

From the Rheumatology Unit,
Department of Medicine, Solna
Karolinska Institutet, Stockholm, Sweden

T CELL SUBSETS AND DISEASE MECHANISMS IN INFLAMMATORY MYOPATHIES

Jayesh Pandya



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T cell Subsets and Disease Mechanisms in Inflammatory Myopathies

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Jayesh Pandya

Principal Supervisor:

Professor Vivianne Malmström
Karolinska Institutet
Department of Medicine, Solna
Rheumatology Unit

Opponent:

Professor Anna Rudin
University of Gothenburg
Department of Medicine

Co-supervisors:

Professor Ingrid E. Lundberg
Karolinska Institutet
Department of Medicine, Solna
Rheumatology Unit

Examination Board:

Associate professor Anna Fogdell Hahn
Karolinska Institutet
Department of Clinical Neuroscience

Dr. Andreas Fasth
Karolinska Institutet
Department of Medicine, Solna
Rheumatology Unit

Professor Francesca Chiodi
Karolinska Institutet
Department of Microbiology, Tumor and Cell
Biology, MTC

Associate professor Gustavo Nader
Karolinska Institutet
Department of Physiology and Pharmacology,
Solna

Professor Lars Larsson
Uppsala University
Department of Clinical Neurophysiology

*To
My mother and family*

ABSTRACT

The idiopathic inflammatory myopathies (IIMs) are a heterogeneous group of chronic muscle disorders, typically displaying infiltrating T cells in skeletal muscle tissue and classified into polymyositis, dermatomyositis and sporadic inclusion body myositis. Several studies involving both humans and animal models point towards a role for T cells in the pathogenesis of IIMs, however, the precise phenotype, functionality and specificity of pathogenic T cells remain elusive. Increased frequencies of a subset of T cells, known as CD28null T cells, in peripheral blood and affected organs in various chronic inflammatory disorders, are reported by several studies. Such CD28null T cells are highly differentiated T cells lacking the co-stimulatory molecule CD28, which acquire expression of other receptors commonly associated with natural killer cells, and display proinflammatory, cytotoxic and apoptosis resistant features. In contrast to CD28null T cells, regulatory T cells are T cell subset critical for maintaining immune tolerance and also described to assist in the muscle repair process.

The aims of this thesis were to investigate CD28null T cell subsets in both muscle tissue and peripheral blood of patients with IIMs, by evaluating frequencies, phenotype, function and clinical relevance of these cells. The cytotoxic mechanisms of CD28null T cells towards autologous muscle cells were investigated using *in vitro* T cell - muscle cell co-cultures. Muscle tissues of patients were investigated for the effects of conventional immunosuppressive therapies on CD28null and regulatory T cell subsets. Glucocorticoid and regulatory T cells mediated immunosuppressive effects on circulating CD28nulls T cells were evaluated using *in vitro* assays.

We demonstrate that muscle-infiltrating T cells are predominantly CD4+CD28null and CD8+CD28null T cells in patients with polymyositis and dermatomyositis. Also in sporadic inclusion body myositis, where the role of immune system is controversial, T cell infiltrates in muscle tissue are dominated by CD28null T cells. Circulating CD28null T cell subsets of both CD4 and CD8 lineage were more common in patients compared to healthy controls, and were associated with human cytomegalovirus infection. These cells displayed oligoclonal expansions, proinflammatory cytokine secretion and degranulation potential, and also contained perforin. Using autologous *in vitro* co-cultures, we showed that the cytotoxic effects of CD28null T cells towards muscle cells are mediated largely via perforin-dependent mechanisms and regulated by IFN γ -induced HLA expression on muscle cells. Interestingly, poor clinical response in patients following immunosuppressive therapy was linked to persistence of CD28null T cells in muscle tissue. CD4+CD28null T cells were also found to be resistant towards glucocorticoid and regulatory T cell mediated immunosuppression in *in vitro* assays.

These findings imply that CD28null T cells represent clinically important effector cells in IIMs, capable of attacking muscle fibers and inducing chronic inflammation mediated pathogenesis. Ineffectiveness of current immunosuppressive therapies appears to be linked with persistent CD28null T cells in muscle tissue as well as their immunosuppression resistant properties; therefore, these cells represent potential target candidates for future therapies.

LIST OF SCIENTIFIC PAPERS

- I. Andreas E. R. Fasth, Maryam Dastmalchi*, Afsar Rahbar*, Stina Salomonsson, Jayesh M. Pandya, Eva Lindroos, Inger Nennesmo, Karl-Johan Malmberg, Cecilia Söderberg-Nauclér, Christina Trollmo, Ingrid E. Lundberg and Vivianne Malmström.
T cell infiltrates in the muscles of patients with dermatomyositis and polymyositis are dominated by CD28null T cells.
Journal of Immunology, 2009, Oct 1;183(7):4792-9
- II. Jayesh M. Pandya, Andreas E. R. Fasth, Mei Zong, Snjolaug Arnardottir, Lara Dani, Eva Lindroos, Vivianne Malmström and Ingrid E. Lundberg.
Expanded TCR-V β Restricted T cells from Sporadic Inclusion Body Myositis Patients are Proinflammatory and Cytotoxic CD28null T cells.
Arthritis & Rheumatism. 2010 Nov; 62(11): 3457-66
- III. Jayesh M. Pandya*, Paulius Venalis*, Lubna Al-Khalili, Mohammad Shahadat Hossain, Vanessa Stache, Ingrid E. Lundberg, Vivianne Malmström, Andreas E.R. Fasth.
CD28null T Cells from Polymyositis Patients are Cytotoxic to Autologous Muscle Cells *In Vitro* via Perforin-Dependent Mechanisms.
Manuscript
- IV. Jayesh M. Pandya*, Ingela Loell*, Mohammad Shahadat Hossain, Mei Zong, Helene Alexanderson, Sukanya Raghavan, Ingrid E. Lundberg and Vivianne Malmström.
Muscle Persistent and Immunosuppression Resistant CD28null T cells in Patients with Polymyositis and Dermatomyositis.
Manuscript

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RELATED PUBLICATION NOT INCLUDED IN THESIS

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Activated LTB4 pathway in muscle tissue of patients with polymyositis or dermatomyositis.

Annals of the Rheumatic Diseases 2013 Feb;72(2):293-9

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LIST OF ABBREVIATIONS

APC	Antigen Presenting Cell
CD	Cluster of Differentiation
CCR	CC Chemokine Receptor
CCL	CC Chemokine Ligand
CMV	Cytomegalovirus
CK	Creatine Kinase
CTLA-4	Cytotoxic T-lymphocyte-Associated Protein 4
CXCR	CXC Chemokine Receptor
DM	Dermatomyositis
DMD	Duchenne Muscular Dystrophy
EBV	Epstein–Barr virus
FACS	Fluorescence-activated Cell Sorting
FI	Functional Index
FOXP3	Forkhead box P3
GCR	Glucocorticoid receptor
GrB	Granzyme-B
HAQ	Health Assessment Questionnaire
HCMV	Human Cytomegalovirus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HTLV-1	Human T-cell Lymphotropic Virus Type-1
ICAM	Intercellular Adhesion Molecule 1
ICOS	Inducible T-cell COStimulator
IE-1	Immediate Early antigen-1
IFN	Interferon
IGF-1	Insulin-like Growth Factor 1
IIM/IIMs	Idiopathic Inflammatory Myopathies
IL	Interleukin
IVIG	Intravenous Immunoglobulins

KIR	Killer Inhibitory Receptors
LFA-1	Lymphocyte function-associated antigen 1
LTB4	Leukotriene B4
MAC	Membrane Attack Complex
mDC	Myeloid dendritic cell
MHC	Major Histocompatibility Complex
MITAX	Myositis Intention To Treat Activity Index
MMPs	Matrix Metalloproteinases
MyHC	Myosin Heavy Chain
NCAM	Neural Cell Adhesion Molecule
NK- κ B	Nuclear Factor kappa B
NK cell	Natural Killer cell
NO	Nitric Oxide
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymarase Chain Reaction
pDC	Plasmacytoid Dendritic Cells
PD-1	Programmed cell death protein 1
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PM	Polymyositis
pp65	Phosphoprotein 65
ROS	Reactive Oxygen Species
sIBM	sporadic Inclusion Body Myositis
SLE	Systemic Lupus Erythematosus
T cell	T lymphocytes
TCR	T cell receptor
TNF	Tumor necrosis factors
tRNA	Transfer RNA
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

1 GENERAL INTRODUCTION

Inflammatory myopathies are a group of diseases characterized by progressive muscle weakness along with chronic muscle inflammation. These diseases are also known as idiopathic inflammatory myopathies (IIMs) or collectively as myositis. "Idiopathic" means that the cause is not known and "myositis" means muscle inflammation. Several findings indicate that IIMs may belong to immune-mediated inflammatory diseases category, where the immune system, which usually protects our body from diseases caused by infections or cancer, cause excessive inflammation in body's organs and tissues, leading to functional impairment. Abnormal and chronic inflammations are the main features of such diseases. Prior studies have reported the abnormal presence of cells linked to immune system including T cells in the skeletal muscle tissue of patients with IIMs. However, the mechanisms underlying the initiation and progression of the muscle pathology and specific role of immune cells are not clearly understood.

T cells (T lymphocytes) are specialized cells of our adaptive immune system, which function as key players in both maintaining immune tolerance against self-tissues and orchestrating multicomponent immune attack on pathogenic foreign microorganisms. Upon encountering a pathogen, T cell expansion takes place, and a subset of T cells known as memory T cells are maintained in the body to allow immediate recognition and effector function upon re-encounter of the pathogen. Memory T cells are thus equipped to kill and eradicate pathogens efficiently. However, increased frequencies of T cell subsets with memory phenotype, known as CD28null T cells, are reported in various chronic inflammatory disorders, indicating that these cells may cause inflammatory damage in various organs and tissue. The studies included in this thesis explore, whether CD28null T cell subsets can be regarded as pathogenic cells in inflammatory myopathies, and if yes, what could be the pathogenic mechanisms. Along with CD28null T cells, regulatory T cells were also investigated, which help to maintain self-tolerance and muscle repair. Furthermore, the effects of currently used immunosuppressive treatments on these T cell subsets were evaluated in patients with IIMs.

The thesis attempts to introduce relevant topics adequately in the background, which would help reader to understand the results and discussion. The suggested pathogenic mechanisms are based upon previous knowledge combined with findings in this thesis. However, hypothesis and suggested mechanisms are my personal view and should be confirmed by future studies before drawing conclusions.

2 BACKGROUND

2.1 IDIOPATHIC INFLAMMATORY MYOPATHIES

2.1.1 Introduction

The idiopathic inflammatory myopathies (IIM/IIMs), collectively named as myositis, are a heterogeneous group of chronic muscle disorders, which are characterized clinically by skeletal muscle weakness and low endurance, and histopathologically by inflammatory cell infiltrates in muscle tissue ¹. Based on clinical and histopathological differences, IIMs have been classified mostly into polymyositis (PM), dermatomyositis (DM) and sporadic inclusion body myositis (sIBM) ^{2,3}. Involvement of other organs than muscles is also frequently seen in DM and PM but less frequently in sIBM. Involvement of skin in DM, and lungs in PM and DM indicate that these myopathies are rather systemic connective tissues diseases ¹.

2.1.2 Classification criteria

Currently, Bohan and Peter ^{4,5} - and Tanimoto ⁶ criteria are the most commonly used criteria for diagnosis and classification of PM and DM. These are based on clinical, histopathological and neurophysiological evaluation in combination with serum levels of muscle enzymes. However, there are several limitations to these criteria because they do not incorporate recently discovered molecular biomarkers such as autoantibodies, which could classify patients more accurately based on their disease mechanisms. The lack of updated classification criteria has also hampered the research and development of novel treatments in IIMs subtypes. Nevertheless, Bohan and Peter classification criteria were used for determining PM and DM included in this thesis work. The above criteria do not distinguish sIBM. Therefore, the criteria suggested by Grigg's et al were used for sIBM classification ^{7,8}, although we did not rely on the compulsion of electron microscopy evaluation.

2.1.3 Epidemiology and clinical manifestations

The PM/DM/sIBM belong to a rare clinical disease category with the incidence of 0.1–1/100 000/year and the prevalence of 1–6/100 000/year ⁹. DM affects both adults and children and is more common in women. PM occurs mostly after second decade of life while sIBM mostly occurs in individuals older than 50-60 years. A male preponderance is observed in sIBM ^{9,10}.

Patients with PM and DM are described to display progressive weakness mainly of proximal limb muscles, while in patients with sIBM, distal muscles are often affected

with pronounced weakness and atrophy in addition to proximal muscles. However, a recent study in our group demonstrated that distal muscles are also affected in patients with PM and DM ¹¹. Proximal muscle weakness presents symptomatically as difficulties in everyday activities such as climbing the stairs, dressing, bathing, lifting objects or combing hair. Distal muscle weakness presents with difficulties in buttoning of shirts, sewing, writing etc. In all forms of IIM, pharyngeal and neck-flexor muscles are usually involved, leading to difficulties in swallowing and holding up the neck. In advanced cases, breathing difficulties may be encountered due to involvement of respiratory muscles. Severe weakness if untreated, almost always leads to muscle wasting. Myalgia and muscle tenderness (pain) may be present but the frequencies are unclear. Weakness in PM and DM progresses sub-acutely over a period of weeks or months and rarely acutely; by contrast sIBM muscle weakness progresses very slowly over years. Other common symptoms include fatigue and weight loss and malaise as a result of inflammation, muscle atrophy, pain and weakness. Some patients can also display arthralgia, arthritis, myocarditis and arrhythmias. Initial symptoms mostly include slow onset of muscle fatigue, but skin and lungs might also be involved. Increased incidences of malignancies are observed in patients with DM, particularly in patients older than 50 years. Symptoms can appear in different organs at different time points as the disease progresses. Involvement of multiple organs may lead to more severe complications, diagnostic difficulties, poor prognosis and reduced life expectancy. ^{9,10,12,13} (*Inflammatory Myopathies. Harrison's Principles of Internal Medicine 16th Edition, Vol II*)

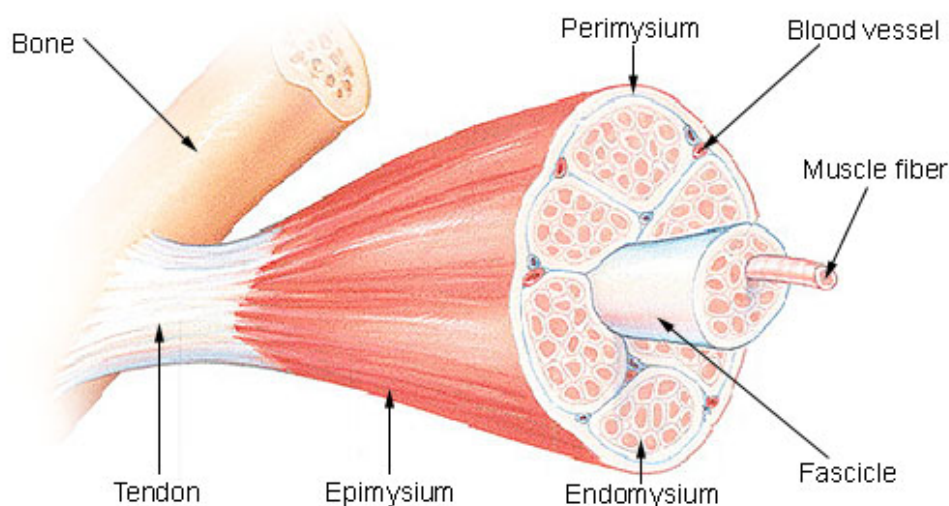


Figure 1: Skeletal muscle structure and connective tissue layers. There are three layers of connective tissue in a skeletal muscle. The epimysium is the outermost layer and surrounds the whole skeletal muscle. The perimysium surrounds the muscle fascicles and contains blood vessels and nerves. A fascicle contains a group of muscle fibers (between 10-100). The endomysium surrounds each single muscle fiber and is a thin, delicate covering of loose connective tissue. Endomysium also contains blood capillaries and nerves. (*Picture and legend adapted from Wikipedia & Gray's Anatomy: IV. Myology*)

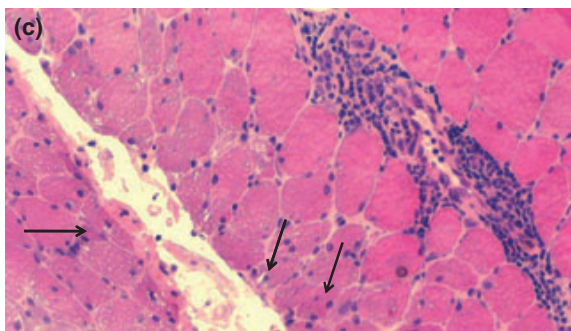
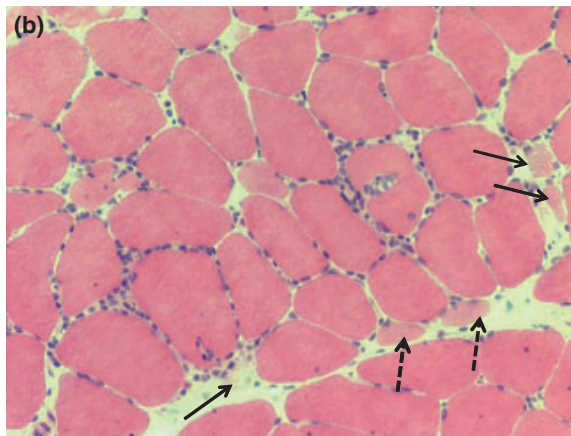
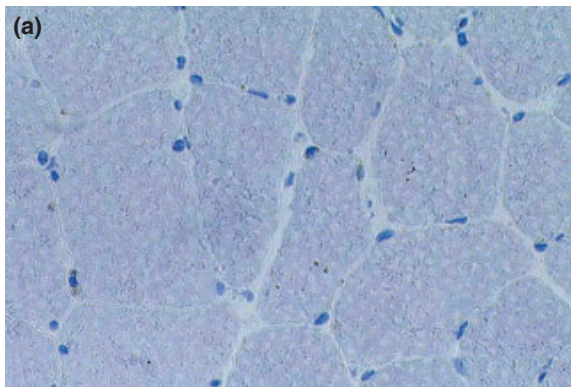


Figure 2. (a–c) Characteristics of healthy muscle and of muscle from patients with polymyositis and dermatomyositis.

(a) Cross-sectional muscle biopsy tissue from a healthy subject (haematoxylin and eosin staining). Image courtesy of Dr Cecilia Grundtman. (b) Cross-sectional muscle biopsy sample from a patient with polymyositis; image shows fiber size variation, several fibres with central nuclei, mononuclear inflammatory cells with an endomysial distribution and surrounding muscle fibres (haematoxylin and eosin staining), degenerating fibres (thick arrows) and regenerating fibres (broken arrows). Image courtesy of Dr Inger Nennesmo. (c) Cross-sectional muscle biopsy sample from a patient with dermatomyositis; image shows a perimysial and perivascular inflammatory cell infiltrate and perifascicular distribution of small fibres (arrows) so-called perifascicular muscle atrophy). Often, these smaller fibres are regenerating fibres. Image courtesy of Dr Inger Nennesmo.

Figure and legend reprinted by permission from John Wiley & Sons. Publication: Loell I & Lundberg IE. Journal of Internal Medicine. 2011 Mar; 269(3):243-57

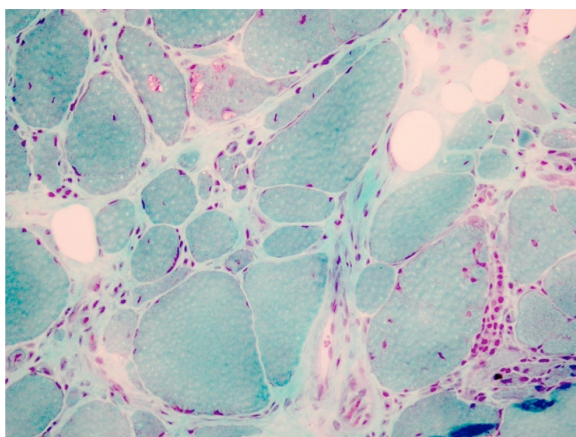


Figure 3: Characteristics of muscle from patients with sporadic inclusion body myositis.

Cross-sectional skeletal muscle biopsy tissue stained with Gomori Trichrome, which allows the visualization of the rimmed vacuoles, the hallmark of sporadic inclusion body myositis. Mononuclear endomysial infiltrates which surrounds and sometime invade muscle fibres, are seen in muscle biopsy section. Fiber sizes vary greatly, among which small fibers may be degenerating and regenerating fibers. (Image courtesy of Dr Inger Nennesmo).

2.1.4 Histopathology of disease subsets

Muscle is the major target organ in the inflammatory myopathies, and distinct histopathological features among different subtypes point towards different disease mechanisms among subtypes. Typically, but not exclusively, DM is characterized by perivascular and perimysial/perifascicular inflammatory infiltrate which is often associated with perifascicular atrophy (**Figure 1 and Figure 2**). Complement and membrane attack complex (MAC) deposition have been detected around the vessels in DM muscle¹⁴. These findings indicate an autoimmune reaction directed against blood vessel leading to microvasculature destruction, followed by various muscle pathologies. On the contrary, PM and sIBM are characterized by endomysial inflammatory infiltrates at multiple foci surrounding muscle fibers (**Figure 1, Figure 2 and Figure 3**), indicating a direct autoimmune attack against the muscle compartment.^{1,10} In all subsets of IIMs, irregular sizes of fibers are observed in cross-sectional area, with many fibers displaying atrophic features (smaller size) (**Figure 2 and Figure 3**). These atrophic fibers often have signs of regeneration, which could reflect chronic degeneration and regeneration processes in these muscles¹³. In sIBM, presence of rimmed-vacuoles is an additional feature (**Figure 3**), along with tiny amyloid deposition in or near those vacuoles, which can be identified by Crystal-violet or Congo-red stains¹⁰.

However, histopathological features of muscle tissue are not always distinct, and many patients display mild or no immune cells/inflammatory infiltrate in muscle biopsies despite pronounced muscle weakness. The absence of immune infiltrates in the muscle biopsies could be due to patchy nature of infiltrate. This problem to a certain extent can be overcome by MRI (magnetic resonance imaging) guided muscle biopsy in the areas with edema, which improve the chance to capture the regions with immune infiltrate^{15,16}. Nevertheless, various findings suggest that both immune and non-immune mechanism could be involved in muscle pathology^{17,18}. Moreover, sIBM muscle show features suggestive of many degenerative phenomena such as unfolded protein response, endoplasmic-reticulum stress, lysosome and mitochondrial abnormalities¹⁹⁻²¹. Although, pathogenic mechanisms in different subtype of IIMs are complex and not clearly understood, the general understanding and proposed immune mechanisms are summarized in **Figure 4**.

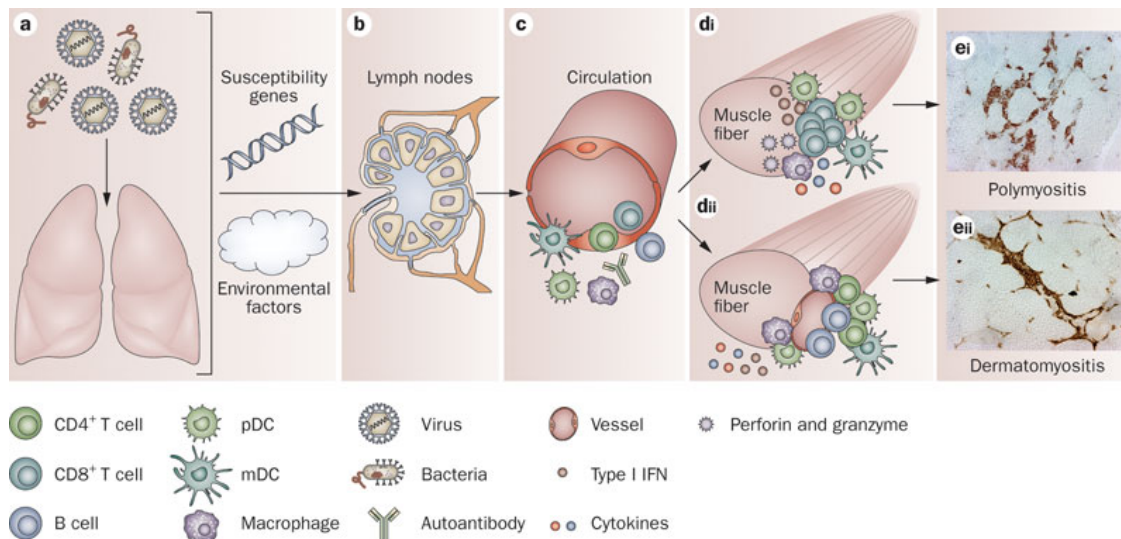


Figure 4: Potential immune mechanisms in myositis pathogenesis. Immune activators, for example, respiratory infection, trigger disease in myositis susceptibility gene carriers. Other environmental factors might contribute. b | Innate and adaptive responses are activated in lymph nodes; macrophages secrete cytokines; mDCs present antigens to T cells; activated T cells assist B-cell activation and proliferation; some B cells differentiate into autoantibody-secreting plasma cells. c | Activated immune cells enter general circulation. Upon encountering specific antigens, immune cells penetrate endothelia, into local muscle. di | In polymyositis (and perhaps sIBM), CD8+ T cells expressing perforin-1 and granzyme invade non-necrotic muscle fibers expressing MHC class I molecules (continuous MHC expression might be induced by pDC-derived type I IFN); mDCs activate T cells, and macrophage-derived cytokines control T-cell homeostasis, activation and proliferation. dii | In dermatomyositis, infiltrates—composed of CD4+ T cells, B cells, DCs and macrophages—usually invade perivascular and perimysial muscle tissue, and might organize into structures resembling lymph node germinal centers. ei | Immunohistochemistry of muscle tissue from a patient with polymyositis, showing CD8+ T cells localized to endomysium. eii | Perivascular localization of CD4+ T cells in muscle tissue of a patient with dermatomyositis, revealed by immunohistochemistry. Abbreviations: IFN, interferon; mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell; sIBM, sporadic inclusion body myositis. (Figure and legend reprinted by permission from Nature Publishing Group. Publication: Zong, M. & Lundberg, I. E. (2011) *Nat Rev Rheumatol.* 2011 May;7(5):297-306)

2.1.5 Role of T cells in IIM pathogenesis

When immune infiltrates are observed in muscle tissue of IIMs, they consist of mainly T cells, macrophages and other immune cells (less frequent). Endomysial mononuclear infiltrates consisting of mainly CD8+ T cells and macrophages which invade HLA class-I expressing non-necrotic muscle fibers, are described as typical of PM and sIBM muscle. While perimysial/perivascular infiltrates consisting mainly of CD4+ T cells with occasional B cells and macrophages are described as typical of DM muscle.²²⁻²⁶ However, above definitions are probably oversimplified in the context of recent findings, where both CD4+ and CD8+ T cells have been reported in endomysial and perimysial infiltrates of all IIM subtypes²⁷.

In addition, several studies describe the presence of cytotoxic and cytolytic molecules in T cells surrounding muscle fibers in IIMs subtypes. The presence of perforin,

granzyme and granulysin have been detected in the T cells which invade muscle fibers^{2,28-31}. Clonally expanded and perforin positive T cells have been detected in both peripheral blood and muscle tissue of patients with IIMs³²⁻³⁴.

The role of T cells in IIMs pathogenesis has also been supported by some recent animal models studies. A murine polymyositis model demonstrated that beta2-microglobulin-null and perforin-null mutant mice display significantly lower number of myositis incidences along with lower muscle inflammation and injury, compared to wild type mice³⁵. The adoptive transfer of CD8+ T cells alone or CD4+ T cells alone, or in combination could induce myositis in naive recipient mice, although the outcome of disease incidence and severity varied depending on the murine models^{35,36}. Murine studies also points towards the role of FoxP3+ regulatory T (Treg) cells in myositis pathogenesis. Recent studies in two different myositis models demonstrate that Treg cells depletion leads to more severe disease, and addition of *in vitro* expanded Treg cells led to disease improvement^{36,37}.

Despite above findings that imply the role of T cells in pathogenesis of myositis, the precise phenotype and functionality of pathogenic T cell subsets are not clearly understood in the setting of human inflammatory myopathies.

2.1.6 Viral triggers

Viruses are known to combat with the immune system and induce interferon productions³⁸. Several viruses are described to be indirectly associated with many chronic and acute myopathy conditions. Specific subtypes of coxsackievirus have been implicated in inflammatory myopathies^{39,40} and RNA from coxsackievirus have also been detected in patients with PM and DM⁴¹. However, this was contradicted by other studies, reporting the absence of any candidate viral genome in the muscle tissue^{42,43}. Additionally, retroviruses such as Human Immunodeficiency Virus (HIV) and Human T-cell Lymphotropic Virus Type 1 (HTLV-1) have been implicated in various chronic and acute myopathies⁴⁴⁻⁴⁷. Infections with retrovirus have been associated with the development of inflammatory myopathies with features of PM and sIBM, in both humans and nonhuman primates⁴⁸⁻⁵². The manifestations of inflammatory myopathies in the retroviral-infected individuals could occur in both early and late stages of infection. More recently, infections with Human Cytomegalovirus (HCMV)^{46,53-57}, Epstein–Barr virus (EBV)^{58,59}, Hepatitis C virus (HCV)⁶⁰⁻⁶⁶ and Hepatitis B virus (HBV)⁶⁷⁻⁶⁹ have also been reported to be associated with the several forms of myopathies. However, the presence of candidate viral genome and viral replication in muscle tissue of myositis patients are controversial^{10,42,54,70}. Nevertheless, association of various viral infections with myopathies suggests that even in absence of viral replication within muscle, chronic viral infection may trigger a persistent inflammatory response, where the virus-specific T cell could play essential role in breakdown of

tolerance towards the muscle compartment. However, the role of viruses and virus-specific T cells in the pathogenesis of IIMs is not clearly understood.

2.1.7 Treatment in IIM subtypes

Due to lack of understanding of disease mechanisms, conventional myositis therapies are largely based on general immunosuppression. There have been very few controlled trials in IIM subtypes. Therefore, the treatment recommendations are largely based on clinical experience and open-label trials^{1,71}. Conventional treatment regimen for PM and DM includes high doses of glucocorticoids 0.75–1.00 mg/kg per day, most often for several weeks. Response to such treatment is seen in many patients, however side effects are common, and the recovery of former physical and muscle functions are rare. Therefore, more recently, many experts recommend combination therapies by including another immunosuppressive/s in order to improve the response and reduce the need for steroids. The frequently used first line drugs for combination therapies are methotrexate at 15-25mg/week or azathioprine at 2mg/kg per day.¹ In case of adverse events, second line drugs are cyclosporin-A, with equal potency as methotrexate⁷² or mycophenylate mofetil based on various case reports^{73,74}. Glucocorticoids interfere with signaling pathways related to immune activation and at high doses, capable of inducing apoptosis in immune cells including T cells^{75 76}. The mechanisms of action of glucocorticoids and specific effects on T cells have been described in section 1.3. Additional immunosuppressives such as methotrexate, azathioprine, cyclosporine-A and mycophenylate mofetil, mainly block the proliferative properties of immune cells⁷⁷. Patients refractory to first line or above treatments are sometime given intravenous immunoglobulins (IVIG)⁷¹. IVIG is derived from large pools of donated human plasma and known to have immunosuppressive effects. However, the response to IVIG treatment is also variable. Some steroid-resistant patients with esophageal involvement show good response, however many patients do not respond to IVIG.⁷⁸⁻⁸¹ In contrast to PM and DM, above-mentioned immunosuppressive treatments usually fails in sIBM patients and currently, there is no effective treatment for sIBM^{10,81,82}.

2.1.8 Treatment resistance and persistence of immune infiltrate in muscle tissue

Ineffectiveness of conventional immunosuppressive therapies in IIMs is also reflected in histopathology, as the inflammatory infiltrates in muscle tissue persist in several patients despite aggressive treatment. The persistent inflammatory infiltrates and inflammatory markers have also been shown to be associated with remaining muscle weakness⁸³⁻⁸⁵.

Although, T cells are implicated in the IIM pathogenesis, whether specific subsets of T cells could persist in IIM muscle despite aggressive immunosuppressive treatment, and may escape apoptotic and immunosuppressive effects of glucocorticoids, is not known. My thesis attempts to address these questions, in the context of conventional immunosuppressive treatments (glucocorticoids alone or combination with additional immunosuppressive drugs). Effects of IVIG are previously studied by our group⁸⁶, and not part of this thesis. Barbasso Helmers and colleagues demonstrated that high doses of IVIG had only limited effects on the molecular markers and immune cells associated with inflammation in muscle tissue, and T cells were present in equal degree before and after IVIG treatment.

2.2 GLUCOCORTICOID EFFECTS

Glucocorticoids belong to a class of steroid hormones, which regulate a variety of important biological functions⁸⁷. Endogenous glucocorticoids exert various kind of immunomodulatory activity including the control of T cell homeostasis⁷⁶. Due to their profound anti-inflammatory and immunosuppressive effect as well as the property to induce lymphocyte apoptosis, synthetic glucocorticoids are among the most commonly prescribed drugs worldwide.

2.2.1 Mechanisms of glucocorticoids action

The effects of glucocorticoids are pleiotropic, dose-dependent and not fully understood. These effects can be either genomic or non-genomic in nature⁸⁸. The genomic effects are largely believed to be responsible for many anti-inflammatory and immunoregulatory effects, however such effects are described to take many hours to many days^{88,89}. Therefore, the immediate therapeutic effects of glucocorticoids are thought to be mediated through non-genomics effects⁹⁰.

2.2.1.1 *Non-genomic effects*

At high doses of glucocorticoids (prednisone-equivalent dose of >30 mg per day)⁹¹, three different mechanisms can lead to non-genomic effects⁸⁸. Non-specific interaction of glucocorticoids with cell membrane or membrane associated proteins, which can hinder the functions of immune cells such as proliferation, migration, phagocytosis and antigen presentation⁸⁹. The specific interaction with glucocorticoid receptors (GCRs), where interaction with membrane-bound GCR can inhibit T cell receptor signaling⁹², or interaction with cytosolic GCR can inhibit arachidonic acid pathway⁹³.

2.2.1.2 *Genomics effects*

Genomic effects of glucocorticoids are better characterized than non-genomic effects. The binding of inactive cGCR with glucocorticoids convert them into active cGCR, which translocate into nucleus and binds to specific DNA binding sites known as glucocorticoid responsive elements. The binding of cGCR-glucocorticoid complex at glucocorticoid responsive elements can either induce or inhibit transcription, the processes known as transactivation or transrepression respectively⁹⁴. Anti-inflammatory effects are believed to occur mostly through transrepression events, which involves interference in binding or functions of transcription factors involved in inflammatory pathways. On the contrary, adverse effects of glucocorticoids are mostly assigned to transactivation events.^{88,95,96}

2.2.2 Immunosuppressive effects of glucocorticoids on T cells

During thymic selection of T cells in mice, glucocorticoid-induced apoptosis is one of the major mechanisms responsible for the negative selection of self-reactive double positive (CD4+CD8+) thymocytes, due to their high sensitivity towards glucocorticoid-induced apoptosis^{87,97}. Mature T cells are considered rather less sensitive to glucocorticoid-induced apoptosis⁹⁸. Nevertheless, murine studies show that glucocorticoids can induce apoptosis in mature T cells also via membrane-bound GCR, however the density of membrane-bound GCR on T cells is critical rather than merely the expression.^{99 100}

Instead, immunosuppressive effects of glucocorticoids on T cells are largely mediated through various other pathways than apoptosis. For example, T cell proliferation upon mitogenic stimuli can be inhibited by glucocorticoids. Glucocorticoids have been shown to interfere with the expression of large number of cytokines, thereby affecting T cell proliferation and functions.⁷⁶ Other effects of glucocorticoids on T cells includes down-regulation of surface adhesion molecules e.g., ICAM-1 and E-selection¹⁰¹; Inhibition of CD40 ligand upregulation on activated CD4+ T cells¹⁰²; Interference with granzyme-B transcription and early T cell Receptor (TCR) signaling events. Most of the data concerning the effects of glucocorticoids on T cells comes from murine studies, so the effects on human cells could vary compared to mice.

2.2.2.1 *Effect of glucocorticoids on different T cell subsets in human*

The role of glucocorticoids in the regulation of human T cell subsets remains controversial. Glucocorticoids induced polarization of naïve T cells towards a Th2 and regulatory T cell phenotype has been reported by some studies^{103,104}. Also, treatment with glucocorticoids has been reported to increase peripheral regulatory T cells and FOXP3 levels in diseases such as asthma and systemic lupus erythematosus (SLE).¹⁰⁵⁻¹⁰⁷ Contrastingly, intra-articular glucocorticoid treatment decreased both the number and the frequency of FOXP3+ regulatory T cells in synovial tissue of rheumatoid arthritis patients¹⁰⁸. Another study describes no effect on regulatory T cells upon oral glucocorticoid treatment in asthmatic patients¹⁰⁹. In general, effects of glucocorticoids on mature and effector human T cell subsets remain largely unclear. Also, it was not known how glucocorticoids based immunosuppressive treatment affects different T cell subsets in IIM muscle tissue.

2.2.3 Adverse effects of glucocorticoids on skeletal muscle and other organs

Prolonged uses of glucocorticoids are associated with several adverse effects such as osteoporosis, weight gain, adrenal suppression leading to steroid-induced Cushing's

syndrome, systemic arterial hypertension, cataracts, metabolic syndromes and catabolic effects on skeletal muscle ¹¹⁰. These adverse effects are dependent on the glucocorticoid dose and the duration of therapy as well as genetic predisposition of patients, which govern the saturation level of glucocorticoid receptors and susceptibility towards these effects ¹¹¹. Importantly, upon prolonged exposure, glucocorticoids may have detrimental effect on the homeostasis of skeletal muscle itself, mainly via inducing catabolic effects and interfering with muscle regeneration process. Such adverse effects could result in exaggerated muscle weakness referred to as ‘steroid myopathy’. The catabolic effects of glucocorticoids on skeletal muscle are mediated largely via affecting two pathways which are linked to regulation of muscle mass, (i) IGF-1-PI3K-Akt pathway, which induces muscle protein synthesis and increase in muscle mass, and (ii) myostatin signaling pathway, which is a negative regulator of muscle mass. Normally, IGF-1 pathway dominantly inhibits the myostatin signaling pathway, however glucocorticoids block the effects of the IGF-1 pathway as well as upregulate catabolic effects of myostatin pathway, leading to a coupled effect on the induction of skeletal muscle catabolism. ^{88,112}

Skeletal muscle holds the capacity to regenerate upon injury, where immune cells play crucial role in muscle repair process (described in section 1.4.5). However, glucocorticoids can negatively affect muscle regeneration process via interfering with differentiation of myoblasts and /or disrupting the immune response involved in muscle repair process ^{88,113,114}.

In conclusion, although glucocorticoids are aimed at dampening the inflammation/immune cell mediated muscle damage in inflammatory myopathies, the adverse effects of glucocorticoids on skeletal muscle itself cannot be ignored. Therefore, development of improved and specific therapies for patients with inflammatory myopathies is highly recommended.

2.3 SKELETAL MUSCLE STRUCTURE, FUNCTION, HOMEOSTASIS AND INTERACTION WITH IMMUNE SYSTEM

2.3.1 Skeletal muscle structure & function

Skeletal muscle constitutes up to 50% of total body mass, making it the largest organ in the human body. Skeletal muscles are the tissue structure specialized to generate mechanical force, and are made up of thousands of skeletal muscle fibers (myofibers), that are bundled together and attached to bones by tendons (**Figure 5**)¹¹⁵. Myofibers are large syncytial (multi-nucleated) cells, generated during development by the fusion of mononucleated myoblasts. Myofibers are covered by a specialized plasma membrane known as sarcolemma, which connects and propagate signals from motor neurons and other external stimuli into muscle fibers. Myofibers are surrounded by a layer of extracellular matrix known as the basement membrane, which constitute both an internal basal lamina and an external reticular lamina¹¹⁶. In the protective niche between sarcolemma and basal lamina, the muscle regenerative cells (satellite cells/muscle stem cells) resides, which can proliferate and differentiate into myoblast helping muscle repair.

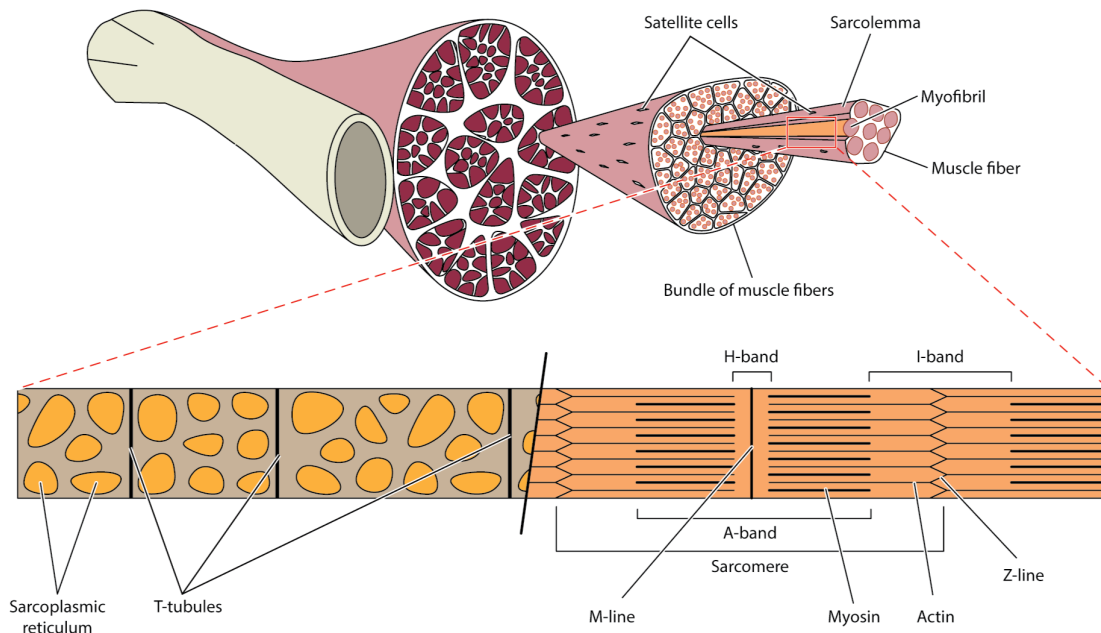


Figure 5. Skeletal muscle structure and composition. Skeletal muscle is composed of numerous bundles of muscle fibers. Each bundle consists of multiple fibers, and individual fibers encompass many myofibrils. Sarcomeres are the structural units of myofibrils and are made up of actin and myosin filaments. Abbreviation: T-tubule, transverse tubule. (Figure and the legend reprinted by permission from Annual Reviews. Publication: Tabebordbar M et al., *Annu. Rev Pathol.* 2013 Jan 24;8:441-75).

The contraction of muscle fibers is calcium-dependent, which is facilitated by a specialized cytoplasm known as sarcoplasm and modified endoplasmic reticulum (ER) known as sarcoplasmic reticulum (SR). The sarcolemma is enclosed with Transverse tubules (T-tubules) facilitating the propagation of action potentials and activation the SR thoroughly. Myofibers contain many myofibrils acting as contraction units and are surrounded by SR. Myofibrils constitute thin myofilaments (actin) and thick myofilaments (myosin) which leads to striated appearance of skeletal muscle in the microscope. Actin filaments make up the light band (I-band), and myosin filaments make up the dark band (A-band) (**Figure 5**). The structure between two Z-lines constitutes one sarcomere, which is the structural unit of the myofibril ¹¹⁷.

The depolarization of the sarcolemma via action potential initiates the muscle contraction process. The depolarization induces opening of sarcoplasmic calcium channels, leading to increase in the intracellular calcium concentration. This triggers the calcium-dependent movement of actin versus myosin filaments leading to contraction of sarcomeres, collectively generating the muscle contraction. The transmission of contraction signals from myofibers to the extracellular matrix and to neighboring myofibers is mediated through protein assemblies known as costameres. ¹¹⁵

2.3.2 Myonuclei and myonuclear domain

Muscle fibers are post-mitotic cells and are unable to divide and replicate. Myonuclei are essential for skeletal muscle homeostasis, maintenance and adaptive responses ¹¹⁸. The theory of myonuclear domain suggests that each skeletal muscle nucleus controls an area of surrounding cytoplasm ¹¹⁹ and supports sufficient protein production in limited area of local “domain” ¹²⁰. Myonuclear domain size is different for fast and slow fiber types due to difference in protein turnover rate and oxidative capacity ¹²¹. Nevertheless, the number of myonuclei can increase or decrease in different situations such as, increase in response to muscle growth, hypertrophy or overload and decrease in case of muscle atrophy. ¹¹⁸.

2.3.3 Muscle injury and cell death pathways

2.3.3.1 Acute and contraction-induced muscle damage

Acute muscle injury can occur due to physical and chemical insults, which can induce rapid myofiber necrosis. Various myotoxins such as in bee or snake venoms mostly disrupts the integrity and function of sarcolemma leading to subsequent necrosis ¹¹⁵. There could be several other causes of acute and contraction-induced muscle injuries, however such mechanisms of muscle damages are beyond the scope of this thesis.

2.3.3.2 *Role of autophagy muscle homeostasis*

In addition to providing mechanical force, skeletal muscles are also one of the most important sites for the control of metabolism in mammals. During catabolic conditions, mobilizations of muscle proteins to liver for gluconeogenesis, functions as the alternative energy source for other organs. In this context, the ubiquitin-proteasome and autophagy-lysosome systems are emerging as the major proteolytic pathways in atrophying muscles ¹²².

The arrangements of various organelles and contractile proteins are different in muscle fibers compared to mononuclear cells. For example mitochondria and sarcoplasmic reticulum and contractile proteins are embedded among the myofibrils in comparison to cytosol localization in other cells. Physical mobility requires enormous energy generated by the muscle mitochondria by production of reactive oxygen species (ROS). The ROS production can have harmful effects on many cellular components. Therefore, muscle cells require an efficient system for the clearance of unfolded proteins, toxic byproducts and dysfunctional organelles. Recent findings indicate that autophagy pathway play important role in muscle homeostasis ¹²².

In autophagy, double membrane vesicles are generated which engulf portion of cytoplasm, organelles, glycogen and protein aggregates ^{123 124}. Autophagosomes are then fused to lysosomes in order to degrade the contents inside. Impaired autophagy either causes accumulation of abnormal organelles and toxic proteins or excessive degradation of muscle proteins, both of these can lead to myofiber degeneration and myopathies ¹²⁵. For example, the accumulation of non-digested autophagosomes forms the morphological structures known as rimmed vacuoles and induces protein aggregation ¹²⁶, similarly to vacuoles in muscle of sIBM patients. A recent study also shows that altered expression of genes involved in autophagy are linked to impaired muscle remodeling in chronic hemiplegic human skeletal muscle ¹²⁷. However, the role of autophagy in the control of muscle cell homeostasis has not been understood substantially.

2.3.3.3 *Apoptosis and necrosis in skeletal muscle fibers*

Apoptosis is one of the most important mechanisms of programmed cell death in mononuclear cells, and is characterized by cytoplasmic and nuclear condensation, DNA fragmentation, and formation of apoptotic bodies and maintenance of an intact plasma membrane during cell death ¹²⁸. An important feature of apoptosis is the rapid clearance of these apoptotic bodies by resident phagocytes, which prevents tissue from harmful inflammatory response. On the contrary, necrosis has been described as uncontrolled release of cellular components due to processes such as swelling and membrane

breakdown, leading to inflammatory response associated with macrophage activity. In the absence of phagocytosis, apoptotic bodies could proceed towards lysis, which is termed as secondary or apoptotic necrosis. The loss of membrane integrity in other processes of cells death such pyroptosis and oncosis could also leads to necrosis ¹²⁸.

In multinuclear cells such as skeletal muscle fiber, presence of apoptosis is less evident and various data are conflicting and unclear. According to some reports, apoptosis generally affects single nuclei rather than inducing the death of the entire myofiber ¹²⁹. Such process has been named as nuclear apoptosis and is suggested to have negative effects on gene expression in the myonuclear domain. In contrast, other reports indicate that myogenic cells such as satellite cells and myoblasts are more sensitive to oxidative stress and pro-apoptotic factors compared to well-differentiated cells, such as myotubes and myofibers ¹³⁰⁻¹³².

While, light microscopic examination reveals muscle fiber necrosis in many disease states, apoptotic cell death is much less evident ¹³³. Although it is not fully understood, autophagy seems to play important role in muscle homeostasis in normal conditions as described above, while the muscle fiber necrosis is mostly indicative of pathological state ¹³⁴. The role of apoptosis in skeletal muscle homeostasis is unclear and whether apoptosis plays any role in pathogenesis of inflammatory myopathies, is not known.

2.3.3.4 Effect of age and systemic environment on skeletal muscle

Studies with single fiber and in vitro motility assays indicate that part of the muscle dysfunction in aged individuals is due to increased post-translational modifications in proteins such as myosin ¹³⁵. The chance of post-translational modifications of proteins increases due to lower protein turnover rates. Also, the impaired regenerative capacity of old muscles has been linked with lower differentiation capacity of satellite cells. Interestingly, the satellite cells from older individual display improved myogenic capacity in the presence of serum from young individuals, indicating the importance of systemic environment in muscle regeneration ¹³⁶. It has also been shown that systemic inflammation and cytokines such as TNF not only suppresses satellite cell differentiation but also induces apoptosis in satellite cells ¹³⁷. Therefore, systemic inflammation such as in patients with PM and DM, and age related factors could negatively affect muscle regeneration process. ¹³⁸

2.3.4 Skeletal muscle cell death in inflammatory myopathies

Necrosis has been reported as cell death mechanism in inflammatory myopathies by a number of studies so far. Although, in PM and sIBM, classical histopathological picture shows the presence of CD8+ T cells attacking the muscle fibers, and therefore one

would expect to see signs of apoptotic cell death in muscle fibers. However, majority of studies do not find any evidence of apoptosis in the muscle fibers of patients with inflammatory myopathies^{132,133,139-144}. There are some contradictory findings, where it is often difficult to distinguish TUNEL staining in myonuclei compared to staining in infiltrating cells in those studies¹⁴⁵ and the positive TUNEL staining could belong to infiltrating cells.

Also, there are contradictory data regarding expression of apoptosis related factors in myositis muscle tissue. Although, expression and upregulation of pro-apoptotic factors such as Fas have been reported in myositis muscle tissue, the co-expression of anti-apoptotic molecules such as Bcl-2, FLICE (Fas-associated death domain-like IL-1-converting enzyme)-inhibitory protein (FLIP) and IAP (inhibitor of apoptosis proteins) explain the apoptosis resistant nature of myofibers in inflammatory myopathies^{132,139,140,143,145-147}.

Proinflammatory cytokines such as TNF, IL-1 β , and IFN γ induce autocrine nitric oxide (NO) production in cultured muscle cells, leading to oxidative damage¹⁴⁸. Indeed, muscle fibers in myositis display increased levels of inducible and neuronal nitric oxide synthase (NOS)¹⁴⁹. The NO induced oxidative stress may not only induce muscle fiber necrosis but may also lead to decreased ATP generation and contractile function¹⁵⁰. In addition, recent findings indicate that impaired autophagy could also play a role in the pathogenesis of myositis, if not in all, at least in some subtypes^{1,19,21,151,152}, which could be linked to ER stress¹. However, the exact mechanisms (immune or non-immune) that are primarily responsible for the pathogenesis of inflammatory myopathies remain unknown.

2.3.5 Muscle tissue repair/regeneration and role of immune system

Muscle fibers, like many eukaryotic cells, have the capacity to repair minor membrane damages using membrane patch. Defects in muscle membrane repair lead to various forms of myopathies, for example, mutation in dysferlin is linked to limb-girdle muscular dystrophy type 2B and Miyoshi myopathy¹⁵³. In inflammatory myopathies too, the leakage of creatine kinase (CK) indicate muscle membrane damage and/or impairment in membrane repair process. However, detailed mechanism and role of the other proteins in muscle membrane repair is poorly understood and out of the scope of this thesis.

In most instances post injury, skeletal muscle repair is mediated via satellite cell muscle regeneration and remodelling processes^{13,115,154}. Satellite cells are unipotent adult muscle stem cells which proliferate and differentiate into myoblasts in response to muscle damage. The myoblasts fuse with damaged myofibers or with one another to

generate new myofibers. Satellite cells contain self-renewal capacity which maintain themselves in the muscle tissue throughout lifespan in order to help muscle repair ¹⁵⁵.

Satellite cells normally are in a mitotically and metabolically quiescent state, however, become activated in response to injury or chronic muscle degeneration. Muscle tissue damage leads to release of various growth factors and cytokines from extracellular matrix and neighboring endothelial, interstitial and immune cells that trigger the proliferation of satellite cells ^{156,157}. In addition to tissue growth factors and cytokines, the infiltrating immune cells and resident fibro-adipogenic precursors (FAPs) also affect the outcome of satellite cell muscle regeneration process. The regenerative capacity of satellite cells is impaired in the absence of infiltrating immune cells ¹⁵⁸, indicating the essential roles of immune cells in muscle repair. However inappropriate or excessive immune infiltration could adversely affect the regeneration process. Among immune cells, neutrophils are recruited first, followed by M1 macrophages and thereafter M2 macrophages ¹⁵⁹. With the interaction with neutrophils, M1 macrophages can further promote muscle damage by producing of high levels of NO and inflammatory cytokines ¹⁶⁰. In contrast, M2 macrophages are involved in tissue repair and are described to be anti-inflammatory. Both neutrophils and macrophages are described to participate in clearance of cellular debris after injury and inflammatory damage. The M2 macrophages in particular induce satellite cell activation and proliferation and further regulate muscle cell regeneration and remodeling ¹⁶¹.

2.3.6 Difference in muscle inflammation between acute and chronic muscle injuries: Breaking of self-tolerance and role of T cells

In response to acute muscle injury, usually a stereotypical inflammatory response is followed by repair process, as described above. In contrast, chronic muscle damage leads to a rather different and more complex process, which is relatively less understood ¹⁶¹. It is believed that the major difference lies in the breakdown of tolerance towards muscle compartment in chronic muscle injuries, which is very unlikely in acute injuries. Most knowledge about muscle-immune system interaction in chronic muscle damage arises from studies on *mdx* mouse model. The *mdx* mouse model is a commonly used animal model of Duchenne's Muscular Dystrophy (DMD). Similar to DMD in human, *mdx* mice have loss of function mutation in dystrophin gene, which leads to progressive muscle degeneration, necrosis and chronic muscle inflammation ¹⁶². Dystrophin is a rod shaped cytoplasmic protein and vital part of protein complex costamere involved in maintaining the integrity of muscle.

The reduction of *mdx* pathology upon depletion of CD4+ and CD8+ T cells and in the perforin mutant mice, and the transfer of muscle pathology from *mdx* mice to healthy mice by adoptive transfer of muscle primed immune cells, collectively demonstrates the significant role of both CD8+ and CD4+ T cells in chronic muscle destruction

^{163,164}. Also, as described in IIM section (1.2.5), both human and murine studies point towards pathogenic role of T cells in inflammatory myopathies.

However, it is not clear, how this breakage of tolerance occurs in patients with myositis, where skeletal muscles are considered normal regarding any genetic defect. Which subsets of CD4+ and CD8+ T cells could play role towards breakage of tolerance and muscle damage in myositis? The studies included in this thesis aim to characterize precise phenotype and functionality of T cell subsets, which could lead to chronic inflammation in muscle tissue.

2.3.7 Impairment of muscle regeneration in chronic muscular degeneration

Chronic inflammation or chronic muscular degeneration may lead to satellite cell exhaustion, thereby impairing the regeneration process. The reason for this exhaustion is most likely due to proliferation-induced telomere shortening and damage associated cell attrition ^{165,166}. A faulty muscle regeneration process usually leads to fibrosis and fat tissue accumulation, which further impairs the regeneration process ¹⁶⁷. In fact, signs of muscle fiber degeneration and regeneration, fibrosis and fat tissue accumulation are commonly seen in myositis ¹³. However, it is not understood, whether a major defect lies in muscle regeneration or immune mediated muscle damage in inflammatory myopathies.

2.3.8 Myotoxic factors in immune system

2.3.8.1 Granzyme-B

Granzymes belong to a family of conserved serine proteases, which are usually stored within cytotoxic granules of cytotoxic lymphocytes and known to be involved in immune cell mediated targeted cell death ¹⁶⁸. There are 11 granzymes in mice and 5 granzymes in humans known so far, among which granzymes-B (GrB) is one of the most abundant and most studied subtype. Previously believed only to be expressed by Natural Killer cells (NK cells) and CD8+ T cells, recent findings show that GrB can be expressed by many other cell types too such as CD4+ T cells, regulatory T cells, neutrophils, basophils, mast cells, macrophages, dendritic cells and many non-immune cells ¹⁶⁸. The protease inhibitor-9 is the only known endogenous inhibitor of GrB, which is expressed mainly by immune cells in order to prevent accidental GrB mediated apoptotic cell death ¹⁶⁹. Conventionally, GrB is known for its involvement in perforin-dependent intracellular apoptosis in target cells. However, recently it has been shown that GrB could also have many extracellular, perforin independent and apoptosis independent roles ¹⁶⁸.

2.3.8.2 *Perforin and its role in granzyme entry into target cells*

It is well established that in cytotoxic lymphocytes, GrB is released towards target cells along with perforin via the polarization of cytotoxic granules in the direction of immunological synapse¹⁷⁰. Perforin is a calcium dependent pore forming protein which can form 5-20nm pores in plasma membrane upon multimerization¹⁷¹. Traditionally, it was believed that perforin directly facilitates the entry of granzymes into target cells by formation of pores in target cell membrane. However, recent models suggest that GrB may be endocytosed by the target cells independent of perforin. Thereafter, GrB is released from endosomes into cytosol in perforin-dependent mechanism and leads to initiation of apoptosis cascade¹⁷²⁻¹⁷⁴. So far, a role of perforin beyond granzyme entry facilitation is not known. Perforin deficiency is associated with impaired lymphocyte cytotoxicity in both human and mice, which confirms the role of perforin in cytotoxic lymphocytes-induced granzyme mediated apoptosis.¹⁶⁸

In the context of perforin-granzyme mediated cell death in muscle fibers, the role of perforin in muscle fiber death has been confirmed by several murine studies^{35,163}. Although, classical apoptosis pathways may be rare or absent in muscle fibers, based on the histological evidences in myositis, it appears that perforin-granzyme could lead to direct necrosis and additional cell death pathways in muscle fibers. However, such mechanisms need to be confirmed by future studies.

2.3.8.3 *Extracellular granzyme-B activities.*

Recently, several groups have reported the presence of GrB in various extracellular matrix (ECM) and body fluids¹⁶⁸. The reported median levels of GrB in the plasma of healthy individuals is 20-40 pg/ml^{175,176}, whereas increased levels of extracellular GrB have been reported in inflamed tissue of various chronic inflammatory conditions such as synovial fluid in rheumatoid arthritis, bronchoalveolar lavage (BAL) in chronic obstructive pulmonary disease (COPD) and lung inflammation, and the cerebrospinal fluid in multiple sclerosis^{177,178}. GrB is suggested to also have various extracellular functions such as cleavage of ECM proteins, which could be linked to various pathologies. The mechanism responsible for higher levels of extracellular GrB is not fully understood, whether it is leaked from immunological synapse spaces or released systemically in response to other stimuli.

2.3.8.4 *Role of granzyme-B in myositis:*

The presence of GrB and perforin expressing cells in the endomysial site of myositis patients suggest a role of GrB in muscle fiber damage and necrosis³¹. In addition, GrB could promote autoantibody production against certain proteins such as histidyl-transfer RNA synthetase and PMS-1, by exposing immunogenic epitopes of autoantigen upon cleavage¹⁷⁹⁻¹⁸². Interestingly, GrB specific cleavage occurs mainly in cases where protein conformation is altered compare to normal. These findings indicate potential role of GrB in regulation of many events *in vivo* such as cell signaling, cell death pathways and tissue homeostasis.

2.3.8.5 *Effect of TNF on muscle damage and repair*

The effect of TNF in muscle inflammation and repair is complex. Impairment of muscle repair in TNF knockout mice point towards an essential role of TNF in muscle regeneration and homeostasis^{183,184}. However, TNF has also been implicated in inducing muscle damage and inhibiting muscle repair process through various mechanisms.¹⁶¹ Several findings demonstrate that TNF effects on muscle cells are mediated mainly via induction of NF- κ B expression^{185,186}. NF- κ B induction in both macrophages and muscle cells directly or indirectly affects muscle cell proliferation and differentiation. In most instances, NF- κ B activation increases proliferation and inhibits differentiation of muscle cells. TNF is also known to induce NO productions by myeloid cells and other immune cells, which can cause muscle fiber damage¹⁸⁴. However, dual effects of TNF on muscle damage and repair processes, could explain the failure of TNF inhibitors in treatment refractory patients with IIMs¹⁸⁷.

2.3.9 Role of skeletal muscle in immune regulation

Muscle tissue is the site for various immunological reactions, which could partly be due to role of immune system in muscle repair. However, other functions could also be possible. Muscle fibers and cultured muscle cells not only express immunological molecules such as HLA (human leukocyte antigen) class I and class II, cytokine and chemokine receptors, co-stimulatory molecules, but also are potent secretor of various cytokines, chemokines and MMPs (matrix metalloproteinases). Muscle cells are described to be facultative antigen presenting cells under certain conditions (for example inflammation). However, the expression of various immune molecules and functions could vary between healthy and disease state. Muscle cells are also reported to express the immuno-protective molecules HLA-G and B7-H1, which can help to control excessive inflammation in muscle tissue¹⁸⁸. Looking at prominent immunological and cytokine secretion capabilities, muscle compartment is likely to play an active role in immune system homeostasis rather than being a passive target of

it. Evidences from anti-inflammatory effects of exercise strongly suggest such mechanisms¹⁸⁹⁻¹⁹¹. Therefore, muscle-immune interaction appears to be two ways regulated process, where both systems could play role in homeostasis of each other. Dysregulation in such interaction could manifest in various pathologies, however such mechanisms are not substantially understood so far.

2.3.10 Muscle cells in culture: Myoblasts and myotubes

From the fresh muscle biopsies specimens (both human and rodents), satellite cells can be extracted and expanded into myoblasts *in vitro*¹⁹². Myoblasts are muscle precursor cells, which by fusion, give rise to muscle fibers *in vivo* and myotubes *in vitro*¹⁹³. In culture, myoblasts can be identified by muscle cells specific protein such as desmin and also by surface expression of neural-cell adhesion molecule (NCAM, also known as CD56). Desmin is an essential muscle cytoskeletal protein and one of the early markers for muscle lineage. NCAM is expressed on various immune cells also such as NK cells and T cells. Nevertheless, NCAM can serve as myoblast specific extracellular marker among the cells, which are grown from muscle biopsy sample and may contain non-myogenic cells such as fibroblasts. When cultured myoblasts reaches high confluency (80%), myotubes can be generated by fusion of myoblasts by adding fusion-supporting medium (such as low serum medium).^{194 195}

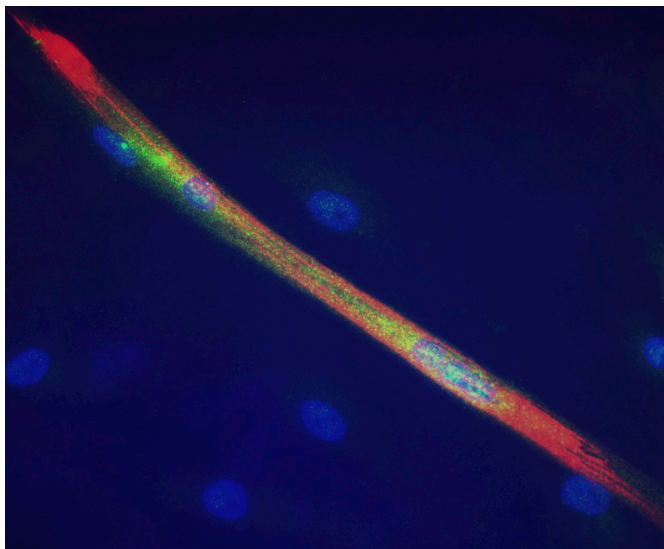


Figure 6: Expression of myosin in a cultured human myotube.

A multinucleated cultured myotube displaying the expression of both slow (green) and fast (red) myosin heavy chain proteins. The myotube was obtained by the fusion of human myoblasts. Expression of myosin heavy chain is very low or none in single nucleated myoblasts. DAPI (Blue).

In contrast to mononucleated myoblasts, myotubes are multinucleated post-mitotic syncytial cells similar to skeletal myofibers *in vivo*. Myotubes express many skeletal muscle specific structural, functional and transcriptional proteins that are seen in skeletal myofibers *in vivo*, therefore resemble more closely to skeletal myofibers than myoblasts¹⁹⁶⁻¹⁹⁸. Myotubes can be identified by the expression of myosin heavy chain (MyHC), an abundantly expressed structural and functional protein in culture **(Figure 6)**.

Both, myotubes and myoblast are described to possess immunological capabilities. Although, myoblasts have widely been used as an *in vitro* system to investigate the immunobiological properties of skeletal muscle cells, their usefulness is limited due to different properties than myofibers *in vivo*. For example, antigenic epitopes expressed by myoblasts are not seen on muscle fiber. Myoblasts also behave differently to various stimuli such as cytokines than myofibers.¹⁸⁸ Therefore, myotubes can serve as better system to understand muscle immune interaction.

The mechanisms of cell death in cultured muscle cells can also depend on their differentiation stage, whether they are in form of myoblasts and myotubes. Like *in vivo* myofibers, cultured myotubes are relatively resistant to apoptosis compared to myoblasts, which could be due to their multinucleated nature, and up-regulation of anti-apoptotic molecules such as FLIC (Fas-associated death domain-like IL-1- converting enzyme) and anti-caspase mechanisms^{129-132,199,200}. Some of the classical apoptotic features may be absent in cultured myotubes due to apoptosis resistant properties, while necrosis can be observed upon extended exposure of cytotoxic agents^{199,201}. Although, allogenic and *in vitro* expanded T cell clone mediated cultured myotube and myoblast death were shown in *in vitro* settings, such T cell cytotoxicity are shown using Chromium release assays indicating overall lysis (membrane damage) of muscle cells, where in depth mechanisms of muscle death were not described^{202,203}.

2.4 FOXP3+ REGULATORY T CELLS

FOXP3+ regulatory T (Treg) cells are naturally occurring regulatory T cells in the immune system, which maintains dominant self-tolerance and immune homeostasis²⁰⁴. The dysfunction/deletion of such cells leads to impaired immune regulation in both human and mice, causing severe autoimmune diseases, immunopathology and allergy. For example, the loss of function mutation in FOXP3 leads to IPEX (polyendocrinopathy enteropathy X-linked syndrome) in human and Scurfy phenotype (fatal lymphoproliferative disease, causing death at around 4 weeks of age) in mice. Among FOXP3+ T cells, most cells are CD4+ and among them CD25^{high} cells (CD4+FOXP3+CD25^{high} T cells) can suppress activation, proliferation, effector functions and antigen presentation functions of wide range of immune cells such as CD4+ T cells, CD8+ T cells, B cells, Natural Killer (NK) cells and dendritic cells²⁰⁵. In contrast to some T cell subsets, which may acquire regulatory function, natural occurring CD4+FOXP3+ Treg cells are developmentally determined in thymus to become a specialized T cell subset to exert suppressive functions. Recent studies have shown that human CD4+FOXP3+ T cells are not a homogenous population in terms of their phenotype, gene expression and mechanisms of suppression. Therefore, molecular features, phenotype and functions of Treg cell subsets, is an intense field of research presently.²⁰⁴ Unless stated otherwise, Treg cells in this thesis refer to FOXP3+ regulatory T cells.

2.4.1 Role of FOXP3+ regulatory T cells in skeletal muscle regeneration

The role of Treg cells in muscle homeostasis and repair was largely unknown, until a recent study by Dalia Burzyn and her colleagues²⁰⁶. Burzyn et al have demonstrated that, in mice models of both acute and chronic muscle injury, a distinct population of Foxp3+CD4+ Treg cells play important role in muscle repair and maintenance of self-tolerance towards muscle compartment. The depletion of Treg cells not only prolongs the inflammatory phase but also impairs the repair process. This study suggests that Treg cells assist the muscle repair process by (i) regulating myeloid cell population by promoting a macrophage phenotype switch from proinflammatory (M1) to anti-inflammatory (M2), (ii) limiting the infiltration of other T subsets (potentially proinflammatory) in muscle tissue, and (iii) also directly promoting the regenerative potential of satellite cells through secretion of a growth factor called Amphiregulin which enhances satellite cell differentiation. Also, as described above in section 1.2.5, Treg depletion in mice models of myositis leads to increased muscle damage and severe disease^{36,37}. However, the histopathological and functional aspects of Treg cells in patients with inflammatory myopathies, is largely unknown. Whether Treg cells are present in adequate numbers to support the muscle repair in IIM muscle tissue? How does the conventional immunosuppressive treatment affect Treg cells, particularly in muscle tissue? Whether Treg cells in patients with IIMs are able to efficiently suppress autoreactive and pathogenic T cells? Whether Treg cells are defective in patients with

IIMs or autoreactive T cells are resistant to Treg mediated immunosuppression? Some of these questions are addressed in my thesis.

2.5 CD28null T CELLS

2.5.1 Definition CD28null T cells

CD28null T cells are highly differentiated T cells lacking the co-stimulatory molecule CD28, in both CD4+ and CD8+ compartment. In the literature, such T cells have been studied based on being CD28⁻ T cells (also written as CD28(-) T cells), and also as terminally differentiated T cells, effector-memory T cells, Tissue-resident memory T cells, co-stimulation independent T cells, etc. Up to what extent, T cells defined by these terms overlap with each other and with CD28null T cells, remains to be clarified. We have preferred to use CD28null term over CD28⁻ or other terms mainly because we want to emphasize on well-differentiated T cells lacking CD28, rather than T cells which may temporarily downregulate surface expression of CD28 during stimulation. The differentiation status of CD28null T cells could vary between CD4 and CD8 compartment. Based on telomere length, it appears that CD28null T cells in CD4 compartment are terminally differentiated T cells (**Figure 7**), whereas in CD8 compartment, CD28null T cells contain both late stage and terminally differentiated T cells (**Figure 8**). Compared to conventional CD28-expressing T cells, CD28null T cells are characterized by the acquired expression of receptors commonly associated with natural killer cells, and also by acquired proinflammatory (IFN γ and TNF secretion), cytotoxic (contain stored perforin and granzymes even in resting state) and apoptosis resistant (upregulation of anti-apoptotic molecules) features.

Note: **Figure 7** and **Figure 8** also relate to the **section 1.6.3** and **section 1.6.5**

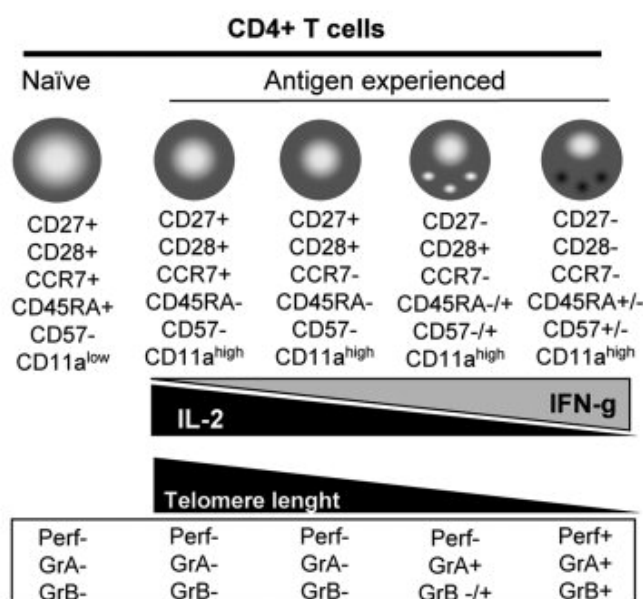


Figure 7. Phenotypic dissection of human CD4 T cells into functionally distinct subsets.

The expression of markers, commonly used to define CD8 T cell subsets, enables also the distinction between several CD4 T cell subpopulations, including CD4 cytotoxic T cells. (*Figure and legend reprinted by permission from John Wiley and Sons. Publication: Appay V et al., Cytometry A. 2008 Nov;73(11):975-83*)

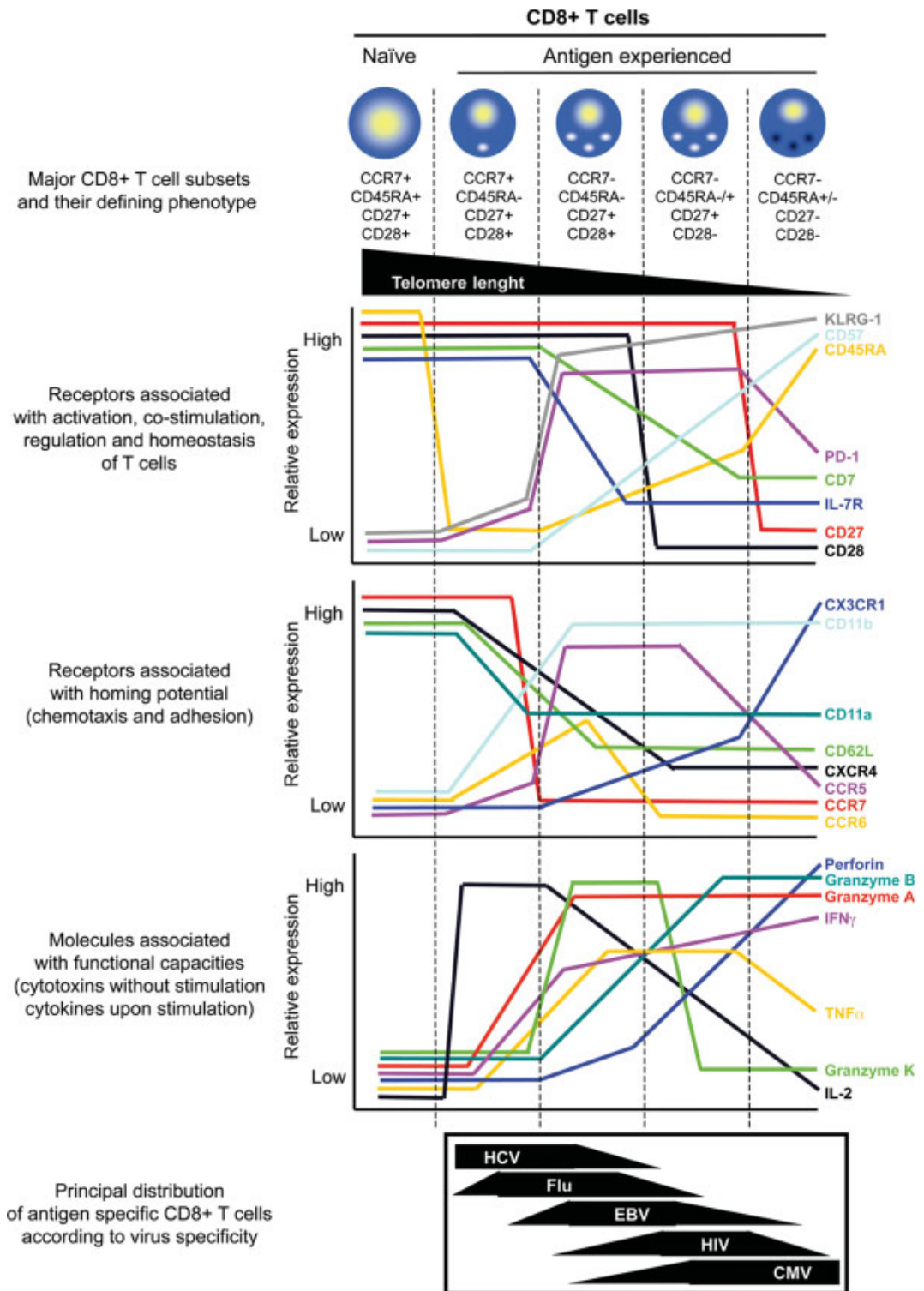


Figure 8. Phenotypic associations within CD8 T cell subsets in humans and relationship with functional attributes. Five distinct subsets of circulating CD8 T cells are defined according to the expression of CD27, CD28, CCR7, and CD45RA. Relative telomere length and expression of a variety of cell surface receptors and intracellular molecules (related to T cell activation, costimulation, regulation, homeostasis, homing potential, and functional capacities) are illustrated in these subsets in a “resting” state according to data from the literature. The common phenotypic distribution of virus specific CD8 T cells is also depicted, after clearance of the virus (Flu) or in latent infection stages (for HCV, EBV, HIV, and CMV). (Figure and legend reprinted by permission from John Wiley and Sons. Publication: Appay V et al., *Cytometry A*. 2008 Nov;73(11):975-83)

2.5.2 Aging and CD28 co-receptor on T cells

It is evident that there is a decline in effectiveness of the immune system with age, particularly in later decades of the human lifespan²⁰⁷. The decrease in the competency of adaptive immune system is mostly assigned to decrease in thymic and bone marrow output of lymphocytes as well as the expansion of memory lymphocytes²⁰⁸. Due to such effects, the resulting T cell repertoire becomes less diverse and displays an altered phenotype. Among various factors, which comprise the phenotypic shift in the T cell system with age, accumulation of CD28null (CD28⁻) T cells is one of most profound and consistent phenomenon.^{209,210}

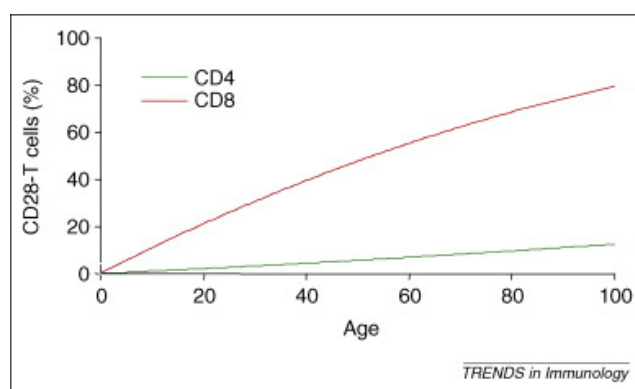


Figure-9 Age-associated accumulation of CD28⁻ T cells. A schematic presentation of the accumulation of CD28⁻ T cells with age for both CD4 and CD8 T cells. The ordinate indicates the frequency of CD28⁻ CD4 or CD28⁻ CD8 T cells. CD28⁻ CD8 T cells show a much more dramatic increase in frequency with age compared with CD28⁻ CD4 T cells.

(Figure and legend reprinted by permission from Elsevier. Publication: Weng et al, Trends in Immunology, Volume 30, Issue 7, 2009, 306 - 312)

The gradual decline of CD28 co-receptor expression is seen in both CD4⁺ and CD8⁺ T cell subsets with age^{207,211} (**Figure-9**). However, this decline is much rapid in CD8⁺ T cell population with 50-60% lacking the expression of CD28 in peripheral blood at the age of 80 years and above. In comparison, CD28 decline on CD4⁺ T cells is less, with only 10-15% of T cells turn negative for CD28 until the age of 80 years²¹¹.

Interestingly, the age related decline in CD28 expression is seen in both human and nonhuman primates but not in mice²¹². Whether the reason for this distinction lies in the prolonged interactions of the immune system with microorganism during the longer life span of primates and humans, or this is an inherent phenomenon of their immune system, remains to be explored.

2.5.3 Co-stimulatory receptors on CD28null T cells

There are mainly five members among CD28 receptor family i.e. CD28, CTLA-4 (CD152), inducible co-stimulator (ICOS), program death-1 (PD-1) and B- and T-lymphocyte attenuator (BTLA)²¹³. Surface expression of CTLA-4, ICOS and BTLA is usually not detected/reported on CD28null T cells in resting state, however, upon activation CD28null T cells are reported to express high levels of CTLA-4 on their surface^{214,215}. Expression of PD-1 is also reported in CD28null T cells (**Figure 8**). PD-1 and CTLA-4 are generally considered inhibitory receptors linked to T cell exhaustion²¹⁶⁻²¹⁸, however the expression of these receptors alone does not indicate that a T cell is exhausted, and despite the expression of these receptors, human T cells are reported to exhibit functional activity^{219,220}. CD28null T cells are also negative for the co-stimulatory molecule CD27²²¹. Besides CD28 family receptors, NK receptors can also function as co-stimulatory molecules in CD28null T cells, which is summarized in section 1.6.5.1.

2.5.4 Origins of CD28null T cells

The restricted T cell receptor repertoire and shortened telomeres in CD28null T cells indicate that these cells are originated from repeated division of CD28+ T cells²²²⁻²²⁵. Repeated division occurs mostly due to antigenic stimulation of TCR, however other mechanisms can also be in place such as cytokine-mediated homeostatic proliferation. IL-7 and IL-15, which are important for homeostatic maintenance of CD8+ T cells in the absence of antigenic stimulation, accelerate the loss of CD28 on the surface^{226,227}. During TCR stimulation, the presence of type-I interferons (IFN α and IFN β) is also known to accelerate the loss of CD28 via telomere erosion^{226,228,229}. These findings suggest that CD28 loss occur in chronic stimulatory and proinflammatory environment, which may lead to generation of terminally differentiated CD28null T cells.

During normal antigenic stimulation, surface expression of CD28 on T cells decreases but is rapidly restored to the same level as before. Therefore, CD28 down-regulation is interpreted as negative feedback mechanism to prevent excessive T cell stimulation²³⁰. However, during continuous T cell stimulation and turnover, CD28 expression is reduced and lost eventually. Initially, CD28 loss can be restored by IL-12²³¹, however, at later stages, CD28 loss is irreversible, suggesting transcriptional silencing of CD28 expression in CD28null T cells. Indeed, studies point towards defects in transcriptional activation of CD28 in CD28null T cells, due to lack of the protein complexes required for CD28 transcription initiation²³²⁻²³⁴. Such protein complexes are rapidly lost in CD8+ T cells upon proliferation but are rather persistent in CD4+ T cells explaining the relative resistance of CD4+ T cells regarding CD28 loss²²⁴. The factors known to accelerate the loss of CD28 or the factors that can restore CD28 expression, both

appears to act on transcriptional assembly level. However, how the transient repression is permanently imprinted, is not known.

2.5.5 Molecular and functional features of CD28null T cells

Although, CD28 is critically important for naive T cell activation, proliferation and survival^{213,235}, the systematic loss of CD28 on T cells is associated with additional features. CD28null T cells acquire expression of additional receptors and functions, and despite the loss of CD28 on surface, have enhanced cytotoxicity and enhanced activation potential, although antigen-induced proliferation response appears to be impaired²³⁶. CD28null T cells also display reduced T cell receptor diversity, a sign of repeated antigenic stimulation and clonal expansion^{237,238}.

CD28null T cells are heterogeneous, and contain different subsets based on the expression of CD45RA, CD27 and other markers^{221,239,240}. Also, the differentiation path for CD4+CD28null and CD8+CD28null T cells appears to vary. Molecular and functional features of CD28null T cells are not fully understood, and also, there are consensus issues regarding definitions of various T cell subsets. Nevertheless, the current understanding of T cell subsets, in the context of surface expression of CD28 is summarized in **Figure-7** for CD4+ T cells subsets, and in **Figure-8** for CD8+ T cell subsets²³⁹.

Gene expression analysis studies showed that the expression of a small set of genes alters between CD28null and CD28+ T cells²⁴¹⁻²⁴³. Interestingly, the molecular and functional features are shared greatly between the CD28null T cells of CD4 and CD8 compartment²⁴⁴. In general, there are four major features, which vary in CD28null T cells compared to CD28+ T cells. The altered expression of CD28 receptor family member is one feature, already described above in section 1.6.3. Other features are described below:

2.5.5.1 Natural Killer cell related receptors

The acquired expression of various receptors, which are commonly expressed on NK cells, is another feature of CD28null T cells. These receptors include CD57, CD244 (2B4), DNAM-1 (CD226), CRACC, NKG2A, NKG2C (CD94/KLRD1), NKG2D, NCR1 (CD355), KIR2DL2, KIR3DL2, KIR2DS2, KLRK1, KLRF1 etc.²⁴⁵⁻²⁴⁹. Expression of various NK receptors indicates that CD28null T cells are capable of NK cell like functions such as cytotoxic killing. Several studies indicate that the final outcome of CD28null T cell cytotoxicity could be regulated by stimulatory and inhibitory KIRs (killer-cell immunoglobulin like receptors) on CD28null T cells^{245,246,249-252}. The activating NK receptors can serve as co-stimulatory receptors on CD28null T cells and could lower the threshold for antigen-specific stimulation. Indeed, a study in our group demonstrate that pair-wise ligation of CD244 with

DNAM-1 and/or NKG2D lead to increased magnitude of activation and effector functions of primary CD4+CD28null T cells from rheumatoid arthritis patients ²⁴⁵.

2.5.5.2 Chemokines, cytokines, and adhesion receptors

The expression of receptors involved in homing, adhesion and cytokine binding on CD28null T cells appears to vary depending on their tissue location, disease condition, and is not adequately understood. Nevertheless, the prominent upregulation of CX3CR1 (Fractalkine receptor) and CD11b, as well as down-regulation of CCR7 is seen on CD28null T cells in both CD8+ and CD4+ compartment ^{239,253-255}. Fractalkine is a chemokine when secreted, as well as an adhesion molecule when membrane bound, and facilitates migration of CX3CR1-expressing lymphocytes into the tissue ²⁵⁶. Interestingly, fractalkine is shown to be involved in the pathogenesis of various diseases such as atherosclerosis, multiple sclerosis, glomerulonephritis and rheumatoid arthritis ²⁵⁷⁻²⁶⁰. The lack of CCR7 expression define that CD28null T cells are not the central memory T cells, but could belong to effector memory and/or tissue resident memory T cells. Interestingly, CD4+CD28null T cells from synovial fluid express enhanced expression CXCR3 and CCR6 compared to peripheral blood in patients with rheumatoid arthritis ²⁶¹. The CXCR3 expression is a feature of Th1 cells, while CCR6 is expressed on the Th17 subset. Expression of both CXCR3 and CCR6 defines a Th1 population, which can secrete both IFN γ and IL-17 ^{262,263}.

CD28null T cells also express high level of LFA-1 (Lymphocyte Function-Associated Antigen 1) adhesion molecule ²³⁶. LFA-1 interacts with its receptor ICAM-1 (Intercellular Adhesion Molecule-1) on various cells. Interestingly, LFA-1 on T cells is also known to lower the T cell activation threshold, which could predispose these cells to become autoreactive ²⁶⁴. Regarding cytokine receptors, CD28null T cells are reported to express lower levels of IL-7 receptor ^{239,265}, however, expression pattern of other cytokine receptors remain largely unknown.

2.5.5.3 Cytokines and effector molecules

CD28null T cells are reported to express elevated levels of cytolytic molecules such as perforin, granzyme-B, granzyme-A and granzyme-H, even in resting state ^{266,267}. In CD8+CD28+ T cells, perforin and granzyme-B levels are very low and increased only after activation, whereas granzyme-H levels are low both in resting and activated stage. On the contrary, higher expression of both granzyme-B and granzyme-H is a cytolytic feature, which is shared by both NK cells and CD28null T cells. In addition, CD28null T cells normally express high level of IFN γ , moderate levels of TNF and low levels of IL-2 upon activation ^{239,255}. Recently, our group demonstrated that, CD4+CD28null T cells from synovial fluid of rheumatoid arthritis patients could secrete both IFN γ and moderate levels of IL-17 ²⁶¹. On the contrary, IL-17 secretion is not seen in

CD4+CD28null T cells from peripheral blood ^{261,268}. This suggests that CD28null T cells can acquire additional functions such as IL-17 secretion in inflamed tissues. In general, CD28null T cells are regarded as Th1 phenotype (profound IFN γ secretion) cells with NK cell like cytolytic features.

As mentioned above, several molecular and functional features are similar between CD8+CD28null and CD4+CD28null T cells. The important differences and similarities between these subsets known so far are summarized subsequent sections.

2.5.6 CD8+CD28null T cells

The accumulation of CD8+CD28null T cells with age in otherwise healthy individuals partly reflects the cumulative infectious burden during the human lifespan. However, frequencies of these cells are further increased in people with chronic viral infections e.g. HCMV, HIV and EBV, possibly due to repeated activation and clonal expansion. Interestingly, such expanded CD8+ T cells, specific for each of these viruses display different status of differentiation (**Figure-8**) ^{239,269}. HCMV reactive CD8+ memory T cells subsets appear to be most differentiated, defined by the lack of expression of both CD27 and CD28.

Phenotypically CD8+CD28null T cells are defined as effector memory population in the CD8+ T cell compartment. However, as shown in **Figure 8**, CD8+CD28null T cells are heterogeneous mix of population. It is suggested that, depending on the setting, CD8+CD28null T cells can either exert increased effector and proinflammatory functions or display signs of exhaustion, loss of cytotoxic ability and suppressive functions due to expression of inhibitory receptors ²⁷⁰⁻²⁷². However, it is possible that different subsets within CD8+CD28null T cell may have different functional features, which remains to be investigated. Nevertheless, several recent reports described CD8+CD28null T cells to be proinflammatory and associated with various inflammatory disorders ^{215,273,274}. Terminally differentiated, CD8+CD28null T cells are described to be resistant to apoptosis ^{209,273,275}. In addition to their proinflammatory and cytotoxic effects, CD8+CD28null T cells seem to have negative impact on immune system through several other mechanisms, such as constraining the adaptive immune system by oligoclonal expansions, and also by competing for available space and resources. Such effects can be reflected in elderly individuals as a form of impairment of immune system. Nevertheless, the functional aspects of human CD8+CD28null T cells and various subsets within this T cell population remain to be explored.

2.5.7 CD4+CD28null T cells

The presence of CD4+CD28null T cells is rare in most individuals until the old age. However, the increased frequency of this subset is seen in individuals with autoimmune and chronic inflammatory diseases, such as Wegener's granulomatosis²⁷⁶, Crohn's disease²⁷⁷, rheumatoid arthritis^{247,255,278,279}, multiple sclerosis²⁸⁰ and atherosclerosis²⁸¹⁻²⁸⁴. The unusual age-inappropriate expansion of CD4+CD28null T cells in patients suffering from these disorders, indicates premature aging of the immune system. This early immunosenescence may reflect chronic immune activation in these patients and, several findings indicate that CD4+CD28null T cells are likely to contribute towards the pathogenesis of these disorders. Although, presence of CD4+ T cells lacking CD28 was first reported by the group of Hansen and Martin in late 1980s²⁸⁵, the function of these cells were not characterized until late 1990s. CD4+CD28null T cells were first identified in the context of a rheumatoid arthritis by the group of Weyand and Goronzy²⁷⁸.

Although, somewhat similar to CD8+CD28null T cells, CD4+CD28null T cells are described to be more strongly associated with HCMV infections^{228,286,287}. In contrast to CD8+CD28null T cells, CD4+CD28null T cells appear to be less heterogeneous (**Figure 7**), and mostly described to be proinflammatory and cytotoxic^{246,288-290}. Similar to CD8+CD28null T cells, CD4+CD28null T cells are described to be resistant to apoptosis *in vivo*^{291,292}. Furthermore, it was shown recently that CD4+CD28null T cells from the peripheral blood of healthy donors were less susceptible to suppression by CD4+CD25^{high} regulatory T cells compared to conventional CD4+CD28+ T cells²⁹³. However, the detail mechanisms of action of CD4+CD28null T cells, their immunosuppression resistant properties and the role in various inflammatory and autoimmune disorders, are not well understood so far.

The literature is growing, which aim to characterize molecular and functional features of CD28null T cell subsets, and whether these cells play pathogenic role in immune-mediated inflammatory disorders. However, CD28null T cell subsets were not investigated in the setting of inflammatory myopathies so far.

3 AIMS

Despite several evidences indicating a role for T cells in the pathogenesis of myositis, precise phenotype and functionality of these cells are poorly understood. Increased frequencies of CD4+CD28null and CD8+CD28null T cells were reported in various chronic inflammatory disorders, however, the presence of these cells in patients with IIMs, particularly in the inflamed muscle, and whether these cells play any role in disease pathogenesis, are not known so far. Resistance against regulatory T cell immunosuppression in CD4+CD28null T cells led to speculation, whether these cells are also resistant to immunosuppression mediated by glucocorticoids, which is the basis of current treatment in IIMs. Moreover, apoptosis resistant properties of both subsets of CD28null T cells suggested that these cells could escape the apoptotic effects of glucocorticoids and could be linked to treatment ineffectiveness in patients with myositis. However, it is not known, how glucocorticoids based immunosuppressive therapies affect CD28null T cells, and also FOXP3+ regulatory T (Treg) cells in muscle tissue of patients with IIMs

The overall aim of the studies performed in this thesis was to characterize T cell subsets in the disease pathogenesis of inflammatory myopathies, with the emphasis to address above questions. The *in depth* understanding of disease mechanisms and role of T cell subsets in IIM pathogenesis may lead us towards developing novel, specific and more effective therapies.

More specific aims were as follow:

1. to investigate whether CD28null T cells could be effector cells in patients with polymyositis and dermatomyositis, and also if these cells are linked with HCMV infection (**Paper-I**).
2. to investigate whether CD28null T cells could be effector cells in patients with sporadic inclusion body myositis, where the role of immune cells in disease pathogenesis is controversial (**Paper-II**).
3. to investigate whether T cells, particularly CD28null T cells from patients with IIMs, are cytotoxic to autologous muscle cells *in vitro* and if yes, what are the mechanisms of cytotoxicity (**Paper-III**).
4. to investigate the effects of conventional immunosuppressive treatment on Treg cells and CD28null T cells in IIM muscle tissue and also if CD28null T cells display immunosuppression resistant properties *in vitro* (**Paper-IV**).

4 METHODS

Methods are described in details in the papers attached. In brief, studies included in this thesis investigate T cells from peripheral blood and muscle tissue of patients with IIMs and healthy individuals. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll separation, from which T cell subsets were obtained using flow cytometry. Flow cytometry based methods were widely used for molecular and functional evaluation of circulating T cell subsets in all studies included in this thesis. Cytokine secretions in culture supernatants (paper-I and paper-III) were measured by a multiplexed method known as BD™ Cytometric Bead Array (CBA) (BD Biosciences). HCMV specific serum IgG and IgM were measured using ELISA and T cell proliferation was measured using thymidin incorporation assay (paper-I).

Muscle biopsies specimens were mostly obtained from the vastus lateralis or tibialis anterior muscle by a “semi-open” technique under local anesthesia^{294,295}. Evaluation of T cells in muscle tissue was done using immunohistochemistry and immunofluorescence techniques. Quantitative assessment of T cell subsets in muscle tissue was done using computer assistant image analysis softwares.

For T cell-muscle cell co-culture studies in Paper-III, satellite cells were obtained from fresh muscle biopsies and expanded into myoblasts, and further differentiated into myotubes. T cell mediated muscle cell death was evaluated using flow cytometry based method and calcein release assay. Evaluation of recombinant cytokine induced muscle cell death and HLA upregulation was done using flow cytometry. The visualization of perforin polarization in T cells towards autologous myotubes, was done using immunofluorescence staining followed by confocal microscopy.

Patient's global disease activity was measured using Visual Analogue Scale (VAS) score in myositis (Paper-I). For clinical-evaluation in paper-IV, muscle performance was measured by the disease-specific Functional Index (FI) of myositis²⁹⁶, and post-treatment 5-years follow up of disease activity was done by "Myositis Intention To Treat Activity Index"(MITAX)²⁹⁷. To measure limitations in daily activities at 5-year follow-up, the Health Assessment Questionnaire (HAQ) disability Index was employed²⁹⁸. Serum levels of creatine-kinase (s-CK) were measured doing routine analyses at the Department of Clinical Chemistry at Karolinska University Hospital.

For most of the graphing and statistical analysis, the software program GraphPad Prism was used. In paper-I, the software program STATISTICA 7.0 was used for linear multivariate regression analyses, JMP for Kruskal-Wallis nonparametric ANOVA and Mann-Whitney analyses. All studies included in this thesis were approved by the local Ethics Committee of the Karolinska University Hospital, and both patients and healthy subjects gave informed consent.

5 RESULTS AND DISCUSSION

This thesis includes four papers. In this section, major findings from all four papers are summarized together with additional unpublished data (related to paper-I) and discussed in the context of current literature.

5.1 CD28NULL T CELLS IN PATIENTS WITH POLYMYOSITIS AND DERMATOMYOSITIS (PAPER-I)

In this study, phenotypic characterization of T cell subsets was done in both muscle tissue and peripheral blood of patients with PM and DM. Furthermore, T cell subsets were investigated in the context of human cytomegalovirus (HCMV) infections and clinical relevance.

5.1.1 Evaluation of frequency, phenotypic and functional potential of CD28null T cells

Using a cohort of 42 PM and 24 DM patients, we have found that CD28null T cells were present in higher frequencies in peripheral blood of patients with PM and DM compared to age and gender matched healthy individuals. Circulating CD28null T cells also displayed proinflammatory and cytotoxic features. To investigate CD28null T cells in the muscle tissue, we needed to identify a positive surrogate marker. With the identification of positive surrogate marker, we can avoid inclusion of T cells, which temporarily downregulate CD28 upon activation. Towards this, we found that majority of circulating CD28null T cells, 89% (median) of CD4+CD28null and 98% of CD8+CD28null T cells express the NK cell receptor CD244 on their surface. In order to investigate CD244 expression on T cells in myositis muscle tissue, we used triple color immunofluorescence staining, and found that in muscle tissue too, CD244 was mainly expressed by cells coexpressing CD3. This indicates that the majority of the CD244-expressing cells are T cells, and not NK cells in myositis muscle tissue. Using CD244 as a surrogate marker in triple color immunofluorescence stainings, we demonstrated that muscle-infiltrating T cells are predominantly CD8+CD28null (CD3+CD4-CD244+, median 79%) and CD4+CD28null (CD3+CD4+CD244+, median 59%) T cells in patients with PM and DM. While comparing the relative abundance, CD4+CD28null T cells displayed significantly higher frequency in muscle tissue compared to peripheral blood of same individuals (median 59% versus 6% $p < 0.0001$), while the frequency of CD8+CD28null T cells were high in both muscle and peripheral blood. Despite the confirmation that the expression of CD244 was mainly on T cells in muscle, there are limitations with the use of CD244 as a surrogate marker, because CD244 can be also expressed by a small proportion of CD8+CD28+ T cells. To further substantiate presence of CD28null T cells in the muscle, we used double immunofluorescence stainings of seven infiltrates in muscle tissue, with CD4 and

CD28 or CD8 and CD28 and confirmed the lack of CD28 on approximately 64% of the CD4 T cells and 60% in CD8+ T cell subsets (**Figure 10**). However, there could be limitations with this method too, because a report demonstrates that muscle fibers and myoblasts can also express costimulatory molecules in inflammatory cytokine milieu, which are known to be expressed on T cells such as CD28 and CTLA-4²⁹⁹. In our study as well, CD28 staining in muscle did not display a characteristic circular membrane staining like CD4 or CD8, but was varied from rather weak to focal granular. Such pattern of expression is suggestive of sarcolemma and sarcoplasmic reticulum/T tubule pattern indicating the expression on muscle fibers themselves, and was similar with the CD28 expression pattern in myositis muscle tissue shown by Nagaraju et al. Therefore, the identification of CD28null T cells with the lack of CD28 staining approach in muscle tissue could lead to underestimation of the CD28null T cell number assessment.

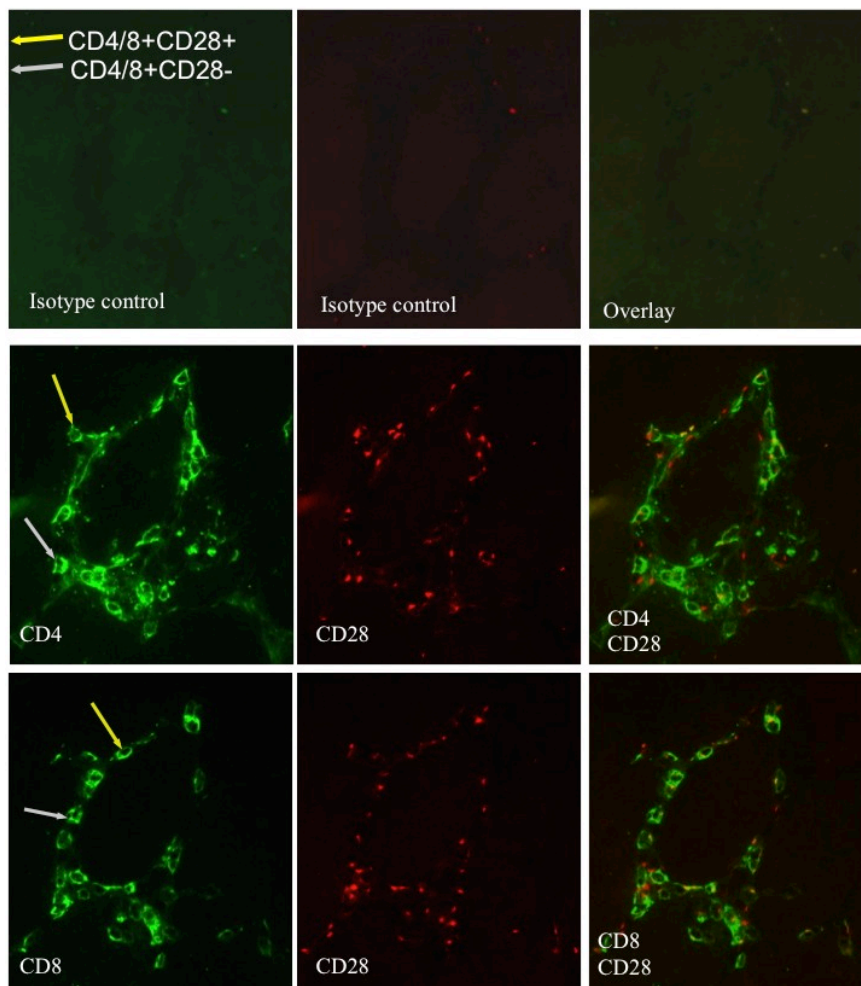


Figure 10: Polymyositis muscle tissue displaying the expression of CD4, CD8 and CD28. Representative staining of skeletal muscle section of polymyositis patient displaying CD4 (green) or CD8 (green) and CD28 (red) (CD28). The staining shows the absence of CD28 on many of the CD4+ and CD8+ cell in muscle tissue. Also, it shows an unusual staining of CD28 in muscle tissue from weak to focal granular, suggestive of the expression on muscle fibers rather than T cells.

5.1.2 Clinical relevance

Subsequently, various subpopulations of T cells were correlated with clinical parameters of the patients. Presence of CD28null T cells at the disease onset in muscle tissue and correlation of muscle infiltrating CD8+ T cells (of which majority were CD28null T cells) with disease activity in patients, support the clinical relevance of these cells. The increase in circulating CD8+CD28null T cell frequency with age indicates a role of age dependent immunosenescence mechanisms in IIM pathogenesis. The decrease in frequency of circulating CD28null T cells subsets with disease duration could be due to the effect of ongoing treatment in these patients, or it is also possible that these cells are consumed while causing inflammatory damage during disease duration. However, these speculations need be confirmed by future studies.

5.1.3 Link with Human Cytomegalovirus

The increased frequency of CD28null T cells in both CD8+ and CD4+ compartment have been linked with chronic viral infections, particularly HCMV. Similarly, we also found that circulating CD28null T cells are predominantly found in patients seropositive for HCMV. To further investigate the association between CD28null T cells and HCMV infection, we investigated whether CD28null T cells display any specific reactivity towards HCMV T cell antigens such as pp65 and IE, using IFN γ production as read-out. We found that both CD8+CD28null and CD4+CD28null T cells from HCMV seropositive patients responded to the pp65 antigen by producing high IFN γ whereas CD28null T cells from HCMV seronegative patients did not display such levels of response. On the contrary, CD28+ T cell subsets from both HCMV seropositive and seronegative patients showed lack of response towards HCMV antigens. Moreover, the minor response in CD28null compartment towards influenza strengthens the association of CD28null T cells particularly with chronic viral infections.

In summary, this study identifies CD28null T cells as the dominating effector T cell subsets in muscle tissue of patients with PM and DM. It further explores the molecular and functional features of CD28null T cell subsets, their clinical relevance and possible link with HCMV infections.

5.2 CD28NULL T CELLS IN PATIENTS WITH SPORADIC INCLUSION BODY MYOSITIS (PAPER-II)

Frequency, clonal expansions and functional evaluation

Although, cytotoxic CD8⁺ T cells invading MHC class I expressing muscle fiber have been implicated in sIBM pathogenesis^{23,24,300,301}, the absence of beneficial effects of conventional immunosuppressive therapies and the lack of signs of the systemic inflammation has questioned the role of immune system and T cells in sIBM pathogenesis^{302,303}. Since CD28null T cells are described as long-lived and resistant to apoptosis *in vivo*^{273,291,292}, it was compelling to investigate presence and functionality of CD28null T cells in the peripheral blood and muscle tissue of patients with sIBM.

In a cohort of 27 patients with sIBM, we found strikingly higher frequency of circulating CD28null T cells in both CD8⁺ and CD4⁺ compartment compared to the healthy controls. In muscle compartment too, CD28null T cells dominated the T cell infiltrate (median 72%, range 42.5-96%). Circulating CD8⁺CD28null and CD4⁺CD28null T cell subsets upon stimulation, not only displayed higher IFN γ secretion potential, but also were capable of degranulating more compared to CD28⁺ T cell subsets.

Clonal expansions in the CD8⁺ T cell populations of sIBM patients, have been reported previously^{33,304}, a feature that is shared with CD28^{null} T cells²⁹². Using TCR-V β repertoire analysis of circulating T cells, we also observed striking clonal T cell expansions in both CD4⁺ and CD8⁺ compartment in patients with sIBM. Importantly, we demonstrated that the clonal expansion observed in the total CD8⁺ and CD4⁺ T cell populations of sIBM were predominantly due to clonally expanded CD28^{null} T cells. Such clonal expansions in CD28null T cells indicate repeated antigenic stimulation scenario. Furthermore, we demonstrated that clonally expanded TCR-V β which were dominant in circulation, were also present in the inflamed muscle of the respective patients, indicating shared antigen-specificity of CD28null T cells between inflamed muscle tissue and peripheral blood.

Persistent antigen-specific activation and clonal expansion is indicative of shifting the balance towards immunosenescence, which may lead to impaired functions of immune cells. Investigating, whether clonally expanded CD28null T cells still retain proinflammatory functions, we have shown that CD28null T cells with dominant TCR-V β are equally capable of IFN γ secretion and degranulating as CD28^{null} T cells with non-dominant TCR-V β . This indicates that these cells were not functionally senescent but potent to exert proinflammatory and cytotoxic functions.

Overall, finding in this study suggests, that clonally expanded T cells in muscle and peripheral blood of patients with sIBM belong to CD28null T cells. These cells

dominate T cell infiltrate in muscle tissue and display proinflammatory and cytotoxic properties.

5.3 MYOTOXIC POTENTIAL OF CD28NULL T CELLS AND THE MECHANISMS INVOLVED (PAPER-III)

After observing the high frequencies of CD28null T cells both in muscle tissue and peripheral blood of patients with IIMs, along with the proinflammatory and cytotoxic features, we were interested to investigate, whether CD28null T cells from patients with IIMs directly exert cytotoxic functions towards autologous muscle cells, and if they do, what are the mechanisms involved. In order to explore myotoxic functions of T cell subsets, we employed a fully autologous *in vitro* human T cell-muscle cell co-culture system, in which the cytotoxic functions of T cell subsets alone towards muscle cells can be explored. To study autologous T cell-muscle cell interactions, we obtained muscle biopsy and peripheral blood samples from 6 patients with PM. Muscle stem cells were extracted from muscle biopsies and differentiated into myotubes, while T cell subsets were obtained from peripheral blood. For co-cultures studies, differentiated cultures with myotubes were preferred because myotube phenotype is more close to *in vivo* muscle fibers such as expression of contractile and structural muscle protein e.g. myosin. Autologous muscle cells were co-cultured with either un-stimulated or stimulated T cell subsets and T cell cytotoxicity towards muscle cells were confirmed by two different methods.

Using above setup, we demonstrated, that CD28null T cells are directly cytotoxic to autologous muscle cells. Moreover, we found that CD28null T cell subsets were spontaneously cytotoxic to autologous muscle cells in culture without any stimulation/reactivation. This indicates that a significant proportion of CD28null T cells is already primed towards muscle compartment in these patients. However, un-stimulated CD28null T cells did not display a dose response (data not shown) or significantly higher cytotoxicity than CD28+ counterparts in co-cultures, indicating the possible requirement of additional factors/immune cells for maximum cytotoxicity potential, which could be absent in our *in vitro* settings. Nevertheless, upon co-culture in stimulatory environment, both CD28null T cell subsets significantly induced more cell death in autologous muscle cells than CD28+ counterparts.

We have also shown here that myotubes were more prone to cell death induced by CD28null T cells compared to myoblast. Myotubes in cultures are considered to have more resistance to chemically induced apoptosis and/or cell-death compared to myoblasts^{130,131,201}. On the contrary, CD28null T cell subsets were able to induce more cell-death in autologous myotubes compared to myoblasts. This suggests that a majority of CD28null T cells could be primed towards structural or functional proteins, which are specifically expressed in myotubes compared to myoblasts, such as myosin.

However, muscle specific autoantigen reactive to CD28null T cells remain to be identified.

Exploring the CD28null T cell mediated mechanism of muscle cell death, we have shown that not only CD8+CD28null but also CD4+CD28null T cells displayed perforin polarization towards muscle cells and secreted higher levels of granzyme-B and IFN γ in co-culture than CD28+ subsets. This determines that both CD8+CD28null and CD4+CD28null T cells can directly kill muscle cell by perforin-dependent mechanisms. To decipher the perforin and cytokine mediated cytotoxic/myotoxic effects, we performed various blocking experiments. By blocking perforin, the myotoxicity was reduced by a median of 52% with CD4+CD28null T cells and by 56% in cultures with CD8+CD28null T-cells indicating that perforin-mediated pathway is one of the major mechanisms of CD28null T cell cytotoxicity towards muscle cells. Nevertheless, blockade of IFN γ and TNF in co-cultures also reduced the level of dead muscle cells by 46% (CD4+CD28null) and 53% (CD8+CD28null). This indicated the essential role proinflammatory cytokines also in the CD28null T cell mediated myotoxicity. However, TNF or IFN γ did not induce death of muscle cells in the absence of T cells, suggesting that these cytokines can have other effects in the context of T cell mediated cytotoxicity such as priming of muscle cells by HLA upregulation. Indeed, we found that cytokines, particularly IFN γ did upregulate both HLA class I and class II on muscle cells. Therefore, the reduced myotoxicity upon blocking of IFN γ and TNF is likely attributed to interference with HLA upregulation, as HLA blockade resulted in a comparable reduction in myotoxicity, 61% for CD4+CD28null and 55% for CD8+CD28null T cell co-cultures.

Overall, the study demonstrates that CD28null T cells are cytotoxic to autologous muscle cell in polymyositis patients. The myotoxic effects of CD28null T cells appear to be predominantly exerted via perforin-granzyme pathway and regulated by IFN γ -induced HLA expression by muscle cells.

5.4 EFFECTS OF IMMUNOSUPPRESSION ON T CELL SUBSETS IN IIM (PAPER IV)

5.4.1 Effects of conventional immunosuppressive treatment on CD28null and regulatory T cell subsets in muscle tissue of PM and DM

Treatment of PM and DM is based on the use of glucocorticoids in high doses over an extended period of time, but treatment outcome is unpredictable.¹ In some patients, the inflammatory infiltrate including T cells persists in muscle-tissue despite aggressive treatment and is associated with remaining muscle weakness.⁸³⁻⁸⁵ Although, we have

identified CD28null T cells as dominant T cell subset in muscle tissue of myositis patients, FOXP3+ Tregs have also been reported in myositis muscle-tissue in close proximity to effector cells.³⁰⁵ Therefore, it was intriguing to investigate whether persistent T cells in affected muscle could be denoted to FOXP3+ regulatory T (Treg) cells or proinflammatory CD28null T cell subsets.

Looking at the relative frequency, CD28null T cells dominated the T cell infiltrates over Treg cells in inflamed myositis muscle-tissue. Overall numbers and frequencies of FOXP3+ cells were very low in our cohort and were similar with findings in the Waschbisch et al report³⁰⁶. However, this is the first study, which investigates the frequencies FOXP3+ Treg cells in relation to the CD28null T cells and how their relative proportion is affected in myositis muscle-tissue by immunosuppressive treatment. Interestingly, after treatment, CD28null T cells were found to be unchanged while the number of Treg cells had declined, leading to a relative increase in the proportion of CD28null T cells over Treg cells. The CD28null T cells have been reported to be long lived and apoptosis resistant *in vivo*,^{273,291,292} which has been linked with the increase in anti-apoptotic protein Bcl-2²⁹¹. Furthermore, CD28null T cells also display replicative senescent properties,³⁰⁷⁻³⁰⁹ and we have also demonstrated this in the setting of myositis³¹⁰. Therefore, it is possible that CD28nulls T cells can escape the apoptotic effects of high doses of glucocorticoids as well as anti-proliferative effects of concomitant immunosuppressive drugs, leading to their persistence in muscle-tissue.

5.4.2 In vitro immunosuppressive effects of glucocorticoids and regulatory T cells on CD28null T cell subsets

How about the immunosuppressive effects of glucocorticoids? Whether glucocorticoids can suppress CD28null T cells efficiently? A report has shown that CD28null T cell proliferation and function could only partly be suppressed by Treg cells in healthy-donors²⁹³. Therefore, we further investigated the immunosuppressive effects of glucocorticoids and Treg cells on CD28null T cell subsets using *in vitro* immunosuppression assays. We have evaluated the suppression effects on the T cell activation (using early T cell activation marker CD69) rather than on T cell proliferation, due to replicative senescent properties of CD28null T cells (as described above). We validated that CD69 is unregulated on both CD4+CD28null and CD8+CD28null T cells upon activation. Interestingly, we found that both glucocorticoids and Treg are less capable of suppressing of CD4+CD28null T cells compared to their CD28+ counterparts.

5.4.3 Clinical relevance of persistent CD28null T cells in myositis muscle tissue

Whether persistence of such proinflammatory and immunosuppression resistant CD28null T cells is related to the post-treatment disease outcomes in IIMs? Indeed, we further demonstrated that the higher number of CD28null T-cells in the post-treatment muscle biopsies correlated with a poor clinical response both in short-term (Functional Index) and long-term (disease activity measurement by MITAX) clinical perspectives.

In summary, in this study, we demonstrated that current treatment regime based on high doses of glucocorticoids in combination with conventional immunosuppressive agents is insufficient to eliminate CD28null T cells in inflamed muscle of patients with PM and DM. CD28null T cells were found to be relatively resistant to both glucocorticoid and Treg mediated immunosuppression and were also linked with poor clinical outcome from conventional immunosuppressive treatments. These findings could provide explanations concerning the inefficacy of current treatment approach, if not for all, at least for a subgroup of myositis patients. Furthermore, it suggests that a specific targeting of proinflammatory T cells could form the basis of better future therapies in patients who are resistant to current immunosuppressive treatments.

6 THESIS SUMMARY AND CUMULATIVE DISCUSSION

Findings in the **paper-I and paper-II**, establish a new angle to look at the T cell phenotype in the pathogenesis of myositis. According to classical dogma, mainly CD8+ T cell were considered to be involved in PM and sIBM pathogenesis, while attack on the microvasculature via CD4+ T cell mediated humoral immunity were considered to play major role in DM pathogenesis. The demonstration of significantly increased presence of CD8+CD28null and CD4+CD28null T cells in peripheral blood as well as in muscle tissues of PM and DM, defines rather precise phenotype of T cells in these compartments, which are potentially pathogenic. The findings in this thesis explains the inadequate expression of CD28 in myositis muscle tissue seen by some other investigations, although such studies did not put emphasis on CD28null phenotype in the muscle tissue^{311,312}. Moreover, our data could also shed light on instances where CD28 expression was completely lacking despite T cell infiltrate in muscle tissue, such as in patients with Duchenne muscular dystrophy³¹¹. Furthermore, previously reported enhanced expression of CTLA-4 on the T cells in contact with muscle fibers could be elucidated by CD28null phenotype because CD28null T cells are described to upregulate CLTA-4 upon activation^{214,215,311,312}.

The phenotypic characterization of CD28null T cell subsets in peripheral blood as well as muscle tissue in **paper-I** provides the tools to explore CD28null T cells in the muscle compartment, which is trickier. The findings in **paper-II** demonstrate that the clonally expanded T cells seen in the muscle tissue and peripheral blood of sIBM are CD28null T cells. Furthermore, the functional characterization CD28null T cells in both **paper-I and paper-II**, reveal phenotype of these T cell subtypes, which are proinflammatory and cytotoxic, and could possibly play role in disease mechanisms and muscle pathogenesis.

Although, we haven't evaluated clonal expansions in PM and DM so far, the results from the **paper-II** may have implications for other subsets of IIMs, where clonally expanded T cells were defined to be auto-aggressive T cells attacking muscle fibers^{34,313}. Of note, in synovial tissue of Rheumatoid Arthritis, CD28null T cells are not enriched despite high levels in peripheral blood²³⁸. This suggests that CD28null T cell phenotype in affected organs is not a general finding in any chronic inflammation, but could be more specific for certain diseases or certain organs such as in inflamed muscles in myositis.

The higher presence of CD28null T cell subsets in HCMV seropositive patients and their reactivity to HCMV derived antigens suggest a possible role for HCMV and/or chronic infections in the pathogenesis of myositis (**paper-I**). The presence and replications of chronic viruses such as HCMV or HIV in muscle tissue is controversial^{10,42,54,70}. Nevertheless, it is possible that CD28null T cells migrate to muscle after

encountering HCMV at other sites in the body. The co-occurrence of both CD4+ and CD8+ CD28null T cells has been suggested to be associated with HCMV-induced IFN α production by plasmacytoid DC ^{226,228,229}. Interestingly, plasmacytoid DCs and the IFN α -induced protein MxA were frequently found in muscle tissue from patients with dermatomyositis and polymyositis ^{314,315}. There are reports describing a link between the long-term use of IFN α , particularly for the treatment of chronic hepatitis C virus infection and development or exacerbation of numerous autoimmune phenomena and spectrum of myopathies ^{316,317}. Also, HCMV specific effector memory CD8+ T cells have been also reported to express high levels of CD244 ³¹⁸. Above findings indicate direct and/or indirect role of chronic viral infections in inflammatory myopathies.

It is still debated whether T cells have a roll in the pathogenesis of sIBM or it is merely a degenerative muscle disease, largely because most patients are refractory to current immunosuppressive therapies. We demonstrate the enhance presence of CD28null T cells in muscle tissue and peripheral blood of patients with sIBM (**Paper-II**). CD28null T cells are described to be resistant to apoptosis *in vivo* ^{273,291,292}, therefore it is possible that conventional immunosuppressive therapies are not able target these cells, particularly in muscle tissue. According to a recent proof-of-principle study involving 13 patients with sIBM, who underwent one series (0.3 mg/kg/day for 4 days) of alemtuzumab (a humanized monoclonal antibody to CD52 that causes depletion or severe reduction of circulating mature lymphocytes) infusions, could slow down disease progression up to six months, improved strength of some patients, and reduced endomysial inflammation in muscle tissue ³¹⁹. Although, these preliminary results are promising towards the targeting of immune cells as sIBM therapies, alemtuzumab is associated with lot of adverse effect to due generalized targeting of lymphocytes. Therefore, specific targeting of proinflammatory and pathogenic T cell subsets such as CD28null T cells should be more appropriate approach towards therapies in sIBM.

To elucidate the myotoxic potentials of T cell subsets and mechanism involved, we established an autologous T cell-muscle cell co-culture approach (**paper-III**). *In vitro* manipulation such as extended cultivation including cloning of T cells can alter their phenotype and functionality, therefore, we choose to use primary T cells from patients without *in vitro* expansion, although, it limited the number T cells, hence the various experimental conditions we could test in co-culture studies. The demonstration of perforin-granzyme pathway in CD28null T cell myotoxicity, including the role IFN γ and TNF, provide better understanding of the role of T cells in muscle pathogenesis.

Finally, in **paper-IV**, we provide evidence that, indeed CD28null T cells are persistent in the muscle tissue of patients despite aggressive immunosuppressive treatment, and associated with poor clinical outcomes. Also, CD4+CD28null were found to be resistant to immunosuppression mediated by both glucocorticoids and Treg cells. The absence of clear pattern in CD8+CD28null T cells regarding immunosuppression

resistance could be due to heterogeneity of this T cell subset, which remains to be dissected in future studies. Interestingly, a recent study in patients with PM and DM shows that after high dose IVIG treatment, the frequency of IFN γ producing cells was significantly higher in muscle tissue of non-responders compared to responders, with an increased IFN γ /IL-17-producing-cell ratio³²⁰. These results further point towards CD28null phenotype in the muscle tissue, especially in patients that are resistant to immunosuppression.

Apparently, there are additional limitations in various studies, which could not be mentioned above. The major limitations with all the studies, was the limited availability of patient samples, both muscle biopsies and peripheral blood due to myositis belonging to rare disease category. Due to that, we could only perform restricted set of experiments in many instances, for example co-culture studies. Also, low patient numbers led to difficulties in finding statistically significant results, which further became difficult in case of clinical parameters correlations, due to inadequate availability of the clinical data. To curtail such limitations, we have pooled the data from different IIMs subtypes in some cases, for example throughout in **paper-IV** and some instances in **paper-I**. Although, we are aware of subtype differences, the pooling of data may provide insight into common pathogenic mechanism in different subtypes. Also, it is time to re-evaluate the old dogmas on IIM subtypes and classify patients according to molecular and cellular markers, where CD28null T cell subsets could serve as proinflammatory T cell markers. Classifications based on molecular and cellular makers are proving better in understanding disease mechanisms and deciding treatment strategies.

6.1 THE PROPOSED MODEL

Based on findings in this thesis, and previous understanding of skeletal muscle-immune system interaction, following model is proposed summarizing the role of T cell subsets in disease mechanisms of inflammatory myopathies (myositis) (**Figure 11**).

Figure 11a and 11b describe that both immune system and skeletal muscle play important role in the homeostasis of each other. In a healthy person, after skeletal muscle injury, cells linked to the immune system including T cells play crucial role in the repair and regeneration process. The neutrophils and M1 macrophages (proinflammatory) cause further transient inflammatory damage in the muscle tissue. This is followed by phenotypic switch in macrophages from M1 to M2 (anti-inflammatory) at the site of injury. M2 macrophages dampen the inflammation and induce/help in satellite cell mediated muscle regeneration and remodeling process. FOXP3+ regulatory T (Treg) cells not only induce phenotypic switch in macrophages from M1 to M2, but also directly promote regenerative potential of satellite cells via secretion of a growth factor called Amphiregulin. In addition, Treg can suppress/limit

occasional autoreactive T cells, which could potentially attack muscle fibers. Towards another direction, a healthy skeletal muscle possesses various immunological and cytokine secretion capabilities and is known to have anti-inflammatory effects on the immune system such as in setting of exercise.

In young and healthy individuals, T cells are efficient in maintaining homeostasis and immune tolerance in skeletal muscle (**Figure 11a**). During human lifespan, various factors promote the accumulation of CD28null T cells such as chronic stimulation (both antigenic and cytokine mediated), chronic viral infections, type-I interferon mediated telomere erosion. Depending on genetic and environmental factors, this process can be rapid in some individuals. T cell repertoire with increased frequencies of CD28null T cells display several

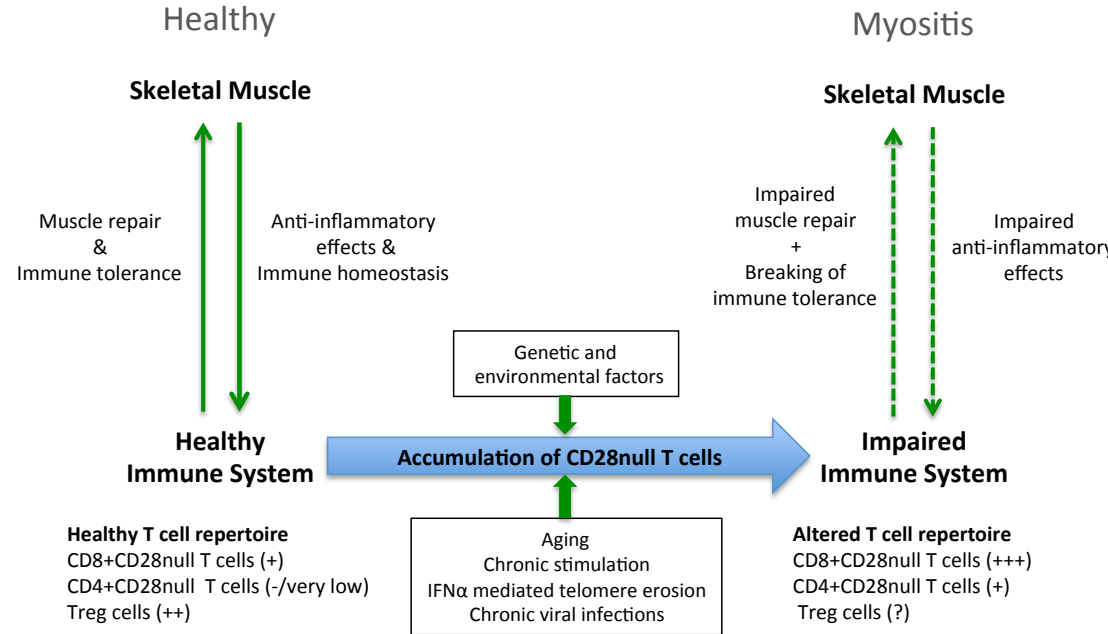


Figure 11a: Differences in skeletal muscle-immune system interaction in healthy verses myositis settings, and effects of T cell repertoire. Green arrow: Inducing/promoting effect, Broken lines: Impaired effects.

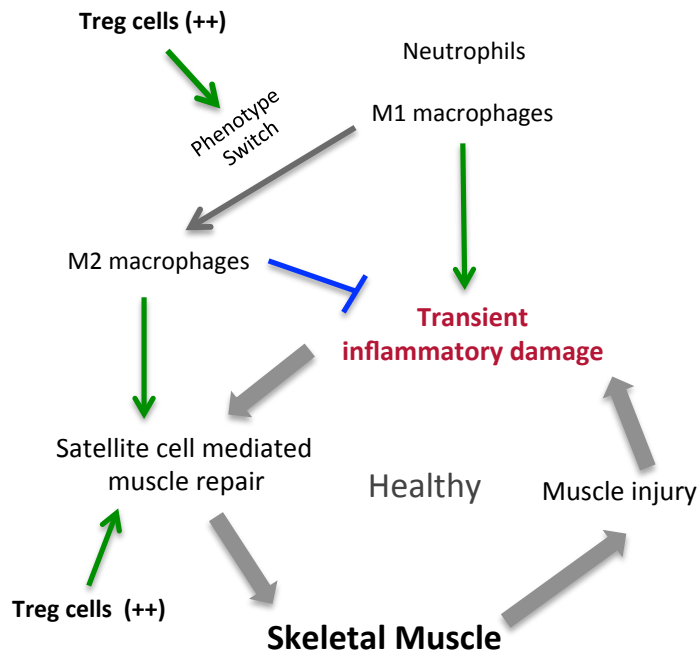


Figure 11b: Role of immune cells in muscle repair and regeneration in a healthy person.
 Green arrow: Inducing/promoting effect, Blue lines with bar: Blocking effect, Broken lines: Impaired effects.

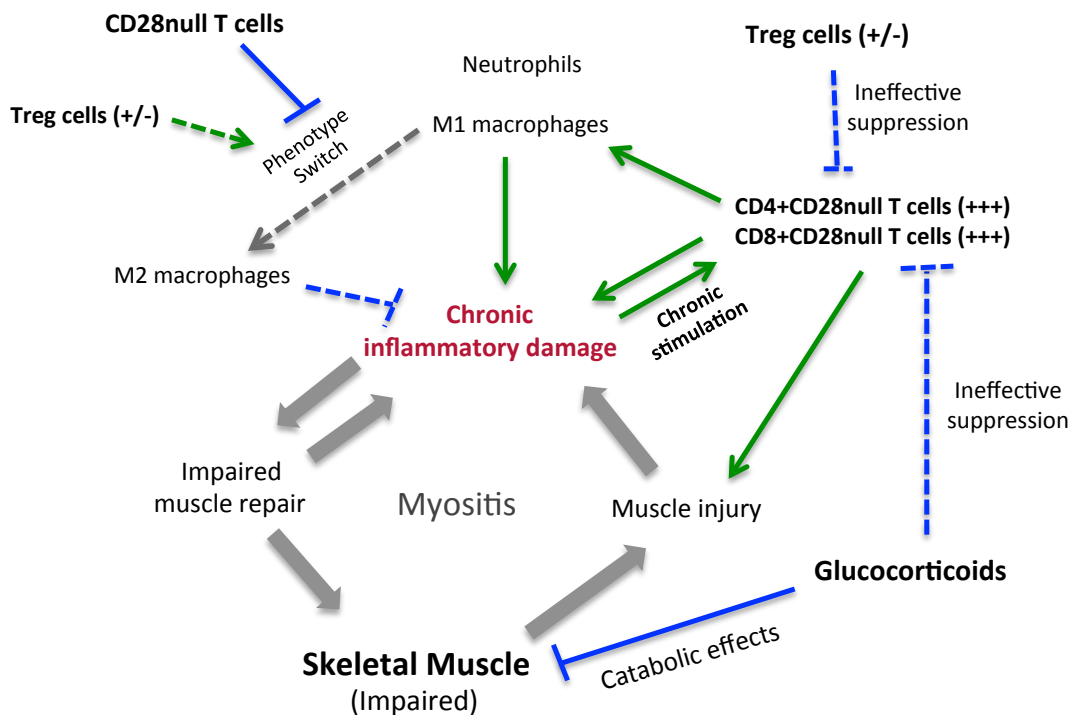


Figure 11c: Role of T cell subsets in the pathogenesis of myositis.
 Green arrow: Inducing/promoting effect, Blue lines with bar: Blocking effect, Broken lines: Impaired effects.

Patients with inflammatory myopathies display accumulation of CD28null T cells in both peripheral blood and muscle compartment (**Figure 11a**). Due to proinflammatory and cytotoxic capacity, CD28null T cells can directly induce muscle damage, and can maintain perpetual chronic inflammation in muscle tissue (**Figure 11c**). Proinflammatory cytokines from CD28null T cells such IFN γ and TNF can promote M1 phenotype macrophages and prevent the phenotype switch to M2. These effects would manifest in chronic inflammatory damage and impaired muscle repair, which can further lead to chronic stimulation and expansion of CD28null T cells and breakage of immune tolerance. Despite massive T cell infiltrates in muscle tissue of myositis, the numbers of FOXP3+ Treg cells are few, which imply that processes induced by Treg cells may be impaired due to their inadequate number. Furthermore, immunosuppression resistant properties of CD4+CD28null T cells could make the regulatory effects of Treg cells and glucocorticoids less relevant in setting of myositis. The long-term use glucocorticoid can also have catabolic effects on skeletal muscle. Overall, the enhanced presence of CD28null T cells and lower number of Treg cells, may possibly explain the pathogenesis and treatment ineffectiveness in inflammatory myopathies.

7 CONCLUSIONS

The results in this thesis provide improved understanding of the phenotype of pathogenic T cells and their role in the disease mechanisms in inflammatory myopathies. The phenotypic characterization of CD28null T cell subsets in all three major subtypes of IIMs equips us to explore these cells for the future inflammatory myopathies research. Furthermore, their mechanism of action towards muscle cell death and role in inefficacy of current therapies provide us with a better understanding of disease pathogenesis and a possible direction for efficient future therapies. In the included studies, we show that:

- CD28null T cell subsets dominate the T cell infiltrate of inflamed muscle, and also display increased presence in circulation, in all three subtypes of IIMs i.e. PM, DM and sIBM.
- CD28null T cell subsets from patients with IIMs display proinflammatory and cytotoxic features.
- HCMV infections are linked with the expansions of CD28null T cells in PM and DM.
- The myotoxic effects of CD28null T cells are mediated predominantly via perforin-granzyme pathways and regulated by proinflammatory cytokines such as IFN γ and TNF.
- The inefficacy of conventional therapies in PM and DM are linked to persistence of immunosuppression resistant CD28null T cells in the muscle tissue.

8 FUTURE PERSPECTIVES

An important future direction is the therapeutic targeting of CD28null T cells in myositis, preferably first in relevant animal models. However, the absence of natural occurrences of CD28null T cells in mice has limited the CD28null T cells research field. Therefore, efforts should be made to develop relevant rodent models of myositis, where pathogenic mechanism of CD28null T cells can be studied further in *in vivo* setting. Also, it would be more appropriate to study CD28null T cells in relevant primates cases of natural inflammatory myopathies, such as myositis associated with natural infections with retrovirus⁵¹.

Nevertheless, some progress can be made with existing rodent models, combined with the growing knowledge of human CD28null T cells. A previous study in our group has demonstrated that circulating CD4+CD28null T cells from rheumatoid arthritis patients display predominantly effector-memory T cell phenotype²⁵⁵. Several other studies also implicate the role of effector-memory T cell population in pathogenesis of various chronic inflammatory disorders, and such effector memory T cells are described to express high level of voltage-gated potassium channel, Kv1.3, particularly in activated state^{280,321-325}. More recent studies describe the selective targeting of CD4+CD28null T cell population (or CD4+ effector-memory T cells), by targeting Kv1.3 channels, which mainly inhibited the effector functions of CD4+CD28null T cells³²⁵⁻³²⁷. The Kv1.3 potassium channel has been reported as an important regulator for influx of potassium ions critical for exocytosis of cytoplasmic granules, including perforin and IFN γ containing granules, on CD4+CD28null T cells³²⁶. The therapeutic potential of Kv1.3 inhibitors has been evaluated in few rat models of autoimmune disorders such as rheumatoid arthritis, type I diabetes mellitus and atherosclerosis, where inhibition of Kv1.3 ameliorated the disease and associated pathogenic features^{325,327}. Interestingly, it has been reported that mouse models of autoimmune disease are not suitable for evaluating the effects of Kv1.3 inhibitors, because Kv channels doesn't seem to regulate the membrane potential of mouse T cells^{325,328,329} and the potassium channel expression pattern of repeatedly activated mouse T cells are very different from that of human T cells^{330,330}. Therefore, I recommend that future studies evaluating the effects of Kv1.3 blockers should be performed in rat models of myositis rather than mice models of myositis.

Moreover, the literature is less clear about the expression and blocking effect of Kv1.3 on CD8+CD28null T cells. Therefore, before targeting CD28null T cells in patients with IIMs by Kv1.3 inhibitors, extensive phenotyping of various T cell subsets, including both CD4+CD28null and CD8+CD28null T cells, should be performed with respect to the specific expression of such potassium channels in patients. Inhibiting ion channels in patients should be carried out with caution, as non-specific binding to other ion channels such as on heart muscles or on neurons can have serious side effects. Moreover, risk of infections should be evaluated before targeting effector-memory or

tissue resident-memory T cells. In this direction, we could benefit from the knowledge on antigen specificity of T cells, where specific targeting of autoreactive CD28null T cells could help us reducing the risk of infections. Overall, the data in this thesis and relevant literature suggest that the selective targeting of CD28null T cells could hold the key to future therapies in myositis.

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