physiopathology of these diseases.

7.1. Surface binding autoantibodies

It could be shown that a significant number of myositis patients have autoantibodies against surface epitopes of muscle cells. This binding was specific to muscle cells since there was no reactivity either in control sera or against fibroblasts. At the same time there was no surface binding to C2C12 mouse muscle cell line either undifferentiated or differentiated or to primary muscle cells SKMC. This might be due to a species specificity of the autoantibodies (Katsumata et al. 2007) and the differences between the primary cells and rhabdomyosarcoma cells. Previous studies have reported that over 50% of the patients have autoantibodies diected against intracellular antigens, although with improved detection techniques, recent studies have shown this frequency to be nearer 80% (Gunawardena et al. 2009 a). These autoantibodies can be categorized into myositis-associated autoantibodies (MAAs) and myositis-specific autoantibodies (MSAs). The MAAs, anti-U1-RNP, anti-U3-RNP, anti-PM-Scl and anti-Ku, are principally seen in myositis-scleroderma overlap syndromes (Koenig et al. 2007), whereas the MSAs (anti-ARS, anti-SRP, anti-Mi-2, and anti-SAE etc.) are highly selective, mutually exclusive and are associated with particular genotypes and clinical phenotypes within the myositis spectrum (Love et al. 1991; Chinoy et al. 2006; O'Hanlon et al. 2006; Gunawardena et al. 2009 a). MSAs target either nuclear or cytoplasmic components of the cell that are involved in gene transcription, protein translocation and antiviral responses. In a recent study Gunawardena et al. have discussed the use of MSAs to define patients into clinical syndromes, which might help in predicting the outcomes and influence the treatment strategies (Gunawardena et al. 2008). Though in our experiments these surface binding autoantibodies could not differentiate among

different myositis groups (DM, PM and OIM) we did see that DM patients showed an increased affinity towards undifferentiated C2C12 mouse muscle cells. The question of whether autoantibodies are simply epiphenomena or directly linked to pathogenesis remains unclear. Several attractive paradigms have been proposed to explain why certain intracellular proteins are selectively targeted by autoantibodies (Helmers et al. 2009; Gunawardena et al. 2009 a).

Statin treatment of muscle cells increased the binding of myositis sera to these cells depending on the different statins used and the cell lines tested. This effect was more pronounced in primary muscle cells SKMC, where mevastatin and simvastatin treated cells showed significantly increased binding to myositis sera compared to untreated cells. DM patients showed the highest binding to simvastatin treated primary muscle cell. This clearly indicates that statins definitely play a role in statins induced myositis.

The availability of MSAs for classifying the patients does not help much in predicting the outcome of disease (Gunawardena et al. 2009 a). The future development of assays that test for MSAs in routine clinical practice is important. Investigating the structure and function of target molecules, and whether autoantibodies themselves have functional roles appears critical for understanding the pathogenic mechanisms in this complex spectrum of diseases. Though these disorders are believed to be mediated by cellular immune mechanisms, several investigators have identified immunoglobulin and complement in the muscles (Whitaker and Engel 1972; Kissel et al. 1986) and focused on vascular deposition of autoantibodies in relation to pathogenesis. Pathogenic role of anti endothelial cell antibodies (AECA) have been implicated in several diseases including autoimmune rheumatic diseases and (Domiciano et al. 2009; Jarius et al. 2009). Konstantin et al. developed a cellular ELISA to detect AECA in patients with IIMs. Fourteen of 19 patients (77%) were shown to have EC-binding activities. Yet, the main message from their study was that EC-specific IgG autoantibodies were more often encountered in MCTD and SS-associated myositis than in idiopathic PM and DM (Salojin et al. 1997). This group contemplated that AECA might be pathogenic through complement activation, antibody dependent cell cytotoxicity, or apoptosis of EC (Cines et al. 1984; Meroni 1994; Bordron et al. 1998). As the involvement of the endothelium is

suggested in the pathogenesis of these IIMs, especially in DM (Nagaraju et al. 2006), I also looked for the presence of anti endothelial cell antibodies. Any surface binding antibodies could not be shown to the endothelial cell lines tested, yet surprisingly these antibodies showed cytotoxicity against endothelial cells. In Wegener's granulomatosis (WG), Holmen et al. showed that WG IgG bind strongly to HKMEC but not to HUVEC and other endothelial cell lines (Holmen et al. 2007), which shows that these autoantibodies are specific for kidney EC and not just any EC. It seems that in our experiments also these autoantibodies are very specific, but then again any surface binding to human dermal micro-vascular endothelial cells (HDMEC) could not be shown.

7.2. Proteomic approach to identify autoantigens

In IIMs, like most autoimmune diseases, several autoantigens participate in the pathogenesis, and epitope spreading is accountable for disease induction, progression and inflammatory relapses. Knowledge of the targeted autoantigens is therefore indispensable for understanding the nature of acute autoimmune responses during recurrent attacks. Furthermore identifying persons at risk to develop several autoimmune diseases is based upon the presence of an autoreactive response to several autoantigens prior to disease state (Verge et al. 1996; LaGasse et al. 2002; Maclaren et al. 2003; Scofield 2004). The accuracy of prediction increases by combining autoantigens to identify potential novel candidate autoantigens in IIMs. In current study the IgG binding profile to the muscle cells proteome was tested.

A proteomic approach based on 2D immunoblotting was used, which combines 2D electrophoresis and immunoblotting. The 2D electrophoresis is a classical method for proteome analysis. It is a powerful tool to investigate differential patterns of qualitative and quantitative protein expression. Theoretically, all the proteins in the cells are separated in one gel. In the present method, 2D immunoblotting was performed and analysis of the western blot images was done with computer systems. The matching procedure allows determining the precise localization of relevant protein spots. Thereafter the selected protein spots were identified by mass spectrometry. Thus, this technical approach of 2D immunoblotting with mass spectrometry also called

“serological proteome analysis” (SERPA) potentially enables the detection and identification of new target antigens. So far, proteome-based technologies have been successfully used in tumor immunology for the identification of tumor-specific autoantigens (Seliger and Kellner 2002; Unwin et al. 2003). In the very last years, 2D immunoblotting with mass spectrometry have been used to identify new antigens targeted by autoantibodies, including myelin protein Po in autoimmune inner ear disease (Cao et al. 1996), heterogeneous nuclear ribbonucleoprotein A2/B1 in autoimmune hepatitis,(Huguet et al. 2004) ATP synthase beta chain in celiac disease,(Stulik et al. 2003) and α-enolase in Behcet’s disease (Lee et al. 2003). In rheumatoid arthritis, autoantibodies directed against α-enolase and its citrullinated molecule have also been detected (Saulot et al. 2002; Kinloch et al. 2005). Another approach, the SEREX method (serological analysis of recombinant cDNA expression library), has been developed for the serological definition of immunogenic tumor antigens (Chen et al. 1997; Fernandez Madrid et al. 2005; Kuboshima et al. 2006). Recent studies indicate that the SEREX approach may also be utilized for the analysis of complex immune responses involved in autoimmune diseases (Krebs et al. 2003). Whereas human proteins are directly used as source of antigens in 2D immunoblotting, proteins are expressed in E. coli in SEREX approach. Although several improvements can be made in the SEREX approach (Fernandez Madrid et al. 2005), the use of human cells as a direct source of proteins seem to us more straightforward and relevant for researches in autoimmune diseases than using proteins expressed in *E.Coli*.

Sera from 26 patients and 15 healthy subjects were analyzed by western immunoblotting with total cell lysate from TE671, C2C12 and HEK 293 cell lines. Seropositivity was based on immunoreactivity with different proteins at serum dilutions of 1/100. With TE671 cell lysate, 12 sera from 26 (46%) myositis patients produced a specific band in the region between 97 and 191 KDa which was not present in healthy controls. This immunoreactivity failed to appear with HEK 293 (Fig. 13) and therefore, was considered to be muscle specific.

These results further show the presence of a specific protein band in western blotting of

muscle cell lysate with patient sera which was not present in control sera and fibroblast cell lysate. Though specific spots in 2-D electrophoresis of muscle cell proteins were found, yet these proteins are not muscle specific instead ubiquitously expressed. The identified proteins include Proteasome subunit beta type 7 precursor proteins. Anti-proteasome antibodies have already been reported in various diseases including SLE, primary sjögren's syndrome, PM and DM (Scheffler et al. 2008). They have also discussed that the autoimmune response against proteasomes is diverse and not disease specific, and due to frequent and extended antibody reactivity, the proteasome is one of the prominent autoantigens in systemic autoimmune disorders. The autoantigens themselves might play a role in disease mechanisms. Enhanced expression of Mi-2 and Jo-1 in myositis muscle compared with normal muscle, that too primarily in regenerating muscles rather than in mature myotubes very nicely supports the hypothesis that the presence of myositis candidate autoantigens during reparative myogenesis can drive induction and propagation of the autoimmune response (Casciola-Rosen et al. 2005). Mi-2, which is essential for the development and repair of the basal epidermis (Kashiwagi et al. 2007), is preferentially expressed in DM muscle rather than PM muscle, supporting the association between DM and Mi-2 (Casciola-Rosen et al. 2005). Certain autoantigenic-tRNA synthetases (histidyl, asparaginyl and tyrosyl) have chemoattractant properties and can induce leukocyte migration through the CCR5 and CCR3 receptors (Howard et al. 2002; Wakasugi et al. 2002).

As the involvement of the endothelium is suggested in the pathogenesis of these diseases (Nagaraju et al. 2006), the presence of anti endothelial cell antibodies has also been analyzed. Any surface binding antibodies could not be shown to any of the endothelial cell lines tested, yet surprisingly these antibodies showed cytotoxicity against endothelial cells.

7.3. Functional effects of autoantibodies

Functional autoantibodies that act at ion channels or receptors and disrupt autonomic or cardiovascular functions have been described in autoimmune diseases including Lambert-Eaton Myasthenic syndrome (LEMS) (Waterman et al. 1997), Scleroderma

(Goldblatt et al. 2002), Sjögren's syndrome (Waterman et al. 2000), and others (Gleicher et al. 2007; Jackson et al. 2008; Maciejewska-Rodrigues et al. 2010). LEMS IgG impairs transmitter release from parasypathetic and sympathetic neurons through down-regulation of one or more subtypes of voltage-gated calcium channels (Waterman et al. 1997). The IgG from patients with primary Sjögren's syndrome show two effects on the submandibular glands. First, it may act as an inducer of the proinflammatory molecule (PGE_2), which then inhibits Na^+/K^+ -ATPase activity. Secondly, it may be involved in dry mouth pathogenesis by abolishing the Na^+/K^+ -ATPase inhibition and the net K^+ efflux stimulation of the salivary glands (Passafaro et al. 2010).

Since disturbances of cell functions often lead to an increased cell death rate, possible cytotoxicity of myositis-IgG on muscle and endothelial cells has been investigated. These autoantibodies showed no surface binding to any of the endothelial cells, but they did show their cytotoxic effects against these cells. Since binding of obviously functional autoantibodies it could not be shown, one might speculate that the antigens responsible for the cytotoxic effect are expressed at low level on the cell surface. Another explanation could be that, the autoantibodies are low-affinity binding IgGs and our method (flow cytometry) is not able to discriminate the specific binding from background. Low-affinity binding autoantibodies have also been recently found in patients with myasthenia gravis where no anti-AChR antibodies could be found in normal tests (Leite et al. 2008).

Calcium is an important messenger in intracellular signalling mechanisms; therefore it has been contemplated that the cytotoxic antibodies might have an effect on stimulated calcium flux. The results show that the purified IgG from patients indeed triggered a rapid calcium flux response in HUVECs while healthy control IgG did not show any effect at all. This change in Ca^{2+} might be responsible for the cytotoxic effects of antibodies.

HUVECs are a well established model to study the changes in calcium flux. A change in calcium flux is the first feature of cell activation, which initiates and coordinates specific cellular activities after a given stimuli. Calcium is an important intracellular messenger

that is involved in modulating a vast array of cellular events. Signalling events through the BCR in SLE B cells are abnormal, as indicated by increased intracellular calcium flux and phosphorylation of multiple proteins. Stimulation of freshly isolated peripheral blood B cells from patients with SLE with BCR ligand led to unusually high calcium responses and increased tyrosine phosphorylation of proteins relative to peripheral blood B cells from healthy individuals and disease controls (Pugh-Bernard et al. 2006). The signalling alterations found in this study did not correlate with disease activity or treatment, and clearly demonstrate the existence of SLE-specific signalling alterations in B cells.

7.4. Skeletal muscle cell MHC I expression in response to statin treatment

The major histocompatibility complex (MHC) genes are located on chromosome 17 (in the case of H-2) and 6 (human leukocyte antigen [HLA]) in mice and humans, respectively. MHC class I molecules are composed of a transmembrane heavy-chain glycoprotein (H), a non-covalently associated soluble protein called ß2-microglobulin, and a short peptide of 8-10 residues derived from endogenous proteins. These genes are constitutively expressed in most adult tissues, although the relative levels of class I expression in different tissues vary widely.

MHC class I over expression is an early event in many autoimmune diseases, particularly in tissues such as muscle, pancreatic ß cells, neuronal cells, and thyrocytes that show little or no constitutive expression (Hanafusa et al. 1983; Bottazzo et al. 1985; Foulis et al. 1987; McDouall et al. 1989). Abnormal high expression of these molecules can occur in the absence of an inflammatory infiltrate, suggesting that it may be independent of, and possibly precede, the effects of cytokines released from infiltrating mononuclear cells. Normal human skeletal myoblasts constitutively express low levels of HLA class I molecules under cell culture conditions (Hohlfeld and Engel 1991; Nagaraju et al. 1998). Muscle fibres in mormal individuals do not express detectable levels of MHC class I antigens (Emslie-Smith et al. 1989; Hohlfeld and Engel 1991). Muscle fibres from patients with IIMs show consistently strong expression of MHC class I molecules (Rowe et al. 1983; Appleyard et al. 1985; Karpati et al. 1988; Emslie-Smith

et al. 1989; McDouall et al. 1989; Bartoccioni et al. 1994). Class I MHC may play multiple roles in myositis serving both to initiate the disease as well as maintain ongoing muscle damage. A big part of IIM immunogenetic research has continued to concentrate on the MHC. In IIM it has long been recognized that HLA status relates to disease specific serologivcal subtypes, which in turn associate with distinct clinical phenotypes (Love et al. 1991).

The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, generically referred to as statins, have been prescribed extensively for their cholesterol lowering properties and efficacy in cardiovascular diseases (Greenwood et al. 2006). Statins also have additional immunomodulatory properties that operate independently of lipid lowering. In some patients, statins can induce necrotizing or inflammatory myopathies (Needham et al. 2007). However, basic mechanisms governing this property are so far unknown. Recently, increased MHC class I expression has been demonstrated in muscle fibers of patients afflicted with statin-induced myopathies. The over-expression of MHC class I molecules is an early event in many autoimmune diseases, since it is a prerequisite for the cytolytic action of cytotoxic T lymphocytes. MHC class I molecules by themselves can have a deleterious effect on cell types that do not constitutively express these molecules (Nagaraju 2005). Normal human skeletal myoblasts constitutively express low levels of MHC class I molecules under cell culture conditions (Hohlfeld and Engel 1991; Nagaraju et al. 1998). Muscle fibres of healthy individuals do not express detectable levels of MHC class I antigens, although these fibres have been shown to express MHC class I in several autoimmune muscle diseases (Appleyard et al. 1985; Emslie-Smith et al. 1989).

The assembly of MHC class I molecules with peptides is orchestrated by several factors including the transporter associated with antigen processing (TAP)(Raghavan et al. 2008). TAP1 and TAP2 gene products are responsible for the transport of peptides from the cytosol to the endoplasmic reticulum where peptides bind to newly synthesized MHC class I (Elliott 1997). The low molecular mass polypeptides 2 (LMP2) and 7 (LMP7) increase the amount of generated peptides available for binding to MHC class I antigens (Driscoll et al. 1993; Gaczynska et al. 1994; Groettrup et al. 1995; Niedermann

et al. 1995). Up-regulation of the TAP and LMP genes by immune regulators, such as interferon gamma (IFN-γ) lead to an increase in MHC class I expression (Yang et al. 1992; Boes et al. 1994; Tanaka 1994).

The mechanism responsible for widespread expression of MHC class I in statin-associated myopathy is unclear (Needham et al. 2007). Therefore, the effect of statins on the MHC class I expression has been investigated *in vitro* on primary cultured muscle cells and the rhabdomyosarcoma cell line TE671, which is often used as a model cell line for skeletal muscle diseases. Surprisingly, statins impaired the IFN-γ-inducible MHC class I expression in primary skeletal muscle cells SKMC while they potentiate the same effect in TE671. In primary skeletal muscle cells, statins alone showed even a decrease in surface expression of MHC class I.

The potential effect of three statins has also been investigated on MHC class I expression and regulation in response to IFN-γ in primary skeletal muscle cell cultures and the rhabdomyosarcoma cell line TE671, the latter being used often as a model for skeletal muscle diseases. In the primary skeletal muscle cells, statins alone not only down-regulated the constitutive MHC class I expression but also impaired the IFN-γ-induced MHC class I expression. In contrast, in the TE671, statins alone did not affect the constitutive MHC class I expression, albeit they enhanced the IFN-γ-induced MHC class I expression. Expression of MHC class I in muscle fibers is a ubiquitous feature of inflammatory myopathies, and it may be a diagnostic marker for inflammatory myopathies (Sundaram et al. 2008). MHC class I is not expressed on the sarcolemma of normal muscle fibers. Its presence has been reported as a marker of immune activation, since it is involved in antigen recognition by CD8+ T cells (Hohlfeld and Engel 1994). Transgenic mice that display up-regulation of MHC class I expression develop a self sustaining autoimmune myositis, supporting the role of MHC class I in the pathogenesis of myositis. Nonetheless, Confalonieri et al failed to find any T-cell cytotoxicity accompanied with up-regulated MHC class I expression in their dysferlinopathic patients (Confalonieri et al. 2003). However, a higher MHC class I expression has also been found in muscular dystrophies that lack the dysferlin protein (Confalonieri et al. 2003). Therefore it may be that the induction of MHC class I is a

general phenomenon in muscle cells affected by different etiologies. Our data show that statins alone are not able to induce MHC class I in primary muscle cells. The results also suggest that TE671 may not be an ideal model of skeletal muscle cells, since the cell line behaves in our study more like a tumor cell as has been seen in other tumor cell lines (Tilkin-Mariame et al. 2005). In the murine B16F10 melanoma cell line and in human melanoma cell lines, these authors demonstrated that statins and geranylgeranyl transferase inhibitors enhance IFN-γ induced expression of MHC class I antigen.

Additionally, the effect of statins was also tested on different gene products associated with MHC class I surface expression, namely TAP1 and 2 and LMP 2 and 7. IFN-γ treatment increased the expression of all four genes. No substantial change could be detected in response to statin treatment; nevertheless, all three statins augmented a further two times increase over IFN-γ induction of TAP1 expression in primary muscle cells but not in TE671. In the latter cells, it seems that increased MHC class I expression induced by the combination of statins + IFN-γ treatment is not due to a reinforcement of the up-regulation of TAP and LMP genes by statins, because I was able to demonstrate the same level of expression as by IFN-γ treatment alone.

In the primary muscle cells, statins increased the IFN- γ-induced mRNA expression of MHC class I, TAP and LMP genes, however the surface expression of MHC I was reduced. Since the proteins of the antigen-presenting machinery (TAP1/2 and LMP2/7) are also induced, the negative effect on the MHC I surface expression could be caused by disturbances in lipid raft formations or an activated control pathway on the protein level. The latter hypothesis is supported by the observation of an increased MHC I expression in patients with dysferlinopathies and dysferlin-deficient mice (Kostek et al. 2002; Confalonieri et al. 2003). In the TE671 rhabdomyosarcoma cells, this mechanism may be defective as a result of the dedifferentiation of the cell. As reported above, tumor cell lines in general seem to behave differently to statin treatment (Tilkin-Mariame et al. 2005).

Our data show a different effect of statins *in vitro* in comparison to previous reported *in*

vivo studies, by showing increased MHC class I expression in muscle biopsies from patients with statin-induced myopathies. This might be due to the presence of other cytokines *in vivo* which are absent in our *in vitro* system. Our findings are partially in agreement with the previous report that statin treatment led to the development of myositis, which was reported in association with an increased MHC class I expression on muscle cells (Needham et al. 2007). Conceivably, the presence of other players like IFN-γ or a different genetic background like association with certain HLA types might also be crucial to the development of statins–induced myopathies. In idiopathic inflammatory myopathies polymorphisms of the IFN-gamma gene have been reported to be a risk factor for the development of myositis. This altered IFN-gamma may have different effects in the MHC expression in patients with myositis and also statin-induced myopathy (Chinoy et al. 2007 b). Recently, a polymorphism in the SLCO1B1 gene, which codes for an organic anion-transporting polypeptide family member (OATP1B1) was shown to be associated with an increased risk for development of statin-induced myopathy (Link et al. 2008). This polymorphism obviously increases the plasma concentration of statins (Pasanen et al. 2007) but there are no data to link the function of OATP1B1 and the MHC class I expression. Additionally the observed effect of the statins on MHC class I expression were not dose-dependent in our study. Investigations about the effect of statins on MHC class I molecules seem to be all the more important, since statins are the leading therapeutic regimen for the treatment of cardiovascular disease (Greenwood et al. 2006) and recently have been contemplated to be used as a novel immunomodulator. Future studies should therefore attempt to investigate cytokine regulation in patients with statin-induced myopathies and to establish the role of MHC I expression on muscle cell lines.

Taken together this data establish that the PM and DM patients have surface binding autoantibodies, which show cytotoxic effects towards endothelial cells and affect the Ca^{2+} influx in these cells as well. Interestingly statin treatment of muscle cells seem to increase the immunogenicity of muscle cells, shown by an increased IgG binding of myositis sera. It proves that the autoantibodies in PM and DM patients do have functional role in the development of these diseases. Further, above data also show that statins alone reduced the expression of MHC I in SKMC and had no effect on MHC I in

TE671. Statins potentiated the MHC I-inducing effect of IFN-gamma in TE671, but not in SKMC, neither on the protein level, nor on mRNA level. This leads to the conclusion that the increased muscle MHC I expression in statin-induced myopathy might not be induced directly by statins themselves.

8. Summary

Polymyositis and Dermatomyositis are both inflammatory muscle diseases. Although different autoantibodies have been reported in DM/PM, pure muscle specific antigens are rarely known.

The current study focuses on identification of proteins, responding to antibodies from inflammatory myositis and screens those proteins using proteomic approach. At the same time the functional effects of the specific autoantibodies has also been investigated by using cytotoxicity assay and calcium imaging.

Flow cytometric antibody binding showed that myositis patients have autoantibodies against surface epitopes of muscle cells but not to endothelial cells. As statins have been implicated in inducing myopathy with increased expression of MHC class I in muscle cells, statin treated cells have been analysed for surface binding of patient sera. Patients' sera showed increased binding to mevastatin and simvastatin treated primary muscle cells but not to rhabdomyosarcoma cells. The 2D approach has been used to identify muscle specific autonantigens but we could not find any specific autoantigens. Interestingly the purified IgG from myositis patients showed cytotoxic effects against endothelial cells but not to muscle cells. In further assessing the functional effects of autoantibodies It has been found that the purified patients' IgG but not control IgG changed Ca^{2+} influx in endothelial cells. When analysed for statins induced changes in MHC class I expression and other proteins (TAP and LMP) at protein and RNA level, statins alone surprisingly reduced the expression of MHC I in SKMC and had no effect on MHC I in TE671. Statins potentiated the MHC I-inducing effect of IFN-gamma in TE671, but not in SKMC, neither on the protein level, nor on mRNA level. This leads to the conclusion that the increased muscle MHC I expression in statin-induced myopathy might not be induced directly by statins themselves. The increased binding of myositis sera to stain treated muscle cells and to primary muscle cells and the functional effects of these autoantibodies point to a pathogenic role of the humoral immune system in inflammatory muscle diseases.

9. Zusammenfassung

Polymyositis und Dermatomyositis sind Erkrankungen aus dem Formenkreis der entzündlichen Muskelerkrankungen. Obwohl verschiedene Autoantikörper bei diesen Erkrankungen beschrieben wurden, sind Muskel- oder Endothel-spezifische Autoantikörper kaum bekannt.

In der vorliegenden Studie wurden Autoantikörper und deren Spezifität mit einem Proteomansatz und ihre möglichen funktionellen Effekte mit Zytotoxizitäts- und Calciumfluxmessungen bei entzündlichen Muskelerkrankungen untersucht.

Mit Hilfe der Durchflusszytometrie konnte gezeigt werden, dass Myositispatienten Autoantikörper gegen Oberflächenepitope von Muskelzellen, aber nicht Endothelzellen haben. Eine Behandlung von Muskelzellen mit Statinen, die vereinzelt Myositiden erzeugen können, führt zu einer erheblichen Zunahme der Bindung von Myositis IgG an primäre Muskelzellkulturen, aber nicht an die Rhabdomyosarkomzelllinie TE671. Die 2D-Gelelektrophorese mit anschließendem Blot führte nicht zur Identifizierung entsprechender muskel-spezifischer Autoantigene. Interessanterweise führte Myositis IgG zu zytotoxischen Effekten bei Endothelzellen, nicht jedoch bei Muskelzellen. Myositis IgG-Fraktionen, aber nicht IgG von Kontrollen beeinflussten auch den Calciuminflux in Endothelzellen.

Aufgrund der Beobachtung, dass bei Statin-induzierten Myopathien MHC Klasse I auf Muskelzellen erhöht gefunden wurde, untersuchte ich den Einfluss von Statinen auf die MHC Klasse I Expression in vitro. Dabei zeigte sich, dass Statine sogar zu einer Verminderung der MHC I Expression in primären Muskelzellen (SKMC) führen, bei TE671 Zellen war kein Effekt nachweisbar. Während Statine die Interferon-γ induzierte MHC I Expression auf der Muskeltumorzelllinie TE671 potenzierten, wurde die MHC I Expression auf SKMC vermindert. Dies ließ sich auf Protein- und mRNA Level zeigen, was zu der Schlussfolgerung führt, dass die MHC I Expression bei diesen Myopathien kein direkter Effekt der Statine ist.

Die von uns nachgewiesenen Antikörper gegen Oberflächenepitope, insbesondere auch die verstärkte Bindung an Statin-behandelte Muskelzellen, und die funktionellen Effekte der Autoantikörper legen eine pathogene Rolle des humoralen Immunsystems bei inflammatorischen Muskelerkrankungen nahe.

10. References

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