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Thesis applying for **Doctoral Degree** in **Medical Sciences** submitted to **School of Medicine and Biomedical Sciences Abel Salazar (ICBAS)**, **University of Porto**.

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LIST OF INTERNATIONAL PUBLICATIONS MADE WITHIN THE SCOPE OF THE THESIS

1. Campar A, Alves I, Santos-Pereira B, Nogueira R, Pinto MM, Vasconcelos C, Pinho SS. **Muscle glycome in idiopathic inflammatory myopathies: Impact in IL-6 production and disease prognosis.** iScience. 2023 Jun 17;26(7):107172. doi: 10.1016/j.isci.2023.107172. PMID: 37404372; PMCID: PMC10316658.

2. Campar A, Alves I, Martins da Silva A, Farinha F, Vasconcelos C. **Idiopathic inflammatory myopathies – the burden of disease: cohort analysis focusing on damage and comorbidities.** Accepted in Autoimmunity Reviews – In Press. doi.org/10.1016/j.autrev.2023.103455.

3. Pereira MS, Alves I, Vicente M, Campar A, Silva MC, Padrão NA, Pinto V, Fernandes Â, Dias AM, Pinho SS. **Glycans as Key Checkpoints of T Cell Activity and Function.** Front Immunol. 2018 Nov 27;9:2754. doi: 10.3389/fimmu.2018.02754. PMID: 30538706; PMCID: PMC6277680.

4. Alves I, Vicente MM, Dias AM, Gaifém J, Rodrigues C, Campar A, Pinho SS. **The Role of Glycosylation in Inflammatory Diseases.** Adv Exp Med Biol. 2021;1325:265-283. doi: 10.1007/978-3-030-70115-4_13. PMID: 34495540.

ABSTRACT

Idiopathic inflammatory myopathies (IIM) are a rare, systemic and heterogeneous group of diseases, which leads to long-term disability, decreased quality of life and a reduced life expectancy.

The origin of these diseases is still not fully understood, but a relationship between environmental triggers and genetic risk factors, with an additional role of faulty immunoregulatory mechanisms seem to play a relevant role in the pathogenesis. Immunopathological mechanisms include adaptive immunity, both humoral and cellular, innate immunity and non-immune mechanisms in a complex interplay, which probably underlies the different clinical phenotypes, disease progression and response to therapy.

The lack of consensus on IIM classification, along with the rarity and complexity of the disease, has hindered the development of significant clinical trials for a directed therapy. However, the discovery of multiple myositis-specific antibodies and their clinical associations provided an insight into subphenotyping these patients, regarding potential involvement of other organs (such as the lung), malignancy risk and, in some cases, response to therapy. Despite survival has improved in recent years, patients with IIM carry a high disease burden, related to damage accrual and comorbidities, which are a reflection, in most cases, of a prolonged steroids exposure. A well-characterized cohort from the “Clinical Immunology Unit” of Porto University Centre Hospital was analysed and the results from 149 patients showed that, both damage and comorbidities were present in more than 90% of cases. Most comorbidities were a consequence of the damage, namely cardiovascular damage. While comorbidities accumulation was the major factor for poor quality of life, damage severity was the main predictor for mortality. Improved therapeutic strategies are needed to reduce the need for steroids and to introduce routine screening and management of comorbidities as an essential partner of immunosuppressive therapy, leading to comprehensive care of myositis patients and effective improvement of their quality of life.

Cumulating evidence suggests a prominent role of glycans in the regulatory circuits that govern immune responses, supporting the impact of complex branched *N*-glycans in the maintenance of self-tolerance. The impairment of *N*-glycan branching and consequent accumulation of less complex high-mannose glycans structures, similar to those present in microorganisms, may therefore contribute to the pathogenesis of various autoimmune diseases, through epitope modification or (glyco) neo-antigen formation that triggers the activation of the immune response. An altered glycosylation (particularly a decreased

expression of branched *N*-glycans) has been related with break of immune tolerance and an enhanced susceptibility to autoimmune disorders, associated with disease severity. In particular, changes in the expression profile of glycans may be the link between innate and adaptive immune responses and autoimmune diseases susceptibility. The cellular glycosignature, besides contributing to a deeper understanding of the pathophysiology of diseases, has the potential to be used as a biomarker for prognosis and patient's stratification and thus as a potential target for directed therapy in IIM as well as in other autoimmune diseases.

Our results showed that muscle biopsies from patients with IIM revealed a deficiency in the glycosylation pathway resulting in loss of branched *N*-glycans. At diagnosis, this glycosignature was able to predict disease relapse and treatment refractoriness, with high sensitivity and specificity. Peripheral $CD4^+$ T cells from active-disease patients showed a deficiency in branched *N*-glycans, linked to increased IL-6 production, which might be used in the future, as an accessible biomarker for individual follow-up and therapeutical approach. Glycan supplementation, restoring homeostatic glycosylation profile, led to a decrease in IL-6 levels. This study highlights the biological and clinical importance of glycosylation in IIM immunopathogenesis, providing a potential mechanism for IL-6 production. Taken together, our results pinpoints muscle glycome as promising biomarker for personalized follow-up and a potential target for new therapies in a patients' subgroup with an ominous evolution.

Keywords:

Inflammatory myopathies, glycosylation, pathogenesis, prognosis, biomarkers, damage, therapy.

RESUMO

As miopatias inflamatórias idiopáticas (MII) são um grupo raro, sistémico e heterogéneo de doenças, que conduz à incapacidade a longo prazo, diminuição da qualidade de vida e redução da expectativa de vida.

A origem destas doenças ainda não é totalmente compreendida, mas uma relação entre "*triggers*" ambientais e factores de risco genéticos, com um papel adicional de mecanismos imunorreguladores defeituosos, parece desempenhar um papel relevante na patogénese. Os mecanismos imunopatológicos incluem imunidade adaptativa, tanto humoral como celular, imunidade inata e mecanismos não imunes, numa interacção complexa, que provavelmente está subjacente aos diferentes fenótipos clínicos, progressão da doença e resposta à terapêutica.

A falta de consenso sobre a classificação das IIM, juntamente com a raridade e complexidade das doenças, tem dificultado o desenvolvimento de ensaios clínicos significativos para um tratamento dirigido. No entanto, a descoberta de múltiplos anticorpos específicos das miosites e suas associações clínicas forneceram uma visão sobre a subfenotipagem destes doentes, em relação ao envolvimento potencial de outros órgãos (como o pulmão), ao risco de malignidade e, em alguns casos, à resposta à terapêutica. Apesar de a sobrevida ter melhorado nos últimos anos, os doentes com MII apresentam uma elevada sobrecarga de doença, relacionada com a acumulação de dano e comorbilidades, que são um reflexo, na maioria dos casos, de uma exposição prolongada aos corticóides. A análise de uma coorte bem caracterizada da "Unidade de Imunologia Clínica" do Centro Hospitalar Universitário do Porto incluiu os resultados de 149 pacientes, que mostraram que tanto o dano como as comorbilidades estavam presentes em mais de 90% dos casos. A maioria das comorbilidades foi consequência do dano, nomeadamente dano cardiovascular. Embora a acumulação de comorbilidades tenha sido o principal factor para a má qualidade de vida, a gravidade do dano revelou ser o principal preditor de mortalidade. São necessárias estratégias terapêuticas melhoradas para reduzir a necessidade de corticóides e para introduzir, de forma rotineira, o rastreio e gestão de comorbilidades como um parceiro essencial da terapêutica imunossupressora, conduzindo a um cuidado abrangente dos doentes com miosite e a uma melhoria efectiva da sua qualidade de vida.

Evidências acumuladas sugerem um papel proeminente dos glicanos nos circuitos reguladores que governam as respostas imunológicas, apoiando o impacto dos *N*-glicanos ramificados complexos na manutenção da autotolerância. O comprometimento da ramificação dos *N*-glicanos e a consequente acumulação de estruturas menos

complexas de glicanos com alto teor de manose, semelhantes aos presentes nos microrganismos, podem, portanto, contribuir para a patogénese de várias doenças autoimunes, através da modificação de epítopos ou da formação de (glico) neoantigéneos, que desencadeiam a activação da resposta imune. Uma glicosilação alterada (particularmente uma expressão diminuída de *N*-glicanos ramificados) tem sido relacionada com a quebra da tolerância imunológica e uma maior susceptibilidade a doenças autoimunes, além da associação à gravidade da doença. Em particular, alterações no perfil de expressão dos glicanos podem ser a ligação entre as respostas imunes inatas e adaptativas e a susceptibilidade a doenças autoimunes. A glicoassinatura celular, além de contribuir para uma compreensão mais profunda da fisiopatologia destas doenças, tem potencial para ser utilizada como biomarcador para prognóstico e estratificação de doentes e, portanto, um alvo potencial para a terapêutica dirigida nas IIM, bem como noutras doenças autoimunes. Os nossos resultados mostraram que as biópsias musculares de doentes com MII revelaram uma deficiência na via de glicosilação, resultando na perda de N-glicanos ramificados. Ao diagnóstico, esta glicoassinatura foi capaz de prever recidiva da doença e refractariedade ao tratamento, com elevadas sensibilidade e especificidade. As células T $CD4^+$ periféricas de doentes com doença activa apresentaram deficiência de *N*-glicanos ramificados, associada ao aumento da produção de interleucina (IL) -6, que poderá ser usada no futuro, como um biomarcador acessível para o acompanhamento individual e a abordagem terapêutica. A suplementação com glicanos, restaurando o perfil de glicosilação homeostática, resultou numa diminuição nos níveis de IL-6. Este estudo destaca a importância biológica e clínica da glicosilação na imunopatogénese das IIM, fornecendo um mecanismo potencial para a produção de IL-6. Em conjunto, os nossos resultados apontam o glicoma muscular como um biomarcador promissor para acompanhamento personalizado e um alvo potencial para novas terapêuticas num subgrupo de doentes com uma evolução desfavorável.

Palavras/chave:

Miopatias inflamatórias idiopáticas, glicosilação, patogénese, prognóstico, biomarcadores, dano, terapêutica.

Acronyms

ACPA – anti-citrullinated protein antibodies

ACR – American College of Rheumatology

ADCC – antibody-dependent cellular cytotoxicity

AID – autoimmune diseases

AMPD – adenosine monophosphate deaminase

APCs – antigen-presenting cells

aSyS – antisynthetase syndrome

BCR – B cell receptors

CADM – clinical amyopathic dermatomyositis

CCI – Charlson comorbidity Index

CD – cluster of differentiation

CK – creatine kinase

cN-1A – citosolic 5'-nucleotidase 1A

CTLA-4 – cytotoxic T lymphocyte antigen 4

DCs – dendritic cells

DC-SIGN – dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

DM – dermatomyositis

ENMC – European neuromuscular centre

ER – endoplasmic reticulum

EULAR – European League Against Rheumatism

Fab – antigen-binding fragment

Fc - crystallisable fragment

FcγRs – Fc-gamma receptors

GNA – *Galanthus nivallis* agglutinin

GNE – glucosamine (UDP-N-acetyl)-2-epimerase/*N*-acetylmannosamine kinase

GnT-V – *N*-acetylglucosaminyltransferase V

HAQ-DI – health assessment questionnaire - disability index

hIBM – hereditary inclusion body myopathy

HLA – human leukocyte antigen

HMGCR – 3-hydroxy-3-methylglutaryl-coenzyme A reductase

IBD – inflammatory bowel disease

IFN-γ – interferon-gamma

Ig – immunoglobulin

IIM – idiopathic inflammatory myopathies

IL – interleukin

ILD – interstitial lung disease

IMACS – international myositis assessment & clinical studies group

IMNM – immune-mediated necrotizing myopathy

IP – interferon-inducible protein

IVIg – intravenous immunoglobulin

JAK – Janus kinase

L-PHA – *Phaseolus vulgaris* leucoagglutinin

MAAs – myositis-associated auto-antibodies

MDA5 – melanoma differentiation–associated protein-5

MDI – myositis damage index

MHC – major histocompatibility complex

MIP-1a – macrophage inflammatory protein 1a

MRI – magnetic resonance imaging

MSAs – myositis-specific antibodies

NXP2 – nuclear matrix protein 2

OM – overlap myositis

pDCs – plasmacytoid dendritic cells

PL – precipitation line

PM – polymyositis

QoL – quality-of-life

RA – rheumatoid arthritis

RNP – antinuclear ribonucleoprotein

ROS – reactive oxygen species

SAE – anti-small ubiquitin-like modifier activating enzyme

SARS-CoV-2 - severe acute respiratory syndrome coronavirus 2

sIBM – sporadic inclusion body myositis

Siglecs – sialic acid-binding lectins

SLE – systemic lupus erythematosus

SRP – signal recognition particle

SSc – systemic sclerosis

STALs – siglec-engaging tolerance-inducing antigenic liposomes

TCR – T cell receptors

Tfh – T follicular helper

Th – T helper

TIF-1γ – transcriptional intermediary factor-1 gamma

TLR – toll-like receptors

Treg – regulatory T cells

tRNA – transfer ribonucleic acid

UIC – unidade de imunologia clínica

CHAPTER I

Introduction

1. Idiopathic Inflammatory Myopathies

1.1 Definition and epidemiology

Immune-mediated or idiopathic inflammatory myopathies (IIM) are a group of rare systemic autoimmune disorders, characterized by proximal skeletal muscle weakness, which leads to long-term disability, decreased quality of life and a reduced life expectancy. IIM are generally characterized by an increase of muscle enzymes, with or without skin involvement and extramuscular organ involvement, predominantly the lungs, with a consequent interstitial lung disease (ILD), but also others, such as the gastrointestinal tract, heart and joints (1-3). Epidemiology is not well known, due to differences in classification criteria, but reported incidence of IIM as a whole ranges from 1.16 to 19/million/year and their prevalence from 2.4 to 33.8 per 100.000 inhabitants (4, 5).

1.2 Pathophysiology

The aetiology of IIM is still unknown, but a relationship between environmental triggers and genetic risk factors, with an additional role of faulty immunoregulatory mechanisms seem to play a relevant role in the pathogenesis of the disease (4).

1.2.1 Environment and genetics

Environmental triggers can be identified in some patients with IIM, some of which are linked to myositis specific autoantibodies. The most frequently mentioned triggers are infection, notably viral, and drug side effects, particularly statins and immune checkpoint inhibitors (6). Recently, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and directed vaccines have also been implicated as triggering some cases of myositis (7, 8). However, the precise relationship between these environmental exposures and IIM development remains unclear. Proposed mechanisms for viral-induced myopathy include the exposure of cryptic epitopes to T-cell recognition (with host transfer ribonucleic acid (tRNA) synthetase used for viral replication), molecular mimicry between pathogens and host proteins, and the activation of auto-reactive B cells leading to the production of autoantibodies. Malignancy has also been implicated in IIM etiopathogenesis, as a paraneoplastic syndrome. Neoantigens expressed by malignancies, such as transcriptional intermediary factor-1 gamma (TIF-1γ), are highly immunogenic and induce a vigorous immune response. Anti-tumour immunity can develop against normal host proteins that are highly expressed on tumour cells, through epitope spreading. On the other hand, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) protein

conformation is altered by statins, exposing cryptic epitopes and trigger autoimmunity against muscle cells, which are rich in HMGCR (6). Immune-related adverse events of immune checkpoint inhibitors have been identified in several organs, including muscle tissue, although the precise mechanism of immune deregulation is not known.

Environmental factors primarily act as disease triggers in genetically susceptible individuals. Geographic location and ethnicity may influence susceptibility to autoimmune diseases. More specifically, human leukocyte antigen (HLA) molecules play a role in T-cell receptor development, peripheral tolerance and immune response to environmental agents. Studies investigating the association of IIM subtypes with HLA suggest that HLA-DRB1*03:01 and HLA-DQA1*05:01 alleles are potential risk factors for myositis in Western populations, while DRB1*08:03 is associated with Polymyositis (PM) among the Japanese population. In northern China populations, HLA-DRB1*04, HLA-DRB1*07, and HLA-DRB1*12 increase susceptibility to Dermatomyositis (DM), while HLA-DQB1*04:01 is a risk factor for both DM and PM. Finally, HLA DRB1*09:01 is linked to DM development in Chinese and Mexican populations (9).

Major histocompatibility complex (MHC) upregulation, both classes I and II, is a typical early finding in skeletal muscle in PM. Additionally, there is an upregulation of interferon-gamma (IFN-γ) transcript expression, involved in MHC class II molecules induction. In contrast, DM exhibits high IFN α and β inducible genes, while antisynthetase syndrome (aSyS) dysplays moderate expression and sporadic Inclusion Body Myositis (sIBM) shows low expression of these genes. These differences highlight variations in etiopathogenesis and suggest potential therapeutic targets (9).

1.2.2 Adaptive immunity

The immune system is a complex and redundant system dedicated to preserving the human body's health. Innate immunity is the archaic and conserved part of the immune system, characterized by a raid and indiscriminate attack to whatever appears dangerous to our organism, whether self or non-self. Adaptive immunity is the eclectic and refined part of the system developing skills across the years, upon presentation of different antigens, by dendritic cells among others, which cause gene rearrangements at the T and B cell receptors, predisposing them to keep memories of these foreign proteins, and respond accordingly. Although classically divided, they can be seen as one single complex system that interplays all steps of the way, since we are born until we die, battling all external and internal fights for promoting our health.

Only a fraction of the real physiologically process is known and only few pathophysiological mechanisms have been identified in immune-mediated diseases. The common denominator in all IIMs is muscle inflammation and, histologically, immune cell

invasion into non-necrotic muscle cells is considered pathognomonic of IIM. To date, the immunopathological mechanisms of this group of conditions remain incompletely understood, but they involve the infiltration of T- and B-cells in muscle tissue, the presence of myositis-specific antibodies (MSAs) and myositis-associated auto-antibodies (MAAs), and the overexpression of MHC class I in myofibers. The etiopathogenesis of IIM includes humoral and cellular immunity mechanisms, triggered by an underlying factor, likely initiated by innate immunity players that provoke the adaptive immune response and break of tolerance (10).

The molecular basis of IIMs in humans is diverse, involving complex cellular components, most probably contributing to differences in disease susceptibility, clinical and histopathological phenotypes, and severity. However, fundamental questions about the triggering events' nature that break tolerance and sensitize T cells, the antigenic muscle peptides recognized by T cells and the role of B cells remain unanswered.

Immunohistochemical studies on muscle biopsies reveal two main types of inflammatory infiltrate: endomysial and perivascular/perimysial. Endomysial infiltrates primarily consist of cluster of differentiation (CD)8^+ T lymphocytes, often surrounding non-necrotic fibers, suggesting an immune response targeting myofibers (Fig. 1). In contrast, perivascular infiltrates are dominated by $CD4^+$ T lymphocytes and macrophages, indicating an immune reaction directed at microvessels. $CD8^+$ T cells are specifically selected and clonally expanded *in situ* by muscle-specific autoantigens, presented by the MHC-I molecule expressed on muscle fibers, suggesting an antigen-driven T-cell response (10).

Activation of T cells and antigen recognition involve co-stimulatory and adhesion molecules, chemokines, and cytokines. Muscle biopsies show the expression of these molecules and cytokines, such as interleukin (IL)-6, 8, 10, monocyte chemoattractant protein 1, macrophage inflammatory protein 1a (MIP-1a), and interferon-inducible protein (IP)-10, in endomysial inflammatory cells and the neighbouring extracellular matrix, enhancing leukocyte recruitment, trafficking and activation. Muscle fibers in the body express MHC-I molecules on their surface and these play a crucial role in presenting antigens to $CD8^+$ T cells as part of the immune response. On the other hand, muscle fibers expressing MHC-I molecules must co-express co-stimulatory molecules from the B7 family, including B7-1 (also known as CD80), B7-2 (also known as CD86) and BB1, in order to interact with $CD8^+$ T cells. Cell-mediated immune response is mediated essentially by these specialized T cells, which can invade or infiltrate tissues, including muscle tissue, in response to specific signals or antigens, and acting essentially in the recognition and eliminating infected or abnormal cells. Autoinvasive $CD8^+$ T cells express specific receptors known as CD28 and cytotoxic T lymphocyte antigen 4 (CTLA-4), which

are essential for interacting with target cells, such as muscle cells or antigen-presenting cells (APCs).

In peripheral blood, the T lymphocyte repertoire differs between active and inactive forms of myositis patients. Expanded T lymphocytes clonally display a memory phenotype, express intracellular perforin, and respond to stimulation by IL-2, suggesting potential for reactivation under appropriate conditions.

This implies a continuous local autoantigen-driven process in the disease, emphasizing the concept of a chronic disorder following a triggering event. In the context of IIM, it is proposed that clonally expanded $CD8^+$ T lymphocytes that have infiltrated muscle tissue may recirculate into the bloodstream. This means that T cells that have initially migrated into the muscle tissue later re-enter the bloodstream. The exact mechanism by which this recirculation occurs is not fully understood. Another possibility is that the same antigen, such as a virus, could independently induce the expansion of specific $CD8^+$ T cell clones in both the bloodstream and muscle tissue. In this scenario, if the antigen is present at both sites (muscle tissue and blood), it could trigger the proliferation of antigen-specific T cells in both locations, leading to the clonal expansion of T cells, sharing the same antigen specificity, in both compartments. Understanding the mechanisms underlying the presence and behaviour of these clonally expanded T cells is important for unravelling the pathogenesis of IIM and may have implications for the development of targeted therapeutic strategies. Further research is needed to elucidate the exact processes involved in the recirculation and expansion of T lymphocytes in IIM.

In IIM patients, T lymphocytes are found not only in inflammatory muscle tissue or blood but also in the lungs, especially in those with ILD, a common extra-muscular manifestation in patients with IIM (found in up to 60 to 70% of these). A restricted accumulation of T lymphocytes expressing specific T-cell antigen receptor V-gene segments is observed in skeletal muscle and lung, but not in peripheral blood, suggesting a common target antigen in these organs.

Humoral immunity and B lymphocytes role in the pathogenesis of IIMs is supported by frequently detected autoantibodies in IIM patients. MSAs are detected in 70-80% of these patients and these have been used for phenotypic classification, disease evolution and prognosis, including malignancy risk, and, in some cases, therapeutical response. However, their role in pathogenesis remains poorly understood as being actually pathogenic or an epiphenomenon (i.e. a marker of disease process).

1.2.3 Innate immunity

Pathological autoimmunity seen in myositis and other autoimmune disorders with diverse autoantibodies can arise not only from the specificity of the autoimmune response but also

from factors such as the location and duration of the triggering stimulus. Evidence suggests a localized antigen-driven response in IIM, indicating that not only T lymphocytes but also other cells like (such as, B lymphocytes and plasma cells) may drive intramuscular antigen-specific autoimmunity (10).

Dendritic cells (DCs) are bone marrow-derived immune cells that bridge innate and adaptive immune systems. They are known as professional APCs and are responsible for initiating and activating naïve T-lymphocytes. In muscle biopsies of patients with DM and PM, both immature and mature DCs have been identified (11).

Within the context of myositis, myeloid dendritic cells, which are specialized in antigen presentation and T cell activation, are notably abundant and infiltrate myofibers, particularly in cases of PM and sIBM. They are often found in close proximity to T cells, suggesting their involvement in presenting antigens and activating T cells within muscle tissue. These cells also appear to regulate the induction of cytokines and chemokines mediated by type I IFN in DM, indicating a potential link between DCs and the type I IFN signatures observed in myositis muscle.

On the other hand, plasmacytoid dendritic cells (pDCs), which share characteristics with plasma cells and express markers like CD4 and myeloid-cell markers such as MHC class II, CD36, CD68, and CD123, are capable of producing type I IFNs and other chemokines in response to viral nucleic acids. They do so through the activation of endosomal Toll-like receptors (TLR-7 and TLR-9), effectively bridging the innate and adaptive immune mechanisms. Plasmacytoid DCs are known to secrete type I IFNs and other cytokines, making them key players in immune responses. Their interaction with innate immune cells and cytokines, as well as their dynamic role in the context of different disease subtypes and stages, highlights the complexity of immune responses in myositis and suggests that these interactions vary depending on the specific type and progression of the disease.

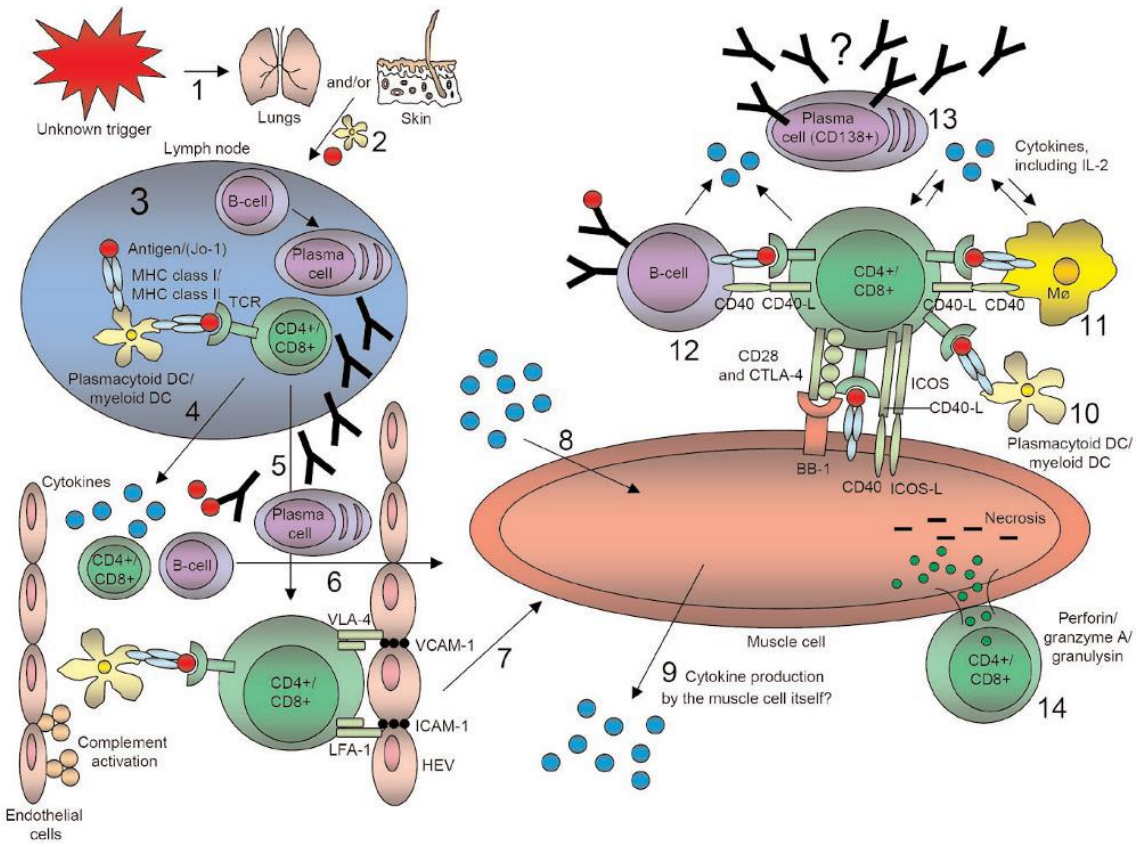

Fig. 1 – Hypothetical involvement of T lymphocytes, B lymphocytes and dendritic cells (DCs) in idiopathic inflammatory myopathies. (1) An unknown trigger (for example viral infection or ultraviolet radiation) in the respiratory tract or through the skin leads to the cleavage of histidyl-tRNA synthetase by granzyme B through antiviral $CD8^+$ T lymphocytes in the lungs. (2) Immature DCs carry receptors on its surface that recognize common features of many pathogens. When a DC takes up a pathogen in infected tissue it becomes activated and migrates to the lymph node. (3) Upon activation, the DC matures into a highly effective antigen-presenting cell (APC) and undergoes changes that enable it to activate pathogen-specific lymphocytes in the lymph node. T lymphocytes become activated and B lymphocytes, with active help from $CD4^+$ T lymphocytes, proliferate and differentiate into plasma cells. (4) Activated DCs, T lymphocytes, and B lymphocytes could release cytokines into the bloodstream. (5) The activated T lymphocyte, on which the DC-MHC–antigen complex is bound, itself binds to specialized endothelial cells called high endothelial venules (HEV). For this purpose it uses the VLA-4 (very late activation antigen-4) and LFA-1 (lymphocyte function associated antigen-1) molecules on its surface to interact with adhesion molecules (vascular cell-adhesion molecule-1 (VCAM-1) and intercellular cell-adhesion molecule-1 (ICAM-1)) on HEVs, where they can penetrate into peripheral lymphoid tissues (6, 7). Naïve T lymphocytes and B lymphocytes that have not yet encountered their specific antigen circulate continuously from the blood into the peripheral lymphoid tissues. (8,9) Various cytokines from the bloodstream or produced locally could affect the muscle tissue or cell in many different ways. However, it is not clear whether the muscle cell itself could produce and release cytokines. (10-12) DCs, macrophages (Mφ), and B lymphocytes can interact with T lymphocytes in various

ways. T lymphocytes could possibly also bind to muscle cells through inducible co-stimulators (ICOS), CD40 ligand (CD40-L), CD28, and CTLA-4 (CD152) on T lymphocytes to ICOS ligand (ICOS-L), CD40, and BB-1 antigen on the muscle cell. In that fashion, the muscle cell would function as an APC. (13) Plasma cells ($CD138^+$) can be found in the muscle tissue of certain subgroups of patients with idiopathic inflammatory myopathy, but whether these cells could produce autoantibodies locally is not yet known. (14) T lymphocytes have been shown to bind in close contact with muscle cells and to release perforin, granzyme A, and granulysin, which may cause necrosis of muscle tissue or cells (**From 10.**).

1.2.4 Non-immune mechanisms

IIM are characterized by three primary histological features: inflammation, fibrosis, and loss of muscle fibers, and these features underlie the clinical symptoms of muscle weakness. While much research has focused on cellular and molecular mechanisms, there is growing recognition of the contribution of non-immune mechanisms to the pathology of IIM. This is crucial because autoimmunity alone cannot explain certain clinical observations, such as:

- Lack of correlation between inflammation and muscle weakness: In some cases, the degree of muscle inflammation does not correlate with the severity of muscle weakness, suggesting that factors beyond immune-mediated inflammation play a role in muscle damage.
- Poor response to immunomodulatory drugs: Some myositis patients do not respond well to potent immunosuppressants, indicating that other mechanisms may be involved in the disease process.
- Limited clinical improvement after immune cell removal: Even after complete removal of inflammatory infiltrates from muscle tissue, clinical disease amelioration is not always observed, implying that non-immune factors may contribute to muscle damage and weakness.

Furthermore, there is evidence from congenital glycosylation disorders, such as hereditary Inclusion Body Myopathy (hIBM), which is originated from a mutation in the glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase (*GNE*) gene involved in sialic acid biosynthesis, which underscores the role of non-immune factors in muscle pathology (11).

One intriguing observation in IIM is the up-regulation of MHC class I molecules in myofibers, which is associated with endoplasmic reticulum (ER) activation of stress responses. This suggests a potential interplay between immunological and non-immunological processes in the development of autoimmunity. The ER has a major role in maintaining musculoskeletal system homeostasis and protein regulation. ER stress relief

mechanisms may be linked to inflammatory processes that trigger a humoral immune response, indicating that ER stress could contribute to the initiation of IIM and the production of associated autoantibodies (12). The ER is highly sensitive to various challenges, including calcium depletion, protein glycosylation alterations, disulphide bond formation, hypoxia, redox conditions, and viral infections, which may result in unfolded or misfolded proteins accumulation, resulting in ER stress and an ensuing inflammatory response. MSAs may also play a role in muscle atrophy by influencing the transcription of specific genes and generating reactive oxygen species (ROS), while reducing the production of anti-inflammatory cytokines, like IL-4 and IL-13 (11).

In addition to immune and ER-related mechanisms, acquired metabolic defects within skeletal muscles have been reported in IIM. These defects often involve deficiencies in glycolytic enzymes and proteins that are more prevalent in fast-twitch muscle fibers (13). One example is the acquired deficiency of the rate-limiting enzyme adenosine monophosphate deaminase (AMPD) 1 in the purine nucleotide cycle. While the precise role of these metabolic pathways in myofiber damage remains unclear, there is a possibility that they are regulated by innate TLR pathways and pro-inflammatory cytokines, like IL-15, in IIM. Consequently, autophagy is proposed as a potential contributor to myofiber damage, though further research is needed to fully understand its role.

Lastly, perifascicular atrophy observed in DM may reflect endofascicular hypoperfusion, which leads to early activation of the complement C5b-9 membranolytic attack complex. This activation results in capillary necrosis, a reduction in endomysial capillaries, ischemia, and muscle fiber destruction resembling microinfarcts, similar to those seen in systemic vasculitis. The binding of C1q to endothelial cells triggers the activation of the membranolytic attack complex, leading to the release of proinflammatory cytokines and upregulation of adhesion molecules on endothelial cells. This process facilitates the migration of activated lymphocytes, including B cells, $CD4^+$ T cells, and pDCs, into perimysial and endomysial spaces (11, 13).

In summary, multiple pathogenic pathways appear to converge to contribute to muscle damage and weakness in IIM. Innate and adaptive immune responses, as long as metabolic abnormalities, appear to be the most important. There is a growing understanding that innate immune pathways may serve as a bridge connecting the adaptive and metabolic aspects of the disease, emphasizing the complexity of IIM pathogenesis.

1.3 Diagnosis *versus* Classification/ Clinical phenotypes

The European League Against Rheumatism (EULAR)/American College of Rheumatology (ACR) classification criteria, published in 2017 (14), updated the IIM classification but have since been highly controversial (15-17). Despite the high specificity, sensitivity is low due to the exclusion of some clinical involvements, namely pulmonary and articular, the exclusion of specific autoantibodies (other than anti-Jo1) and the high reliability on very specific parameters of muscle biopsy (which is increasingly rarely performed in clinical practise), that do not contain histological features suggestive of necrotising myopathy (18-20). Failure to recognise Anti-Synthetase Syndrome (aSyS) as an individual disease was also highlighted as a weakness (21). As a result, many patients with myositis are not considered when applying these criteria and a significant proportion is classified as polymyositis, which is currently considered the rarer subtype of IIM. In summary, these criteria should be used in clinical trials, but their role in clinical diagnosis is of less importance.

Finally, diagnosis is a clinical act, based on medical knowledge of the disease symptomatic presentation and evolution, most probable pathophysiology and exclusion of mimickers in differential diagnosis.

At the present, most clinicians consider as main subtypes of IIM in adults: DM, Clinical Amyopathic DM (CADM), Overlap Myositis (OM), aSyS, Immune-mediated Necrotizing Myopathy (IMNM), PM, sIBM, Paraneoplastic Myositis and Non-Specific Myositis.

One of the remarkable clinical features of IIM is the presence of MSAs (Fig. 2), which has been used to predict manifestations of the disease, beside the typical muscle weakness, such as ILD and the possibility of malignancy (22, 23). However, a long-standing debate continues to persist as to the extent to which the presence of the various autoantibodies usually associated with IIM represents accurate biomarkers for recognizing myositis subcategories and if they may actually have a pathogenic role in the disease process (24). On the other hand, around 20-30% of patients are seronegative, i.e. do not display any autoantibody. Therefore, there is an urgent need in the clinical setting of accurate and reliable biomarkers for improving IIM subsets classification, since this lack of diagnostic and prognostic biomarkers has been hindering major advances in the treatment of these disorders.

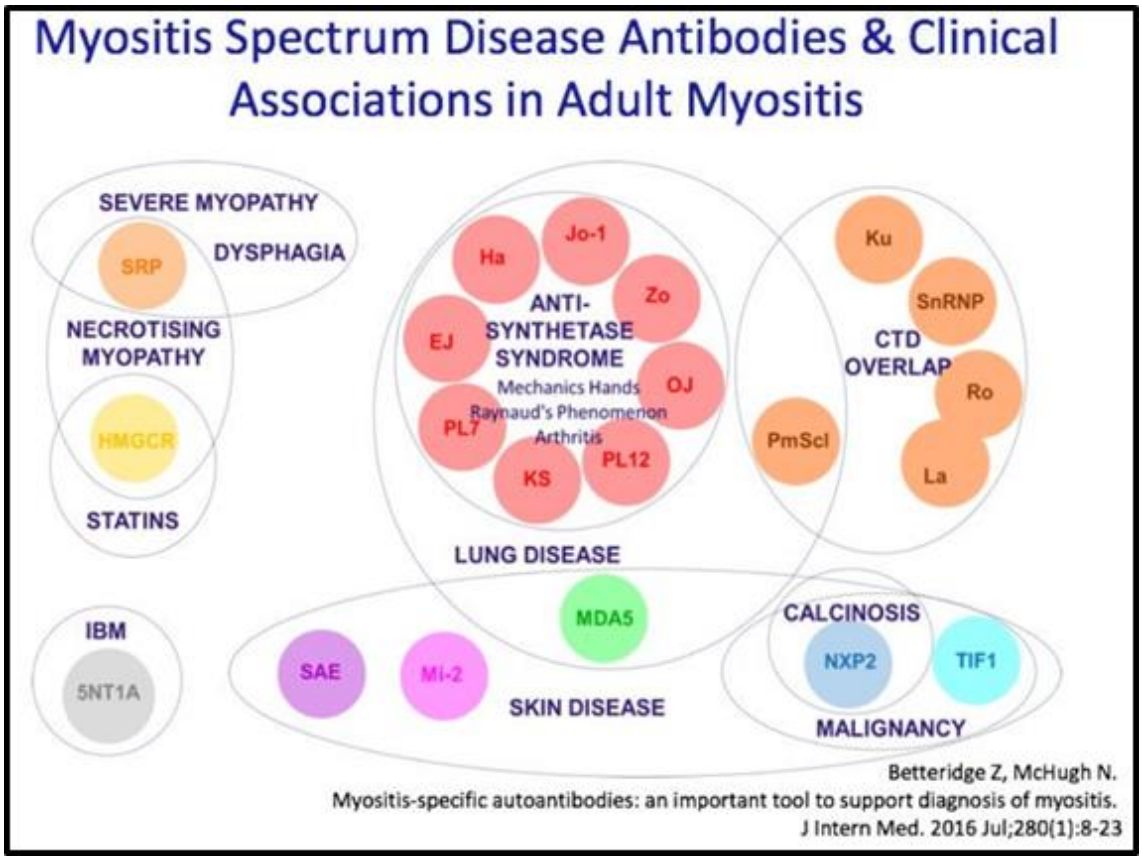

Fig. 2 – Myositis specific and associated antibodies and respective clinical associations (**From 23**).

Dermatomyositis

Dermatomyositis is one of the most frequent subtypes of IIM. Patients present with typical cutaneous lesions, predominantly at ultra-violet-radiation exposed sites, and usually associated with pruritus, which might be severe. Gottron's papules and heliotropic rash are the most common and considered pathognomonic by most experts. However, several other cutaneous changes may be present, such as shawl and V signs, Holster lesions, peri-ungueal erythema, nail-fold telangiectasias and cuticular overgrowth. Psoriasiform lesions at extensor surfaces or the scalp and linear extensor erythema are less frequent. Diffuse erythroderma has been associated with paraneoplastic DM. Finally, ovoid palatal patches have been associated to anti-TIF1-γ positive DM (25).

Usually, cutaneous lesions appear before or at the same time of proximal muscular weakness, although they can appear after this. When concomitant, particularly if specific antibodies are also present, such as anti-Mi2, anti-TIF1-gamma, anti-nuclear matrix protein 2 (anti-NXP2) or anti-small ubiquitin-like modifier activating enzyme (anti-SAE), diagnosis can be assumed without the need for muscle biopsy.

Clinical Amyopathic Dermatomyositis

In CADM, muscular weakness is absent or is minimal. Most patients are positive for anti-melanoma differentiation–associated protein-5 (anti-MDA5) antibodies. Ulcerated lesions, which may evolve to necrosis, at extensor surfaces of the hands, elbows, ear lobes or calcaneus, typically associate with rapidly progressive lung disease in anti-MDA5 positive CADM (26). At the palmar sites, distal erythema plaques can also be present in these patients. This is the most serious form of myositis, as it is usually very refractory to treatment and progresses rapidly to respiratory failure and multi-system degradation with high rate of early mortality. Ulcerated cutaneous lesions associated with rapidly progressive ILD are a synonymous of anti-MDA5 positive CADM and immediate treatment should not be delayed by the confirmation of the antibodies or muscle histology (which is not necessary in this setting). One of the characteristics of thoracic alterations in these patients is the presence, along with rapidly progressive ILD, of spontaneous pneumothorax and/ or pneumomediastinum (27).

Anti-Synthetase Syndrome

The classic triad of aSyS comprises interstitial lung disease, present in 85 to 90% of patients at some point of disease evolution, myositis and arthritis in up to 65% of patients (21). However, other manifestations are typically present in this setting, such us Raynaud phenomenon, mechanic's hands skin lesions and sometimes fever (in the most acute presentations). Mechanic's hands lesions, which typically appear at the first 2 or 3 fingers of the hands, and less frequently hiker feet lesions (the equivalent but in feet fingers), are particularly important as they usually associate with active ILD. The diagnosis is made when anti-aminoacyl-tRNA synthetase (anti-synthetase) antibodies are confirmed at the circulation and preclude muscle histology confirmation (28). At the present 8 different anti-synthetase antibodies are described, although histidyl-transfer RNA synthetase (anti-Jo1) is clearly the most prevalent, followed by anti-precipitation line (PL)12 and anti-PL7. Of notice, the concomitant presence of anti-Ro52 and anti-Jo1 portends a more severe ILD and consequently worst prognosis.

Some authors consider that aSyS should be regarded as a separate entity and not part of IIM, mainly because ILD can be the only manifestation for several years before myopathy appears in up to 50% of patients. On the other hand, symmetric and bilateral polyarthritis of the small joints of the hands (most commonly of the interphalangeal joints), similar to rheumatoid arthritis (RA), may also be the first and isolated manifestation for years in

around 25% of the patients, and some of these actually receive the diagnosis of RA as the initial classification (29, 30).

Immune-mediated Necrotizing Myopathy

IMNM is now considered as the most frequent subtype of IIM (7), and consists of a serious myopathy, presenting usually as an acute or subacute proximal muscle weakness and characterized by a severe rhabdomyolysis (creatine kinase (CK) levels ranging from 100-400x the normal lower limit). It might be triggered by viral infections, drugs (such as statins or immune check point inhibitors) or malignancy. Neck extensors, pharyngeal and respiratory muscles can be involved and the heart can also be affected (as a serious cardiomyopathy), particularly in patients with anti-signal recognition particle (anti-SRP) antibodies (31). At the present, two thirds of patients have circulating antibodies, divided between anti-HMGCR positive and anti-SRP positive; one-third of the patients has no detectable antibodies (seronegative). Muscle biopsy continues to be essential for the definitive diagnosis, essentially for the differential diagnosis with toxic myopathies and less frequently congenital myopathies. This subtype of IIM is usually more refractory to immunosuppressive treatment. Until a few years ago the form associated with anti-HMGCR was considered as a result of statin exposure, but nowadays it is clear that these antibodies may be present in patients without pharmacological history at all.
Finally, is worthy to emphasise that isolated rhabdomyolysis, even when severe, should not lead to an immediate diagnosis of IIM, as it is almost always associated with other manifestations in the context of IIM (32).

Sporadic Inclusion Body Myositis

Contrary to the other forms of IIM, sIBM is more frequent in males, especially over 50 years-old and along with proximal myopathy courses with distal myopathy, mostly asymmetrical (33). Usually sIBM is slowly progressive with most patients showing muscle atrophy and dysphagia at diagnosis. Rhabdomyolysis and systemic inflammatory markers are absent or minimal, and most patients are seronegative. Recently, anti-citosolic 5'-nucleotidase 1A (cN-1A) antibodies were identified and are described in 30-50% of sIBM patients (7). However, these are not specific for myositis as they can be found in patients with Systemic Lupus Erythematosus (SLE) and Sjögren's syndrome. Finger flexors and/or foot extensors weakness is a quick and practical way to recognise these patients. Atrophy

of the forearms and quadriceps muscles as long as frequent falls due to quadriceps weakness are also clues to early clinical diagnosis (7). However, muscle biopsy remains essential for diagnosis (34). Haemotoxin-eosin analysis reveals rimmed vacuoles in muscle cells, and the typical inclusion bodies can then be confirmed with electronic microscopy. Of note, in up to 30% of patients with the typical clinical sIBM-phenotype, the biopsy does not show vacuoles or amyloid deposits but only inflammation (more commonly at the early course of the disease); leading to erroneous diagnosis of polymyositis. Most patients with sIBM are refractory to immunosuppressive therapy (35).

Overlap Myositis

OM represents a connective tissue overlap syndrome, in which myositis can be the first manifestation or it may appear along the disease course of other systemic autoimmune diseases (AID) (36). It often presents with systemic sclerosis-like lesions and the most frequent associated entity is Systemic Sclerosis (SSc). However, Mixed Connective Tissue Disease, RA, Sjögren's syndrome or SLE are other AID that may be overlap with IIM. MAAs are frequently present, namely anti-Ku, antinuclear ribonucleoprotein (anti-U1 RNP), anti-Ro, among others. The presence of anti-PM-Scl antibodies is particularly important, as it is associated with ILD development (37), which not rarely evolves to fibrosis.

Polymyositis

PM is now considered the rarer subtype of IIM, with some authors even considering that is actually almost an extinct entity (38-40). With the description of MSAs, most patients previously diagnosed with polymyositis are now classified as aSyS, OM or IMNM.
The few that remain are usually women, presenting with an insidious and progressive proximal weakness, which spares para-spinal and oculomotor muscles, and might complicate later with dysphagia. Constitutional symptoms and manifestations of other organs may be present and rhabdomyolysis is typical. Non-specific antibodies may be present and muscle biopsy remains essential for diagnosis confirmation.

Paraneoplastic Myositis

Myositis is one of the most frequent paraneoplastic syndromes, more frequently with DM presentation (up to 15% of patients) and it may be the “tip of the iceberg” for an early and potential curative stage of malignancy diagnosis, before cancer symptoms are apparent. Paraneoplastic Myositis is considered when associated with malignancy diagnosed at the same time of IIM or three years preceding or after myositis diagnosis. Malignancy associated with IIM follows the most frequent types in general population, i.e. lung, breast, colorectal, prostate, with the exception of ovarian cancer which is much more common in paraneoplastic myositis. If at myositis diagnosis an exhaustive cancer screening is mandatory in all patients, during-follow-up the frequency of rescreening can be guided by the presence of some autoantibodies. Anti-TIF1-γ antibodies are associated with high risk of malignancy (up to 40%), followed by anti-NXP2 antibodies (41). Therefore, myositis patients who carry one of these antibodies should have a tighter vigilance regarding malignancy (42). Anti-HMGCR has been associated with malignancy, although less frequently. Seronegative myositis and the CADM subtype are also considered as having higher risk of malignancy. Finally, when a patient becomes refractory to treatment, it is also important to rescreen for malignancy before considering therapy escalation.

Non-specific Myositis

Non-specific Myositis is considered in patients with proximal muscle weakness and rhabdomyolysis, without cutaneous lesions or specific antibodies. Histologically, there are muscle alterations, mostly inflammatory, with MHC-I upregulation, but without any of the specific patterns associated with PM, DM, aSyS, IMNM or sIBM (43).

1.4 Therapy

The therapeutic resources available for the treatment of IIM are very limited, with the choice of the various immunotherapeutic drugs being mainly empirical and based on the same principles applied to other autoimmune diseases (44, 45). There are no on-label drugs for IIM patients, with the exception of the recently approved use of high-dose intravenous immunoglobulin (IVIg) for refractory DM (46). The reasons include the rarity of IIM, their heterogeneous clinical phenotypes and absence of consensual classification criteria, as well as the small number of randomized, double-blind controlled clinical trials.

The main objective of the treatment is remission, through elimination of inflammation, restoration of muscle performance and prevention of muscle disability as well as damage at the several organs that may be affected. It is important to stress, that, both pharmacotherapy and physical rehabilitation combined from the diagnosis are essential to achieve these objectives.

The first therapeutic guidelines were published in 2022 (47). Immunosuppressive therapy depends on the severity of the disease and clinical phenotype, considering affected organs. Acute dysphagia, hypercapnic respiratory failure and acute ILD are considered emergencies among IIM patients. Therapy usually implies combination of potent immunosuppressive drugs (cyclophosphamide or rituximab) with other classic drug such as mycophenolate mofetil, calcineurin inhibitors (mainly tacrolimus) or azathioprine, after bolus of methylprednisolone. IVIg is particular effective in dysphagia and might be very useful in patients with active infection besides myopathy. Organ support in an intensive care unit is necessary in most of these serious presentations.

Oral corticosteroids are used in less serious presentations, but should be always combined from the diagnosis with an immunosuppressor (methotrexate, azathioprine, mycophenolate mofetil). Considering the several, serious and debilitating complications of prolonged and/or high-dose steroid therapy, associated to the fact that steroids are myotoxic, nowadays we tend to use lesser doses in bolus and in oral therapy and accelerate the weaning to zero or the lowest possible dose.

As already stressed out, non-pharmacological therapy is a key factor to achieve control of the disease, to recover muscle performance and to promote autonomy and quality of life. Physical therapy performed by trained and expert personnel must be regular and adequate to the level of weakness, starting to be inactive and moving to active (48). In active phases, non-invasive ventilation (for hypercapnic respiratory failure) and specific nutritional support (for all patients, considering the high catabolic potential of these diseases) are also essential.

1.5 Refractory disease in IIM

Different definitions have been used to designate refractory myositis in clinical trials (49-51).

However, de Souza *et al* definition is one of the most consensual (52). Refractory disease is considered in patients that present an inadequate response to the combination of two immunosuppressive agents at their maximal tolerated dosage, sequentially or concomitantly, for at least 3 months, and precluding steroids weaning.

Nevertheless, refractory disease should not be assumed before: checking therapeutical compliance, excluding malignancy, confirming that clinical manifestations are driven by active inflammatory disease, and not damage, and, in some cases, revise the diagnosis.

The first strategy is to add-on a different immunosuppressor to the one the patient is on.

Except for refractory DM, no therapy has been specifically approved for refractory patients.

Most common drugs used are rituximab (or, less frequently, cyclophosphamide), IVIg, particularly for refractory dysphagia or skin lesions, abatacept, and more recently, Janus kinase (JAK) inhibitors. Refractory ILD is one of the most difficult to treat manifestations and usually implies association of one biological agent and a classic immunosuppressive (mycophenolate mofetil or tacrolimus) plus IVIg (53). Combination of rituximab and cyclophosphamide is considered by some authors in rapidly-progressive ILD associated with anti-MDA5 antibodies (54, 55). In desperate cases, plasma-exchange therapy and stem-cell transplantation are also regarded as options (56).

1.6 Burden of disease

IIM share many of the features of autoimmune diseases such as RA and SLE, being a chronic, life-long and recurrent disorder that mainly affects professionally active populations with significant morbidity, economic burden and mortality (57-59). Although survival of these patients has improved since the widespread use of corticosteroids and immunosuppressive agents, patients with IIM continue to have increased mortality, with a reported 5-year survival rate of 60%, particularly in the first year after the diagnosis (60). Increased morbidity is primarily due to severe muscle weakness and visceral involvement. Recent reports indicate that only 20% to 40% of treated patients will achieve remission, whereas 60% to 80% will experience a polycyclic or chronic active, continuous course of the disease, resulting in great impact on quality of life in medium- and long-term follow-up, as up to 80% of treated patients are still disabled (57). Evolution in determining mortality

and the causes of cumulative morbidity of this chronic disease extends beyond a description of its epidemiology, though.

As long-term, chronic conditions, IIM are often associated with comorbidities, mainly resulting from damage accrual, which place an additional burden on patients' lives and limit their productivity and well-being (61, 62). Analysis of damage and comorbidities has rarely been the main objective in the published literature on IIM, and correlation with quality-of-life (QoL) scores is not described, but their impact on patients' disease evolution is clear.

1.7 Unmet needs

From the pathophysiology point of view, several issues remain unknown. In particular, the nature of the triggering events that break tolerance and sensitize T cells, the type of the antigenic muscle peptide recognized by the T cells and the role of B cells are poorly understood. Moreover, innate immunity is increasingly being implicated in IIM etiopathogenesis, but the precise molecular and cellular pathways are still not clear, and the putative role in linking it with metabolic acquired defects and other non-immune mechanisms remains poorly understood.

Diagnostic tools still need to be improved, as most testing is not specific (63). Electromyography is operator dependent and the irritable myopathy shown can originate from autoimmune, traumatic, and genetic, among other causes. Magnetic resonance imaging (MRI) is useful to differentiate between muscular edema and atrophy, but edema from inflammation can also derive from several different origins (autoimmune, traumatic, and genetic). Myositis is a patchy disease, so muscle biopsy may not even catch inflammatory/ diseases sites, most patients have a mixture of the described patterns and, in reality, perifascicular atrophy appears to be the only pathognomonic finding. The recent description of myositis-specific antibodies and the clinical correlations developed are actually the most specific tools for diagnosing these patients, giving us important information on prognosis and phenotype. However, until 20-30% of patients are seronegative and the real pathogenic role of a lot of these autoantibodies remains to be proved (64). On the other hand, we still do not understand why patients with the same clinical-serological phenotype have such different disease evolution and prognosis, including strikingly diverse treatment responses.

Furthermore, consensus is needed about classification in order to develop proper clinical trials. Targeted, and preferentially personalized, treatment relies on identifying and characterizing key molecular parameters/biomarkers that may help, early in the disease

course, to the proper diagnosis of the disease, and importantly, to the stratification of patients that are at risk for developing an aggressive disorder and consequently premature mortality and/or higher damage accrual (2, 44, 45, 65). Survival has surely improved in last years, but patients remain with poor QoL, mainly resultant of damage accrual and comorbidities.

2. Glycosylation

2.1 Definition and importance

The surfaces of all cells are covered with a dense coat of carbohydrates (sugars), and this sugar coating, or glycocalyx, is the main interaction pathway between cells and the microenvironment that includes microorganisms and the immune system.

Glycosylation is a fundamental post-translational modification process that occurs in essentially all cells. It involves the enzymatic addition of glycans (sugar chains) to other saccharides, proteins, and lipids, resulting in a diverse and abundant repertoire of glycans collectively known as the glycome. Glycans play a crucial role in various biological processes and as such are implicated in most known diseases. Approximately 50% of genes encode proteins that are glycosylated, highlighting the fundamental importance of glycans in biology (66).

Glycans are assembled sequentially through the coordinated actions of multiple enzymes, resulting in the formation of a heterogeneous array of carbohydrates with distinct structures and functions, which makes their full structural and functional analysis a challenging endeavour.

Glycans serve as key molecules not only in intracellular processes but also in cellular communication and, as such modulating the interactions between cells. Glycans can exist in various forms, including polysaccharides like glycosaminoglycans, as well as lipid-conjugated and protein-conjugated forms (66).

The process of glycosylation involves the attachment of glycans to specific sites on proteins or lipids. In the ER, a precursor glycan is co-translationally or post-translationally attached to asparagine residues on proteins (Fig. 3). This process includes the removal of glucose residues, which serves as a quality control step for proper protein folding. Subsequently, the glycoprotein enters the Golgi apparatus. In here, high-mannose structures, characterized by glycans comprising five to nine mannose residues, can undergo enzymatic trimming and extension. This modification is orchestrated by a

combination of glycosidases and glycosyltransferases, resulting in the formation of complex *N*-glycans (67).

In contrast, *O*-linked glycans are added exclusively post-translationally within the Golgi apparatus, attaching to specific serine (S) or threonine (T) residues found within complex sequence motifs. The composition of these glycans is influenced by several factors, including the availability of nucleotide sugars, the expression levels and subcellular localization of glycosyltransferases, glycan transporters, glycosidases, and the accessibility of specific consensus sequences.

The metabolic state of the cell also plays a role in determining glycan composition by influencing the trafficking of glycoproteins within the ER and the Golgi apparatus. It is important to note that nearly all proteins destined for secretion, membrane integration, or other cellular functions, and which pass through the ER, undergo glycosylation. This includes critical immune receptors such as T cell receptors (TCRs), B cell receptors (BCRs), secreted immunoglobulins, as well as MHC and MHC-like molecules. Each of these proteins carries unique glycan modifications that can significantly impact their structural properties, functional characteristics and interactions with other molecules in the cellular environment.

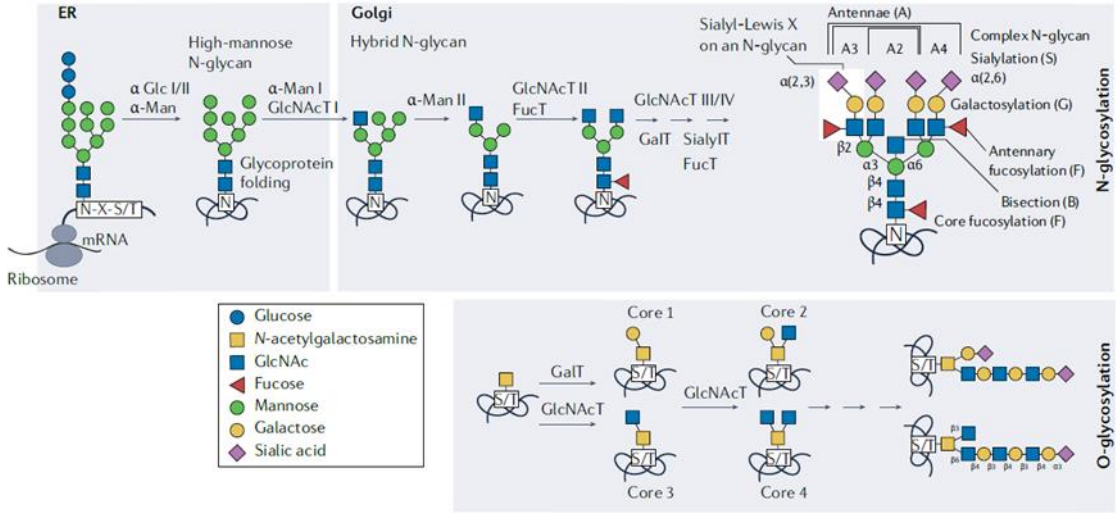

Fig. 3 – Biosynthesis of protein glycans (simplified). *N*-linked glycans are attached to asparagine (N) residues in N-X-S/T motifs, where 'X' is any amino acid except for proline. A precursor N-glycan is co-translationally or post-translationally attached in the endoplasmic reticulum (ER). After the removal of terminal glucose and mannose residues (folding control) by α-glucosidase (α-Glc) and α-mannosidase (α-Man) enzymes, and addition of *N*-acetylglucosamine (GlcNAc) by GlcNAc transferase (GlcNAcT), the protein enters the Golgi apparatus, where the high-mannose structure is trimmed down and subsequently built up to complex-type di-antennary (A2), tri-antennary (A3) and tetra-antennary (A4) *N*-glycans that may carry sialyl-Lewis X terminal motifs. *O*-linked glycans

are attached post-translationally in the Golgi apparatus to serine (S) or threonine (T) amino acids, and can be further extended (**From 68.**).

Glycans play critical roles in various cellular functions, encompassing the maturation and turnover of proteins, as well as facilitating cell adhesion, trafficking, and receptor binding and activation. Glycan linkages are indispensable for maintaining normal cell function and viability, and their regulation is highly intricate, particularly within the immune system (68). Changes in the glycome often occur in response to environmental and genetic cues, frequently associated with the development of altered cellular characteristics, such as malignancies and chronic inflammatory conditions (69-71).

Glycosylation profoundly influences the structure and function of proteins, primarily through steric effects involving interactions both between molecules and within the same molecule. Moreover, glycosylation can facilitate the production of glycan ligands recognized by specific lectins specialized in carbohydrate recognition (72). The interaction between lectins and glycans serves as a mechanism for molecular recognition, enabling organisms to discern the biological information within their own cellular glycomes and those of other organisms. This process serves as a means of distinguishing self from non-self, playing a pivotal role in immunological activation.

Remarkably, the process of glycosylation has been intricately linked with the evolution of living organisms (66). Evolution has witnessed a reduction in the utilization of mannose linkages and the introduction of novel glycosidic bonds carrying different saccharides, resulting in the emergence of more complex, branching glycans. These evolutionary changes primarily aimed to diversify and enhance the functionalities of proteins and lipids, while also serving as a means to distinguish between different organisms (i.e. self from non-self). It is noteworthy that many prokaryotic organisms, including bacteria, viruses, and fungi, lack the sophisticated ER and Golgi apparatus machinery required for synthesizing complex glycans. Consequently, at their cell surfaces predominantly feature less complex, highly mannosylated glycans. Over millions of years of evolution, the human immune system has adapted to recognize these molecular structures as potential signals of danger, finely tuned to respond as if facing an invasion by pathogenic organisms when encountering these structures on cell surfaces.

2.2 Among immune system: cellular glycans as major interplayers

The glycocalyx serves as a central regulator of immune responses and inflammation, exerting its influence either by directly modifying the activities and functions of immune cells or through recognition by glycan-binding receptors expressed by other immune cells. Alterations in the glycome of immune cells occur during various processes such as cell differentiation, activation, and apoptosis. These changes have been linked to both the normal maintenance of immune cell functions and the mechanisms underlying immune-related diseases (Fig. 4), including immune cell trafficking, differentiation, antigen and cytokine receptor activation, autoimmunity, and the initiation of leukocyte apoptosis (73).

Notably, our research and that of others have underscored the critical role of glycans as master regulators in multiple immunological processes, with a particular focus on adaptive immunity, especially concerning T cells (69). In this context, glycosylation has been shown to impact the immune system through several mechanisms (73-75).

Glycans have been identified as key modulators of the rate of TCR endocytosis, thereby influencing cellular responses through interactions with lectins such as galectins and their ligands (72). They are also involved in cellular mechanisms controlling the threshold for TCR activation. Importantly, a reduction in the branching of *N*-glycans on T cells, often associated with a malfunction or deficiency of *N*-acetylglucosaminyltransferase V (GnT-V) glycosyltransferase, leads to increased clustering of T cell receptors. Consequently, this results in a lower threshold for T cell activation by directly promoting TCR clustering (70). Conversely, the presence of branching *N*-glycans on TCRs enables interactions with surrounding galectins, reducing TCR clustering and localization with other co-receptors. Enhanced branching of TCR *N*-glycans, therefore, decreases the activation status of T cells and their susceptibility to inflammatory and autoimmune diseases (75).

In line with these findings, research conducted by our group and others has demonstrated a connection between reduced *N*-glycan branching, often due to glycosyltransferase dysfunction or deficiency, like GnT-V, and increased TCR clustering, resulting in a lowered activation threshold in chronic inflammatory conditions such as inflammatory bowel disease (IBD) (75).

Furthermore, evidence indicates that glycosylation plays a role in the differentiation of immune cells, particularly in generating and determining the phenotypic profiles of T helper (Th) cell subsets, including activated $CD4^+$ effector T cells, which differentiate into Th1, Th2, Th17, and regulatory T cells (Treg). This differentiation is partly influenced by

the production of ligands for various lectins, with *N*-glycan branching as a major player in the role of promoting the differentiation of anti-inflammatory Th2 cells over pro-inflammatory Th17 cells (76).

Lastly, glycosylation contributes to apoptosis in T cells and neutrophils by generating ligands for endogenous lectins, including galectins. These lectins bind to cellular glycans, forming glycoprotein complexes that initiate intracellular signalling cascades promoting cell death (72).

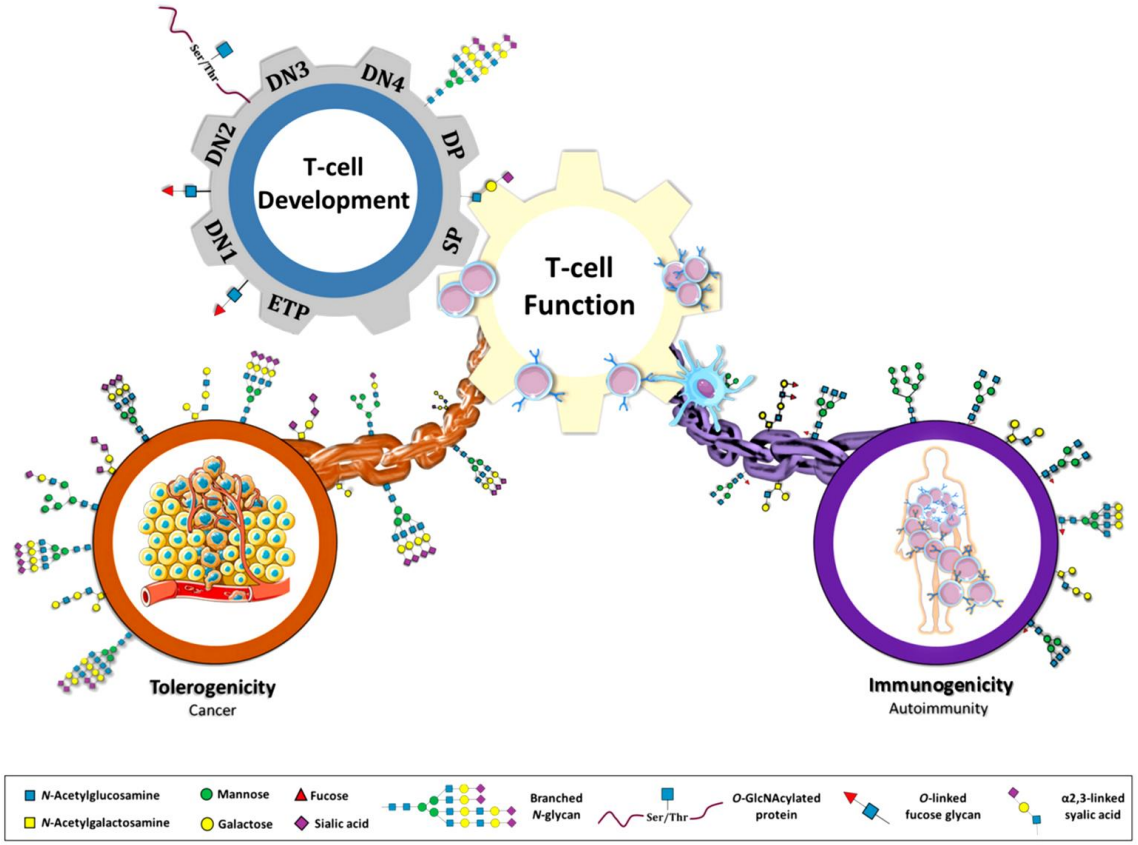

Fig. 4 – Glycans as a major connective chain that controls T cell response in either a tolerogenic or immunostimulatory scenario. Glycosylation appears to be central in regulating several steps of a T cell's life. During T cell development, different population of T cells (ETP, early thymocyte progenitor; DN1, 2, 3, and 4, double negative; DP, double positive; SP, single positive) display specific glycosylation patterns. The normal glycosylation of SP population results in an educated T cell function. However, by genetic, environmental or metabolic constrains, T cell glycosylation can be compromised re-directing immune system toward an immunostimulatory or tolerogenic response. Glycans are proposed here as key players in immune-unbalanced diseases, such as autoimmunity and cancer (**From 70.**).

Antibodies, particularly Immunoglobulin G (IgG) molecules, are abundant glycoproteins in human serum. All IgGs circulating in the body carry *N*-glycans within the fragment crystallisable (Fc) region's CH2 domain. These *N*-linked glycans are predominantly of the complex type, di-antennary glycans, typically featuring core fucose, variable amounts of antennary galactose, and occasionally bisecting *N*-acetylglucosamine (GlcNAc) (GlcNAc linked to the core β-mannose residue) or terminal sialic acids. Fc *N*-glycans significantly impact the Fc domain's structure and can modulate IgG effector functions (67).

For instance, Fc *N*-glycans can stericly interact with *N*-glycans found on Fc-gamma receptors (FcγRs), influencing FcγR-mediated processes like phagocytosis and antibody-dependent cellular cytotoxicity (ADCC). The significance of IgG Fc *N*-glycan fucosylation in biology is underscored by observations that afucosylated anti-spike-protein IgG levels correlate with corona-virus disease (COVID)-19 severity (77). Afucosylated anti-spike IgG has an enhanced ability to induce inflammatory responses through FcγRIII-expressing immune cells. Less complex IgG Fc glycans, such as fucosylated ones devoid of terminal sialic acids and galactose (agalactosylated Fc glycans), are linked to inflammation and disease progression in autoimmune diseases, although their functional implications remain to be fully elucidated.

In addition to Fc glycans, certain autoantibodies possess *N*-glycans in their antigen-binding (Fab) domains. Compared to Fc glycans, Fab *N*-glycans exhibit higher levels of galactosylation, sialylation, and bisection, with reduced fucosylation. Fab *N*-glycosylation sequences are primarily introduced during antigen-specific immune responses following somatic hypermutation, a process that appears to be selective. Similar to Fc glycans, Fab glycans can influence antibody stability, affect IgG's propensity to form immune complexes or aggregates, and potentially modulate IgG effector functions, including complement activation. Moreover, Fab glycans exert differential effects on antigen binding, contingent on the number and location of glycosylation consensus sequences. Fab glycans were proven (in mice studies) to prevent binding to self-antigens while maintaining cross-reactivity to foreign antigens, enabling BCRs to avoid autoreactivity and escape negative selection (67). Fab glycans also play a role in the survival of autoreactive B cells by affecting antigen binding and their activation threshold. Sialic acid-binding lectins (Siglecs) may provide a competitive advantage to autoreactive cells, as glycan-Siglec interactions are known to regulate B cell activity, such as in fetomaternal tolerance. Furthermore, the glycosylation status of secreted autoantibodies, such as anti-citrullinated protein antibodies (ACPA), anti-myeloperoxidase, and anti-proteinase 3 antibodies, alters their important immunomodulatory functions. *N*-glycans in the Fab region also contribute to transforming IgG into more stable structures, enhancing C1q binding and subsequent complement-dependent cytotoxicity. Notably, antidrug antibodies that emerge in patients

treated with adalimumab or infliximab also often feature high Fab *N*-glycans. These observations suggest that the introduction of *N*-linked glycosylation sites into the immunoglobulin variable domain results from chronic and systemic antigen exposure in conjunction with the helper activity of $CD4^+$ T cells, which is essential for somatic hypermutation in autoreactive B cells. Indeed, T-cell cytokines, such as those secreted by T follicular helper (Tfh) cells, including IL-6 and IFN-γ-positive Tfh1 cells, influence germinal centre processes (67) and IgG glycosylation. In light of these findings, it appears that Fab glycosylation may serve not only as a potential biomarker for disease prediction but also assist autoreactive B cells, which have pivotal roles in disease development, in circumventing the stringent control mechanisms typically in place to prevent autoimmunity.

2.3 In autoimmune diseases: implication of glycans in the loss of immune tolerance and autoimmunity

Glycans play crucial roles in various immunological processes and are likely to have significant implications in the regulation of immune responses underlying autoimmune diseases. Alterations in glycosylation have been observed in both peripheral (blood) and tissue cells in some systemic AID.

Patients with autoimmune diseases often exhibit the presence of autoantibodies, primarily immunoglobulins like IgG, with atypical Fc glycan profiles characterized by the absence of terminal galactoses and sialic acids, while fucosylation remains relatively stable. This distinct glycan signature is associated with the initiation and resolution of inflammation. These glycosylation changes may result from the inflammatory environment and differential expression of glycosyltransferases, potentially without directly affecting the pathogenicity of IgG.

RA was the first autoimmune disease associated with specific glycosylation patterns (67). However, a disease-specific increase in the prevalence of these IgG agalactosylated Fc glycans has also been observed in patients with psoriatic arthritis, juvenile idiopathic arthritis, SLE, active Spondyloarthritis, anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), or SLE associated with Sjögren syndrome. This suggests that such glycan alterations are common features of autoimmune diseases. Other inflammatory conditions, including IBD, multiple sclerosis, and Lambert–Eaton myasthenic syndrome, are also associated with a shift towards less complex Fc glycans (67).

Despite detailed analyses of Fc glycans in total IgG, the Fc glycan profile has only been characterized for a few rheumatic-disease-specific autoantibodies. For instance, Fc galactosylation and sialylation can also influence IgG's effector functions, although the precise role of Fc-glycan galactosylation is still debated. ACPA in RA patients exhibit reduced galactosylation compared to healthy individuals, and agalactosylated Fc glycans in ACPA IgG are associated with disease progression and systemic inflammation. Similarly, myeloperoxidase antigen-specific antibodies in AAV patients also display agalactosylated Fc glycans compared to the total IgG glycan profiles of healthy individuals, although no direct link with disease activity has been established in this case.

Notably, tissues and cells exhibit characteristic disease-related glycosylation patterns that can influence immunity. For example, cytokine-induced desialylation can transform synovial fibroblasts into an inflammatory phenotype, contributing to the progression and persistence of RA. Synovial fibroblasts in particular exhibit a high-mannose glycan-rich *N*-glycome (78). Differences in terminal sialylation between healthy and diseased individuals are evident, with TNF stimulation inducing a reduction in synovial fibroblast sialylation.

Our research group demonstrated that patients with Ulcerative Colitis exhibited a deficiency of branched glycans on intestinal T cells, which was associated with T cell hyperactivity and increased disease severity (75). Additionally, GnT-V-deficient mice displayed heightened delayed-type hypersensitivity responses and increased susceptibility to experimental autoimmune encephalitis (EAE) (76) and IBD (79).

In SLE, characterization of epithelial cell glycosylation patterns revealed a unique and unusual high-mannose tissue glycan signature (80), which could be linked to the loss of tolerance. Since the immune system typically responds to high-mannose glycans on the surface of foreign pathogens, the overlap in glycosylation patterns between host and pathogen might contribute to the loss of self-tolerance and the development of SLE (Fig. 5).

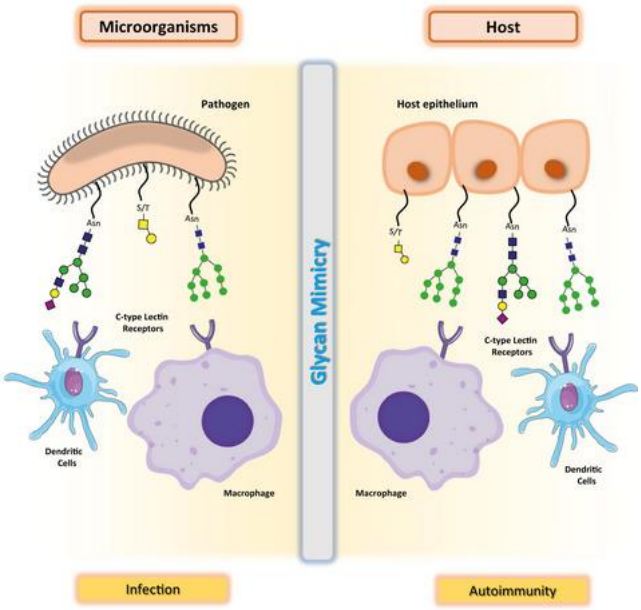

Fig. 5 – Molecular mimicry at surfaces of pathogens and host tissues as one of the mechanisms for infections to trigger autoimmune diseases. (**Adapted from 73.**)

Our recent research has unveiled an abnormal overexpression of a mannose-enriched glycan signature in lupus nephritis (80). This unusual glycosylation pattern stems from a deficiency in the complex *N*-glycosylation pathway, characterized by the downregulation of α-mannosidase 2 (a key player in regulating *N*-linked glycan branching) expression and the upregulation of *O*-mannosylation through mannosyl-transferase expression. The precise pathways leading to disease in these patients remain elusive, but these findings strongly suggest that aberrant mannosylation contributes to the risk of SLE development. In fact, studies involving mice with mutations in the gene encoding α-mannosidase 2 have demonstrated the development of a systemic autoimmune disease resembling SLE (81). Loss of α-mannosidase 2 disrupts *N*-glycan branching and impairs the immune system's ability to maintain self-tolerance, as indicated by the production of autoantibodies and kidney function failure. Although the molecular mechanisms behind such transformations are not yet fully understood, the accumulation of high-mannose *N*-glycans on the surface of various pathogens suggests that these pathogens may trigger the activation of antigen-presenting cells via mannose-glycan-binding receptors like dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), which is notably present in the glomeruli of SLE patients (80).

Further research from our team has led to the characterization of a glyco-signature associated with lupus nephritis development in a mouse model, particularly associated with a specific subgroup of T cells. We discovered that the presence of microbial-associated mannose structures on the kidney's surface triggers the recognition of DC-

SIGN-expressing γδ T cells, leading to a pathogenic IL-17a-mediated autoimmune response (82). Mice lacking Mgat5, which have a higher abundance of mannose structures in the kidney, exhibited increased γδ T cell infiltration into the kidney and spontaneously developed lupus as they aged. Similar pathogenic roles for γδ T cells have been observed in previous studies related to autoimmune anti-myeloperoxidase glomerulonephritis (83).

Alterations in tissue glycosylation have also been observed in other diseases, such as GNE myopathy. In this condition, muscle cells exhibit low levels of sialic acid due to mutations in the *GNE* gene, which encodes a bifunctional enzyme responsible for synthesizing the sialic acid precursor *N*-acetylneuraminic acid (84).

Most recently, we have shed light on the role of *N*-glycans in central tolerance and T-cell development associated with disease (85). High-mannose restricted *N*-glycomes in thymocytes globally disrupt their normal development, particularly in beta-selection, Treg cell generation, and gamma-delta-cell lineage choice and selection, leading to spontaneous autoimmune phenotypes (Fig. 4). In summary, our research demonstrates that the glycan signature of thymus structure can influence T cell development, priming these cells for different functions upon release into the general circulation.

2.4 Potentials applications of glycans in medical practice

2.4.1 Glycanbiomarkers for disease diagnosis/ classification and prognosis

Glycan signatures specific to IgG are associated with rheumatic diseases and can be used as prognostic markers. By analysing these glycan patterns, it becomes possible to identify more homogeneous patient groups within these diseases.

Agalactosylation of IgG Fc *N*-glycans is mentioned as a specific glycan alteration associated with disease activity, progression, and relapse in diseases like RA. This glycan modification has the potential to complement existing biomarkers for RA (67).

Research conducted by our group and others has demonstrated the potential of altered protein glycosylation as a biomarker for diagnosis and prognosis, particularly in refractory cases of IBD (86) and other chronic inflammatory gastrointestinal diseases, as well as liver diseases (87).

Finally, lupus nephritis is highlighted as having a unique mannose-enriched glycan signature. This glycan pattern serves not only as a marker for lupus nephritis but also predicts, with high specificity, chronic kidney disease development. As a result, it may function, at diagnosis, as a potential prognostic biomarker in the context of lupus nephritis (80).

2.4.2 Glycans as therapeutical targets

Various therapeutic strategies related to glycosylation could potentially be used to intervene in autoimmune processes and manage autoimmune diseases.

Studies in mice have suggested that altering the Fc glycosylation of antibodies could be a strategy for therapeutic interference in autoimmune processes. Enzymes like Endoglycosidase S have been tested in preclinical models and have shown potential in reducing joint inflammation and modulating IgG effector functions (67). This approach has implications for the treatment of conditions like arthritis.

Transferring sialylated, anti-inflammatory immune complexes has been explored as a potential therapeutic strategy. This approach aims to attenuate the development of arthritis, reduce pathogenic Th17 cells, and suppress autoantibody responses (67). It focuses on influencing the glycosylation profiles of autoantigen-specific IgGs to intercept pathogenic T cell and B cell responses.

Altering the surface glycosylation of cells, particularly synovial fibroblasts and other cell types involved in inflammation, is suggested as a promising therapeutic intervention (67). This modification could be achieved through cytokine-mediated modulation of the enzyme machinery responsible for sialylation or galactosylation. This approach may help reduce inflammation in autoimmune diseases.

An alternative therapeutic approach involves directly administering glycans or altering the metabolite supply for glycan biosynthesis. For example, the administration of *N*-acetylglucosamine (GlcNAc), which enhances the hexosamine pathway, has been shown to reduce chronic inflammation and autoimmunity (67). GlcNAc supplementation increases *N*-glycan TCR branching, reducing T cell hyperactivation. Treatment of GnT-V-deficient mice with high concentrations of GlcNAc increases the GnT-V-mediated N-glycan and inhibits autoimmune responses in mouse models of EAE, IBD (79) and type I diabetes. In humans, this approach is being explored in clinical trials for conditions like juvenile IBD (88).

In a recent study from our group, GlcNAc supplementation, which promoted biosynthesis of tolerogenic branched *N*-glycans in the kidney, was found to inhibit γδ T cell infiltration and control lupus nephritis development (82).

Glycoengineering has been used to modify monoclonal antibodies for therapeutic purposes (67). For example, the anti-CD20 antibody obinutuzumab was glycoengineered to reduce fucosylation, resulting in improved therapeutic efficacy.

Supplementation with sialic acid precursors has shown promise in preventing muscle atrophy in a mouse model of inflammatory myopathy. It has also been tested in a phase II study for patients with GNE myopathy (89).

Finally, glycan-based interventions can also be used for the targeted degradation of autoreactive B cells or the antibodies they produce (67). Siglec-engaging tolerance-inducing antigenic liposomes (STALs) are mentioned as a method to recruit Siglecs, which are negative regulators of B cell receptor signalling, to autoantigen-specific B cells' immunological synapses (90).

CHAPTER II

Goals and Aims of the project

GOAL AND AIMS OF THE PROJECT

Major goal of this broad thesis: to assess the impact of glycan changes in the regulation of both adaptive and innate immune response in IIM, aiming to propose an adjunctive physiopathological mechanism in etiopathogenesis and a potential novel targeted-specific therapeutic strategy in IIM patients, focusing on characterization of a poor prognosis subgroup.

Specific aims to validate the specific glycosignature in IIM as:

I) A new mechanism of action in the pathophysiology
II) A potential biomarker for improving classification and prognosis stratification
III) A potential novel target for directed therapy

Main questions:

1. What is the real disease burden, defining a poor prognosis subgroup, through full characterisation of Unidade de Imunologia Clínica (UIC) cohort of Idiopathic Inflammatory Myopathies (Chapter II: Campar A, et al. Idiopathic inflammatory myopathies – the burden of disease: cohort analysis focusing on damage and comorbidities. Autoimmun Rev 2023; doi.org/10.1016/j.autrev.2023.103455. In Press.

2. What are the levels of expression and types of glycans in muscle biopsies from IIM patients comparing with controls? Is there a difference between patients with severe versus non-severe disease? Is there a correlation with myositis' specific autoantibodies, serious visceral involvement or malignancy? Can this be used to propose a prognostic assessment at the time of diagnosis? (Chapter III: Campar A, et al. Muscle glycome in idiopathic inflammatory myopathies: Impact in IL-6 production and disease prognosis. iScience. 2023 Jun 17;26(7):107172. doi: 10.1016/j.isci.2023.107172. PMID: 37404372; PMCID: PMC10316658.)

3. How the altered expression of glycans in muscle does influence the local and systemic immune response in these patients, namely the T cell response? (Chapter III: Campar A, et al. Muscle glycome in idiopathic inflammatory myopathies: Impact in IL-6 production and disease prognosis. iScience. 2023 Jun 17;26(7):107172. doi: 10.1016/j.isci.2023.107172.)

4. Can we target this mechanism by using a glycan-based immunomodulatory strategy to promote self-tolerance? (Chapter III: Campar A, et al. Muscle glycome in idiopathic inflammatory myopathies: Impact in IL-6 production and disease prognosis. iScience. 2023 Jun 17;26(7):107172. doi: 10.1016/j.isci.2023.107172. PMID: 37404372; PMCID: PMC10316658.)

CHAPTER III

Results

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Idiopathic inflammatory myopathies – The burden of disease: Cohort analysis focusing on damage and comorbidities

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ABSTRACT

Introduction/Background: Idiopathic Inflammatory Myopathies (IIM) continue to be a major clinical challenge worldwide. The exact aetiopathogenesis of this chronic and disabling disease remains elusive, preventing the development of novel and effective therapeutic strategies and leading to a high incidence of damage. The complexity of treating these diseases is even greater due to the numerous comorbidities that affect these patients.
Methods: Retrospective review of the cohort of patients diagnosed with IIM and followed in a dedicated unit of a tertiary hospital between 1971 and December 2022, with particular attention to damage and comorbidities. Damage was assessed using the Myositis Damage Index. Comorbidities were recorded and analysed as a whole and also assessed using the Charlson Comorbidity Index. Health Assessment Questionnaire (HAQ) Disability Index (DI) was performed by phone call in December 2022, to all patients actively followed-up in the Unit.
Results: Analysis of 149 patients with a mean follow-up of 9 years (range 0–51) revealed >90% with damage and comorbidities. Most comorbidities were a consequence of the damage and were particularly related to prolonged steroid therapy. Cardiovascular damage, which occurred either as cardiovascular risk factors or as end-organ sequelae (cardiovascular disease and chronic kidney disease), was the main cause and a major contributor to death. Depression was also high on the list of associated comorbidities. Median HAQ was 2.09 representing high negative impact in quality of life.
Conclusions: Although survival rates have increased in recent decades, patients with IIM carry a high burden of disease with poor quality of life, mainly caused by damage and comorbidities.
While comorbidities accumulation is the major factor for poor quality of life, damage severity is the main predictor for mortality. Improved therapeutic strategies are needed to reduce the need for steroids and to introduce routine screening and management of comorbidities as an essential partner of immunosuppressive therapy, leading to comprehensive care of myositis patients and effective improvement of their quality of life.

1. Introduction

Idiopathic inflammatory myopathies (IIM) are a rare group of heterogeneous disorders. Although usually considered inflammatory pathologies of muscles and/or skin, recent descriptions confirm their true systemic nature, with frequent involvement of the lungs, joints and heart [1,2].

Identification of myositis-specific antibodies (MSAs) and correlation with clinical phenotypes have helped to increase knowledge of the disease regarding diagnosis, prognosis and, in some cases, response to therapy. However, there is still much debate about classification issues [3–5] that justify the diversity of cohorts and limit clinical trials.

There is still no on-label therapy for most patients. Immunosuppressive therapy is administered individually according to the same principles as for other systemic autoimmune diseases (SAID). However, it is neither targeted nor tailored to the different clinical phenotypes and

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rarely takes comorbidities into account [6,7]. Nevertheless, some efforts are being made with regard to the latter aspect [8].

As long-term, chronic conditions, IIM are often associated with comorbidities, mainly resulting from damage accrual, which place an additional burden on patients' lives and limit their productivity and well-being. Analysis of damage and comorbidities has rarely been the main objective in the published literature on IIM [9,10], and correlation with quality-of-life (QoL) scores is not described, but their impact on patients disease evolution is clear.

The aim of this study is to provide a detailed and complete characterisation of all types of morbidity associated with IIM, including the impact on QoL and mortality, and to correlate these with other morbidity outcomes, in a cohort of patients with a long follow-up period.

2. Methods

Retrospective analysis of all patients with IIM followed up at our Unit since its onset by reviewing paper and electronic records of clinical notes and results of complementary diagnostic investigations (blood analysis, imaging, electromyographic investigations and neuropathology). Demographic and clinical parameters were analysed, as well as treatment and evolution. Refractoriness was considered in patients who did not respond to ≥2 immunosuppressive drugs administered concomitantly for at least 3 months [11]. European League Against Rheumatism (EULAR)/American College of Rheumatology (ACR) criteria [12] were applied to all patients.

Comorbidities were recorded at 2 different time points: before IIM diagnosis and at the end of follow-up (December 2022). Total number of comorbidities is the sum of those present before IIM diagnosis plus those acquired through damage.

Damage was calculated and analysed in December 2022 using the Myositis Damage Index (MDI) [13] for all settled areas. In addition to "Death", 4 other parameters were added under "Other" damage, according to the prevalence in the cohort, namely: "Chronic kidney disease", "Obstructive sleep apnoea", "Depression requiring treatment" and "Dementia". Charlson Comorbidity Index (CCI) [14] was applied to all living patients (i.e. deceased patients were excluded) (total $n = 124$).

Health Assessment Questionnaire (HAQ)-Disability Index (DI) [15] was collected by telephone in December 2022 from all patients who were under active care in the department ($n = 99$).

Statistical analysis was performed using Statistical Software version 24 (IBM Corp., IBM SPSS Statistics for Windows, version 24.0, Armonk, NY; published 2016). Threshold for statistical significance was p-value <0.05.

3. Results

Analysis included 149 patients (114 women, representing 76.5%) followed-up at our Unit from 1971 to December 2022. Ninety-nine patients are actively followed-up, 25 have been lost for follow-up and 25 (16.8%) have died. Patients were lost for follow-up because they changed residence, migrated or, in a small proportion, were in remission without medication and were discharged. Median follow-up time was 9,41 years (range: 0–51 years) (Table 1).

Most patients had Dermatomyositis (DM) ($n = 41$; 27.52%), followed by Overlap Myositis (OM) ($n = 26$; 17.45%), Anti-Synthetase Syndrome (aSS) ($n = 24$; 16.11%), Paraneoplastic Myositis ($n = 17$), Immune-mediated Necrotizing Myopathy (IMNM) ($n = 9$), Polymyositis ($n = 8$), Juvenile Myositis ($n = 7$), Non-specific Myositis ($n = 6$) and Inclusion body myositis (IBM) ($n = 5$) (Table 2). Juvenile Myositis corresponded to patients transferred from paediatrics due to persistent active disease. Non-specific Myositis was considered in patients with proximal myopathies who had no cutaneous lesions, were seronegative and did not consent to muscle biopsy. EULAR/ ACR criteria yielded an IIM probability of 83.23%, with mean maximum score of 23.61 points. These figures relate to the fact that a significant proportion of patients had only lung and/or joint involvement, which are not included in the criteria. The median age at diagnosis was 51,5 years and that for first symptoms was 49,8 years, with a median delay in diagnosis of 26 months.

Muscle and/or skin involvements were the most frequent, but a significant proportion had concomitant lung involvement (52.32%) and joints were also commonly involved (40.94%). In 5 patients, isolated polyarthritis was the sole manifestation for several years and, in another 8, isolated Interstitial Lung Disease (ILD) was the only manifestation until aSS was diagnosed. MSAs were present in >55% of patients, and associated autoantibodies in >50% of the 128 patients tested (Table 2).

Table 1
Demographic and global evolutionary characteristics of patients from the cohort.

Total of patients	149
Gender (female/ male) (%)	114/35 (76,5/ 23,5)
Age at diagnosis (median, years)	51,5
Age of first symptoms (mean, years)	49,8
Delay in diagnosis (median, months)	26
Time of follow-up (median, years)	9,41
(range, years)	(0–51)
Remission without drugs, n (%)	9 (6,04)
Remission on drugs, n (%)	6 (4,03)
No damage (MDI = 0), n (%)	13 (8,90)
No comorbidities, n (%)	11 (7,38)
Refractoriness, n (%)*	47 (37,90)
Treatment with biological, n (%)	31 (20,80)
Treatment with small molecules, n (%)	11 (7,38)
HAQ-DI** (median)	2,09
Lost for follow-up, n (%)	25 (16,8)
Mortality, n (%)	25 (16,8)
Survival rates, %	
At 1 year of follow-up	96,58
At 5 years of follow-up	92,59
At 10 years of follow-up	86,55
At 15 years of follow-up	79,17
At 20 years of follow-up	71,62

HAQ-DI - Health Assessment Questionnaire-Disability Index.
* Relative to patients with active follow-up in December 2022 plus dead patients ($n = 124$).
** Relative only to patients with active follow-up in December 2022 ($n = 99$).

Table 2
Clinical parameters of patients from the cohort.

Diagnosis	
Dermatomyositis, n (%)	41 (27,52)
Clinical Amyopathic Dermatomyositis, n (%)	6 (4,03)
Overlap Myositis, n (%)	26 (17,45)
Anti-synthetase Syndrome, n (%)	24 (16,11)
Immune-mediated Necrotizing Myopathy, n (%)	9 (6,04)
Polymyositis, n (%)	8 (5,36)
Inclusion body myositis, n (%)	5 (3,36)
Juvenile Dermatomyositis, n (%)	7 (4,70)
Paraneoplastic Myositis, n (%)	17 (11,40)
Non-specific Myositis, n (%)	6 (4,03)
EULAR/ ACR classification	
IIM probability (mean, %)	83,23
Maximum score (mean, points)	23,61
Phenotypic characteristics	
Muscle involvement, n (%)	125 (83,9)
Cutaneous involvement, n (%)	92 (61,74)
Pseudobulbar involvement, n (%)	65 (43,62)
Lung involvement, n (%)	78 (52,35)
Articular involvement, n (%)	61 (40,94)
Heart involvement, n (%)	24 (16,11)
Laboratory characteristics	
Rhabdomyolysis, n (%)	108 (72,48)
Specific autoantibodies, n (%)	75 (58,59)
Associated autoantibodies, n (%)	70 (54,79)
Electromyographic study (positive), n (%)§	50 (43,48)
Muscle histology (positive), n (%)§	42 (41,18)

§ relative to the total of procedures performed.

The low percentage of seropositive patients is justified because a significant proportion of patients were diagnosed before MSAs were described and routinely analysed. Confirmatory muscle histology, e.g. showing the features included in the EULAR/ ACR histological criteria, was only achieved in 41.18% of patients who underwent muscle biopsy ($n = 102$).

Overlap with other SAID occurred in 49 patients (33.89%), mainly with Systemic Sclerosis ($n = 16$) followed by Sjögren's syndrome ($n = 14$).

Corticosteroids were used in 95.97% of patients. Methotrexate (45.64%) was the most used immunosuppressive drug, followed by intravenous Immunoglobulin (39.6%) and Azathioprine (35.57%). Around 60% of patients required combination of 2 immunosuppressive drugs, in addition to steroids, to control disease (data not shown). Therapy refractoriness was noted in 37.9% of patients ($n = 47$) (mainly related to pulmonary involvement). Biological therapy was used in 31 patients (mainly rituximab) and small molecules (JAK inhibitors) in 11, all due to refractoriness. Remission, with or without therapy, was achieved only in about 10% of patients (Table 1).

Damage analysis included 146 patients (3 were not included because they died within 2 months of initial diagnosis). >90% of patients had some type of damage, with only 13 (8.9%) recording an MDI of zero. The 3 main causes were: muscle (80.82%), gastrointestinal (79.45%) and lung (72.60%) damage, closely followed by endocrine (63.70%) and cardiovascular (60.96%) damage (Table 3). Global damage was assessed by extent (mean score of 5.81 points) and severity (mean score of 34.58).

Mean age at death was 70.2 years with median follow-up time of 9.32 years. The main cause of mortality was malignancy (32%), closely followed by cardiovascular disease (24%) and infection (16%). Active IIM was the cause of death in only 2 patients (8%), both due to respiratory failure, secondary to rapidly progressive ILD (data not shown). One young man died by suicide, highlighting the importance of psychological assessment and treatment in these patients. Survival rates are up to 90% up to 5 years follow-up, after which they decline steeply (Table 1).

>90% of patients had comorbidities and only 11 (7.38%) had no comorbidities. Most of the patients (78.52%) had no comorbidities prior to IIM diagnosis. Consequently, most comorbidities are the result of damage, especially secondary to high cumulative steroid doses, reflected for example in acquired cardiovascular risk factors. Cardiovascular risk factors (including almost 15% with metabolic syndrome), psychiatric disorders (especially depression, occurring in 54.36% of patients) and chronic lung disease (in 55.7%) were the most common comorbidities (Table 4). A total of 727 comorbidities could be taken into account, resulting in an average of 5.27 comorbidities per patient.

Table 3
Damage analysis of patients from the cohort.

Myositis Damage Index domain	Result
Muscle damage, n (%)	118 (88,72)
Skeletal damage, n (%)	58 (43,61)
Cutaneous damage, n (%)	16 (12,03)
Gastrointestinal damage, n (%)	116 (87,22)
Pulmonary damage, n (%)	106 (79,70)
Cardiovascular damage, n (%)	89 (66,92)
Peripheral vascular damage, n (%)	28 (21,05)
Endocrine damage, n (%)	93 (69,92)
Ocular damage, n (%)	37 (27,82)
Infection, n (%)	16 (12,03)
Malignancy, n (%)	19 (14,28)
Other damage, n (%)	136
Death	25 (18,80)
Chronic kidney disease (≥ stage 2)	21 (15,79)
Obstructive sleep apnea	5 (3,76)
Depression requiring treatment	78 (58,65)
Dementia	7 (5,26)
Global damage	
Extension (mean)	**5,81**
Severity (mean)	**34,58**

Table 4
Comorbidities analysis of patients from the cohort.

Cardiovascular risk*		156
	Diabetes Mellitus	15
	Hypertension	50
	Dyslipidaemia	53
	Obesity	16
	Metabolic syndrome**	22
Cardiopathy		71
	Cardiomyopathy	43
	Heart Failure	22
	Chronic disrrythmias	6
Chronic renal disease (≥ stage 2)		23
Chronic pulmonary disease		83
	Pulmonary fibrosis	23
	Restrictive syndrome	24
	Chronic respiratory failure	13
	Secondary pulmonary hypertension	14
	Obstructive sleep apnea	9
Hepatic steatosis		52
Metabolic bone disease		45
	Osteoporosis	33
	Osteoporosis with fracture	12
Cerebrovascular disease		15
	Dementia	9
	Cerebrovascular accident sequelae	6
Endocrinopathy	Hypothyroidism	12
Gastrointestinal disorders		54
Cataract/ Glaucoma		35
Musculo-skeletal degenerative disease		24
Psychiatry disorders		92
	Depression	81
	Anxiety	11
Malignancy		19
Chronic venous insufficiency with thrombotic complications		25
Other		21
	Inflammatory arthropathy	4
	Psoriasis	6
	Immunoglobulins deficiency (IgA and/or IgA + IgG)	11
Total		**727**
Comorbidities by patient (mean)		**5,27**

Ig – Immunoglobulin.
* 40% of patients had >1 cardiovascular risk factor.
** considered when patients had ≥3 cardiovascular risk factors.

According to CCI, most common comorbidities (Table 5) were connective tissue disease (124 patients), diabetes mellitus (25 patients) and chronic kidney disease (18 patients). Mean total score was 3.89, resulting in a 10-year survival rate of 55.26%.

Median HAQ was very high (2.09). Highest HAQ scores were registered in IBM, followed by aSS and PM (Supplementary data 1). Although all other morbidity outcomes, except refractoriness, correlated with HAQ, multivariate analysis described the accumulation of comorbidities ((odds ratio (OR) 33.49, p-value <0.0001)) as the only independent variable that affected Quality of Life (QoL).

Highest damage severity (> 25), was the only independent predictor for mortality (OR 10.152, p-value 0.015) (Supplementary data 2).

In-depth analysis among IIM subgroups showed higher clustering of comorbidities in aSS, followed by IBM and PM. Damage extension and severity was higher in aSS. Most refractory subgroups were founded in Non-specific Myositis followed by IMNM and aSS. Mortality was higher in Paraneoplastic myositis, as expected, followed by aSS and CADM (Table 6).

4. Discussion

As far as we know, this is the IIM cohort with the longest follow-up

Table 5
Charlson Comorbidity Index of living patients from the cohort.

Total of patients (n)		124
Age (mean, years)		58,15
Myocardial infarction (n)		1
Cardiac Heart Failure (n)		15
Peripheral vascular disease (n)		14
CVA or TIA (n)		3
Dementia (n)		5
COPD (n)		5
Connective tissue disease (n)		124
Peptic ulcer disease (n)		17
Liver disease (n)		13
	Mild	13
	moderate-severe	0
Diabetes mellitus (n)		25
	Uncomplicated	12
	end-organ damage	13
Hemiplegia (n)		0
Moderate to severe CKD (n)		18
Solid tumor (n)		9
	Localized	9
	Metastatic	0
Leukemia (n)		0
Lymphoma (n)		0
AIDS (n)		0
Total (points) (mean)		**3,89**
Estimated 10-year survival (%)		**55,26**

CVA – Cerebrovascular accident with minor or no residual; TIA –Ttransient ischemic attacks; COPD – Chronic obstructive pulmonary disease; CKD – chronic kidney disease; AIDS – Acquired Immunodeficiency Syndrome.

time reported in the literature. Time is of critical importance in chronic diseases as it allows us to get a picture of natural and conditional (therapy-related) evolution and, most importantly, to quantify and qualify the damage accrual.

Demographic characteristics are broadly similar to other reported cohorts [9,10,16,17].

The EULAR/ ACR classification criteria published in 2017 updated the IIM classification but have since been highly controversial [3–5,18,19]. Despite high specificity, sensitivity is low due to exclusion of some clinical involvements, namely pulmonary and articular, exclusion of specific autoantibodies (except anti-Jo1) [20] and high reliability of very specific muscle biopsy (increasingly rarely performed in clinical practise) parameters that do not include histological features suggestive of necrotising myopathy. Failure to recognise aSS [21,22] as an individual disease was also highlighted as a weakness. As a result, many patients are not considered when applying these criteria and a significant proportion are classified as polymyositis, currently considered the rarer subtype of IIM [23,24]. In summary, these criteria should be used in clinical trials, but their role in clinical diagnosis remains of minor importance.

The EULAR/ ACR classification criteria used in our patients confirmed a moderate probability of IIM and a mean maximum score, as previously determined [25], and consistent with other cohorts [3–5,26]. In some patients, clinical diagnosis was completely different from the results of classification criteria, particularly regarding IMNM, aSS and OM, most of them classified as Polymyositis (PM). This confirms the increasingly accepted subclassification of IIM patients according to clinical phenotype and positive MSAs, also referred to in other cohorts [3,5].

IIM phenotypes of all patients were reviewed based on clinical and serological features and supplemented by histological results when available, according to criteria proposed by Anthony Amato and approved by European Neuromuscular Centre (ENMC) workshop [27,28]. This resulted in reduction of DM subgroup, and most importantly, an almost elimination of PM diagnoses, with most of them diagnosed in the IMNM, OM and aSS subgroups.

Muscle biopsy, once considered the gold standard, was found to be less accurate for diagnosis in our cohort, which has recently been described by others, especially without an accompanying electromyographic study [29–31]. IIM is a patchy muscle disease, which makes it difficult to determine the pathological location of immune-mediated changes at a specific site corresponding to the site at which the biopsy is performed. On the other hand, according to EULAR/ ACR criteria, only some very specific changes are considered significant for myositis, so histological findings of IMNM, for example, are not considered. Finally, muscle biopsy is preferably performed on upper limbs, but diagnostic accuracy is higher for lower limb samples [29]. Nevertheless, it remains today an indispensable tool essentially for differential diagnosis, especially in refractory cases when the diagnosis needs to be revisited.

In our cohort, diagnosis was made with an average delay of >2 years after the first symptoms. This is justified for several reasons, but mainly because of the rarity and heterogeneity of this disease group, which is hardly known to most general practitioners. The other, no less important aspect is the nature of the presentation in a considerable number of patients, such as isolated ILD or arthritis. Knowledge of the different clinical features these diseases may present with is crucial for timely diagnosis and initiation of treatment. Delayed diagnosis also leads to accumulation of potential damage by the time of diagnosis, contributing to disability and poor QoL, and may also contribute to refractoriness (~38% in our cohort).

Survival of IIM patients has increased in recent decades, as confirmed in our and some other cohorts [15,32,33], although reports vary widely. Heterogeneity in subgroup classification, disease involvements staging, inclusion or non-inclusion of cancer-associated myositis are some of the reasons that make comparison between

Table 6
Global morbimortality analysis by IIM subgroup.

IIM subgroup		HAQ*	Comorbidities	Damage (mean)		Refractoriness**		Mortality	
	N	Mean	Mean	Extension	Severity	N	%	N	%
Dermatomyositis	41	2,02	6,27	5,73	32,07	10	30,30	6	14,63
CADM	6	1,42	4	2,83	10,83	2	33,33	1	16,67
Overlap Myositis	26	2,09	6,35	5,61	32,61	5	25,0	3	11,54
Antisynthetase syndrome	24	2,29	7,83	6,79	40,04	10	45,45	5	20,83
IMNM	9	2,18	5,44	3,67	16	5	55,56	1	11,11
Polymyositis	8	2,27	7,38	5,63	29,13	4	50	0	0
Juvenile Myositis	7	1,9	4,57	4,71	30	3	42,86	0	0
IBM	5	2,63	7,8	6	35,2	2	40	0	0
Paraneoplastic	17	2,1	7,24	5,41	33,18	2	11,76	9	52,94
Non-specific	6	1,92	7,33	4,17	25,5	4	66,67	0	0

IIM – Idiopathic Inflammatory Myopathies; HAQ - Health Assessment Questionnaire Disability Index; CADM – Clinical Amyopathic Dermatomyositis; IMNM – Immune-mediated Necrotizing Myopathy; IBM – Inclusion Body Myositis.

* relative only to patients with active follow-up in December 2022 (n = 99).

** relative to patients with active follow-up in December 2022 plus dead patients (n = 124).

different studies difficult. Worldwide, reported mortality rates vary between 2 and 45% [34]. In our cohort, mortality was 16.8%, which is higher than in general population and in most other SAID, except for Systemic Lupus Erythematosus (SLE) with nephritis [35–37]. We chose to include cancer-associated myositis because the relative number of patients was small. The causes of mortality in our cohort are similar to those described by others, including patients with paraneoplastic myositis. Active myositis contributes to few deaths, with the leading cause after malignancy being cardiovascular disease, as described by others [33,38], which is a direct consequence of the accumulation of cardiovascular risk factors, as shown by our analysis of comorbidities.

Therapeutic strategies are based on the clinical assessment of the patient's condition. In this context, the distinction between activity and damage is crucial in order to neither undertreat, leading to disease progression, nor overtreat, which may lead to severe morbidity.

Damage accrual was enormous in our cohort, affecting >90% of patients. Similar figures are reported by others [9,39]. Muscle, gastrointestinal and lung damage, the most common, are mainly a direct consequence of IIM disease. However, >60% of patients had endocrine (essentially diabetes and dyslipidaemia) and cardiovascular damage, which is a consequence of corticosteroid therapy and a major contributor to death. Indeed, recent studies show an increased incidence of coronary heart disease in myositis patients [40–42], among other cardiovascular events [43]. Dermatomyositis patients appear to have the greatest cardiovascular risk. Some studies have shown that the risk is similar to that of diabetes, and parallel to SLE [44]. All this is strong evidence for the importance of cardiovascular monitoring, prevention and treatment in patients with IIM.

CCI is the most commonly used index for assessing comorbidity worldwide. However, it has several limitations [45] and does not fully reflect the heterogeneity, life-threatening and QoL-impairing comorbidities in IIM patients. Chronic lung disease and psychiatric disorders are examples. Cardiovascular risk factors in CCI are limited to diabetes, not including other factors (hypertension and dyslipidaemia) and especially their combination as a metabolic syndrome, which carries a different and higher risk of end-organ damage. In our cohort, CCI evaluation yielded an estimated 10-year survival rate of just over 50%. Nevertheless, it differs from the calculated survival rates in our cohort and others, confirming the low accuracy in assessing morbidity in this context.

Given the limitations of CCI, we decided to analyse all important comorbidities in IIM patients. The overall picture shows a high prevalence of comorbidities (> 90% of patients) added to IIM itself. Although comparison with other cohorts is limited as this analysis is not presented as a whole in most series, other studies report similar figs. [9,10,36]. Cardiovascular risk factors come first, with about 80% of patients having at least one factor and almost 15% having a metabolic syndrome. This is followed by end-organ consequences, with structural cardiopathy and chronic kidney disease being the most important consequences, directly contributing to death and morbidity. Our and other observations are clear as to the cause of these serious comorbidities [9,45]: a cumulative high dose of steroids, combined with ongoing active disease contribute to accelerated atherosclerosis. Accumulation of comorbidities places an enormous burden on patients' daily lives. It not only shortens life expectancy, but also complicates the management of basal autoimmune disease. Polypharmacy and drug-drug interactions are main problems, as well as restrictions on the use of immunosuppressive drugs when thinking of kidney and/or liver failure, heart problems and infection risks when, for example, diabetes is also present. The number of tablets took daily increases with the accumulation of comorbidities, which lead to additional side effects and intolerances and affect the patient's psyche.

Depression is very common in IIM patients [10,47], which was also confirmed in our study, although it is rarely addressed in most series. Moreover, mood alterations are not included in the MDI, which makes it more difficult to record in routine care. Depression contributes to deterioration in functioning and increased absenteeism, and affects patients' adherence to medication and non-pharmacological interventions, such as physical rehabilitation programmes. In our cohort, depression was only considered if it required treatment, which means additional medication, drug interactions and side effects.

HAQ-DI is one of the most commonly used scores to assess health-related QoL and is part of the International Myositis Assessment & Clinical Studies Group (IMACS) Core Set of Domains and Measures, although it is not fully validated for IIM patients [48]. HAQ-DI was very high in our cohort of myositis patients (median 2.09) and describes a low QoL compared to other cohorts [49]. The prevalence of depression and the proportion of patients who still had active disease at the time of evaluation (~40%) may have influenced the results. HAQ was higher in IBM due to muscle damage and dysphagia, and in aSS, mainly due to lung involvement and arthritis. A positive correlation was found with other outcome measures of morbidity, with the exception of refractoriness, but poor QoL is independently influenced by the clustering of comorbidities, highlighting their importance.

Mortality, on the other hand, is influenced in an independent way by damage severity, but not by other morbidity measures. Curiously, delay in diagnosis was inversely correlated with mortality, which is contrary to findings by others [33], with the exception of Bronner et al. [50], who also describe a lower interval between symptoms initiation and diagnosis as a predictor of death. We believe this is due to the severity of disease at presentation in some patients, particularly in aSS and CADM with rapidly progressive ILD, which ultimately leads to death when it is refractory to treatment induction.

Analysis among IIM subgroups in our cohort revealed aSS, after paraneoplastic myositis, globally as the most ominous sub-entity, with highest scores in all morbidities outcome measures, particularly concerning damage and comorbidities, and the second most deadly after cancer-associated myositis. Although it is usually therapy responsive, attention should be paid to aSS, as control of disease activity often implies long-term heavy immunosuppressive therapy [50], which carries pharmacological side effects. With respect to lung involvement in aSS, most times, therapy only serves to avoid/ slow down progression [51], which actually means slower but progressive damage accrual, with consequent lost in QoL and higher mortality.

To the best of our knowledge, this is the first report to concentrate information on global morbimortality, correlating different outcomes with mortality and QoL, in the same cohort. Different parameters correlate with death and, in those who survive, with QoL. In order to deliver the best care for myositis patients, all these issues need to be accounted for, concerning diagnosis and management, in routine daily practice. Comprehensive care of IIM patients, as for other SAID, goes far beyond controlling inflammation and disease activity. Damage, in all its dimensions, and comorbidities, in all its extension, are as or even more important. A specific morbidity index for myositis patients, able to be implemented in routine clinical practice, and prospectively validated, may pave the way for a more rational, homogeneous and effective way of caring for IIM patients, as well as facilitating significant clinical trials design.

Our study has several limitations, such as its retrospective nature and the absence of some information in the medical records (especially for those patients who were followed for a longer period), which prevented us from performing a more detailed analysis. A cohort from a single centre (such as ours) is formally another limitation, although, taking into account the rarity of IIM, this also led to greater homogeneity in diagnosis subgrouping and treatment strategy among patients. Finally, MSAs have only been routinely analysed in our laboratory in the last 10 years, which limited a more precise diagnosis, especially in patients diagnosed more antiquely.

5. Conclusions

Although survival rates have increased in recent decades, myositis

patients continue to have a poor QoL. Damage and comorbidities, as extensions of chronic autoimmune diseases, are particularly common in IIM patients. While comorbidities accumulation is the major factor for poor QoL, damage severity is the main predictor for mortality. High cumulative doses of steroids play an important role in both damage and comorbidities, contributing in particular to cardiovascular risk factors and subsequent cardiovascular end-organ damage and death. A therapeutic strategy without steroids or a lower dose and/or temporary use of steroids needs to be validated in larger and prospective cohorts. On the other hand, screening and management of comorbidities and damage are as important as immunosuppressive treatment in the comprehensive care of myositis patients to improve QoL and promote a genuine resumption of patients' active lives, both professional and personal.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.autrev.2023.103455.

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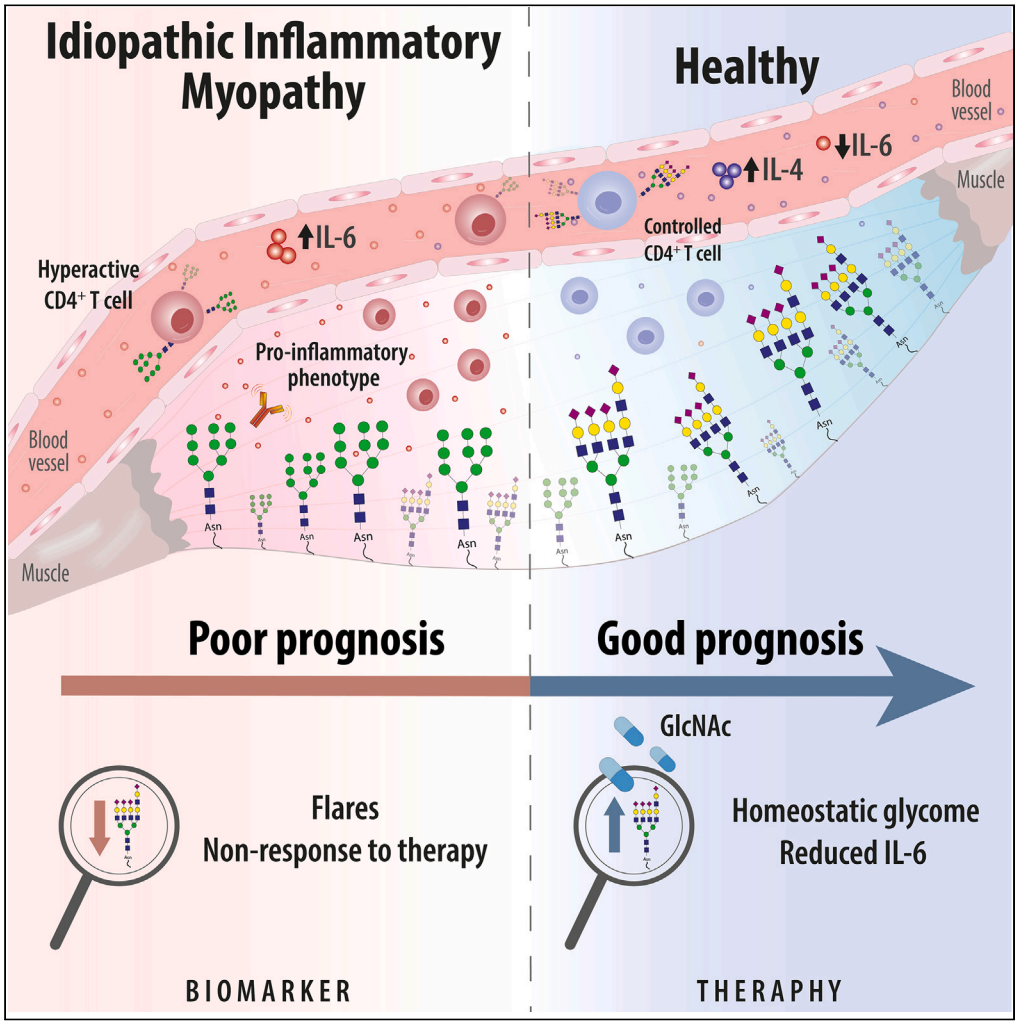

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Highlights

A specific tissue glycosignature in patients with IIM predicts poor disease course

Loss of branched *N*-glycans in peripheral T cells associates with IL-6 production in IIM

Ex vivo glycan supplementation of IIM biopsies results in decreased IL-6 levels

Muscle glycome acts as a prognostic biomarker and a potential therapeutic target

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Muscle glycome in idiopathic inflammatory myopathies: Impact in IL-6 production and disease prognosis

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SUMMARY

Idiopathic inflammatory myopathies (IIM) are a group of chronic autoimmune diseases mainly affecting proximal muscles. Absence of meaningful prognostic factors in IIM has hindered new therapies development. Glycans are essential molecules that regulate immunological tolerance and consequently the onset of autoreactive immune response.

We showed that muscle biopsies from patients with IIM revealed a deficiency in the glycosylation pathway resulting in loss of branched *N*-glycans. At diagnosis, this glycosignature predicted disease relapse and treatment refractoriness. Peripheral $CD4^+$ T cells from active-disease patients shown a deficiency in branched *N*-glycans, linked to increased IL-6 production. Glycan supplementation, restoring homeostatic glycosylation profile, led to a decrease in IL-6 levels.

This study highlights the biological and clinical importance of glycosylation in IIM immunopathogenesis, providing a potential mechanism for IL-6 production. This pinpoints muscle glycome as promising biomarker for personalized follow-up and a potential target for new therapies in a patients' subgroup with an ominous evolution.

INTRODUCTION

Immune-mediated or idiopathic inflammatory myopathies (IIM) are a group of rare systemic autoimmune diseases characterized by proximal skeletal muscle weakness leading to long-term disability, decreased quality of life, and reduced life expectancy. IIM are generally characterized by an increase in muscle enzymes, with or without skin involvement. In addition, other organs may also be affected in a large proportion of patients, namely the gastrointestinal tract, lungs, and heart, often resulting in a poor prognosis.[1] Notwithstanding recent advances in the classification of the disease,[2] clinically the most important subtypes of IIM are dermatomyositis, namely the subgroup of amyopathic dermatomyositis, inclusion body myositis, immune-mediated necrotizing myopathy, and overlap myositis (which includes the subgroup of anti-synthetase syndrome). Polymyositis appears to be an increasingly dying entity.[3]

The etiology of IIM is still unknown, but a relationship among environmental triggers, genetic risk factors, and defective immunoregulatory mechanisms appears to play an important role in the pathogenesis of the disease, particularly with an important, albeit unexplored, role for the interleukin (IL)-6 cytokine.[4,5] IIM share many features with other systemic autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus. It is a chronic, lifelong, and recurrent disease that primarily affects occupationally active people and is associated with significant morbidity, economic burden, and mortality.[6,7] Although survival in these patients has improved since the widespread use of corticosteroids and immunosuppressants, mortality remains elevated in patients with IIM. The 5-year survival rate has been reported to be 60%, especially in the first year after diagnosis.[8] The increased morbidity is primarily due to severe muscle weakness and visceral involvement. Recent reports suggest that only 20%–40% of treated patients achieve remission, while 60%–80% experience a polycyclic or chronic continuous disease progression, which has a major impact on quality of life at mid- and long-term follow-up, as up to 80% of treated patients remain disabled.[6]

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One of the notable features of IIM is the presence of myositis-specific antibodies, which have been used to predict the manifestations of IIM. At our center, we have found that the presence of Ro52 antibodies and the overlap with other autoimmune diseases appear to be independent variables associated with the development of damage and refractoriness in IIM and act as predictors of the worst prognosis (data not published). However, there has long been debate about the extent to which the presence of the various autoantibodies commonly associated with IIM may represent accurate biomarkers for the detection of IIM subcategories or behave as epiphenomena of the disease process.[9] On the other hand, about 15%–20% of patients are seronegative, i.e. they do not display significant levels of autoantibodies. Therefore, there is an urgent need in the clinical setting for accurate and reliable biomarkers to improve the classification and diagnosis of IIM subgroups and to obtain significant reliable prognostic information about disease progression, as this lack of diagnostic and prognostic biomarkers hinders major advances in the treatment of these diseases.[10–12]

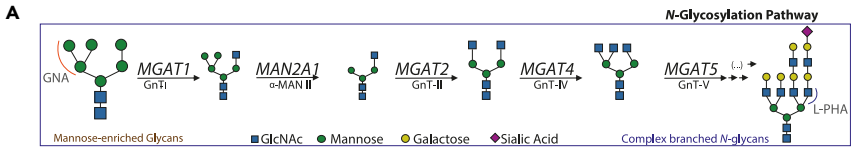
Furthermore, the therapeutic tools available for the treatment of IIM are limited. The choice of different immunotherapeutic drugs is mainly empirical, and standard treatment guidelines are not conservative and not disease/subgrouping-directed. The reasons for this include the rarity of IIM, its heterogeneous clinical phenotype and lack of consensual classification criteria, as well as the small number of randomized, double-blind controlled clinical trials.[13]

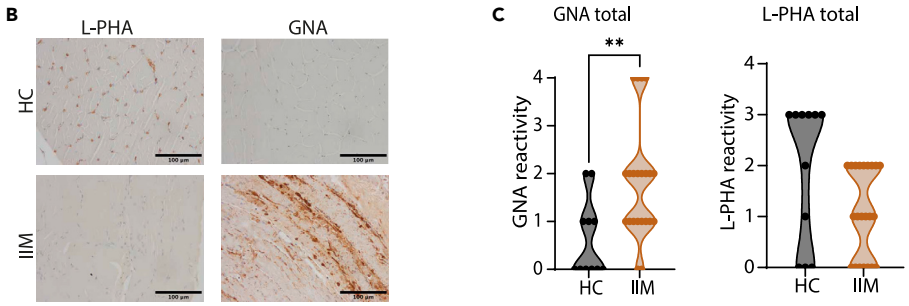
Given these major problems in the clinical and therapeutic management of IIM and the significant medical and societal impact of the disease, there is an urgent need in clinics for the identification and characterization of key molecular parameters/biomarkers that could contribute to the correct diagnosis of the disease subgroup at an early stage of disease progression and, more importantly, to the stratification of patients at increased risk of developing aggressive disease and premature mortality. This knowledge will certainly improve the course of the disease and the rate of therapeutic success, paving the way for the development of novel targeted therapeutic strategies that will ultimately have a positive impact on patient survival and quality of life.

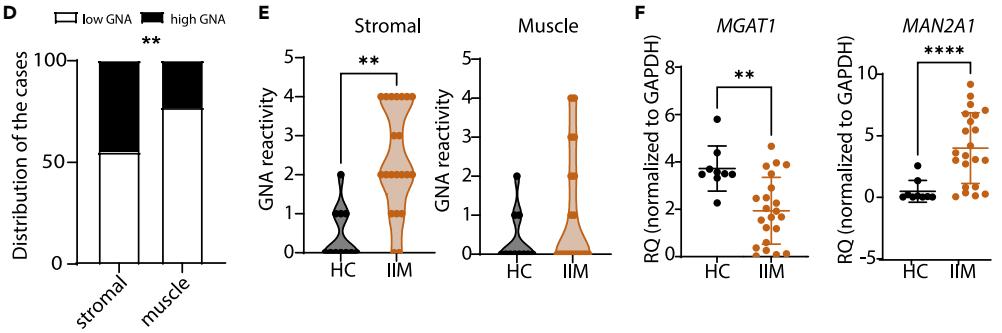
Glycosylation is a major post-translational modification that occurs in essentially all cells. It is characterized by the enzymatic attachment of glycans (sugar chains) to other saccharides, proteins, and lipids, resulting in a diverse and abundant repertoire of glycans on the cell surface collectively known as glycome. Glycans have important biological functions within a cell[14] and are extremely important as master regulators of the immune system. Changes in cellular glycome, including immune cell glycome, can occur in response to environmental and genetic stimuli and are often associated with the acquisition of altered cellular phenotypes such as malignancy and chronic inflammation.[14–17]

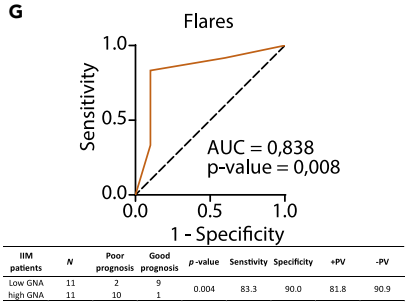
In particular, we and others have demonstrated the importance of glycans as regulators of various immunological mechanisms, including adaptive immunity, especially involving T cells.[18,19] Glycans have been reported to control the rate of T cell receptor (TCR) endocytosis and modulate cellular responses through interaction with lectins (such as galectins) and their ligands.[20] A reduction in the branching of *N*-glycans (in the context of a malfunction/deficiency of *N*-acetylglycosaminil transferase V (GnT-V) glycosyltransferase) on T cells has been shown to lead to an increased clustering of TCRs and consequently a lower threshold for T cell activation.[16] This was demonstrated by our group in patients with ulcerative colitis, in whom a deficiency of branched glycans on intestinal T cells was associated with T cell hyperactivity and increased disease severity.[19] In addition, GnT-V-deficient mice were shown to have an enhanced delayed-type hypersensitivity response and increased susceptibility to experimental autoimmune encephalitis (EAE)[18] and inflammatory bowel disease (IBD).[21] Treatment of these mice with high concentrations of *N*-acetylglucosamine (GlcNAc) increased GnT-V-mediated *N*-glycan branching and inhibited TCR activation and autoimmune responses in mouse models of EAE, IBD, and type 1 diabetes. Furthermore, there is evidence for a role of glycosylation in immune cell differentiation, e.g. in the generation and phenotypic profiles of T helper (Th) cell subsets, particularly activated $CD4^+$ effector T cells (which differentiate into TH1, TH2, TH17, and regulatory T cells), in part through its effect on the production of ligands of various lectins. In particular, *N*-glycan branching has been shown to play an important role in autoimmunity by promoting the differentiation of anti-inflammatory TH2 cells over pro-inflammatory TH17 cells.[22]

In addition to the role of glycans as direct regulators of immune cell function, we have also shown that alterations in epithelial cell glycosylation have an impact on the loss of self-tolerance. We have shown that





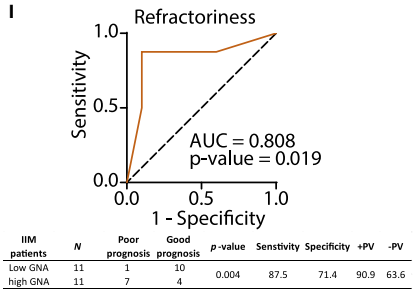




IIM patients	N	Poor prognosis	Good prognosis	p-value	Senstivity	Specificity	+PV	-PV
Low GNA	11	2	9	0.004	83.3	90.0	81.8	90.9
high GNA	11	10	1					

H

		N	Good prognosis	Poor prognosis	OR (95%)	p-value
GNA reactivity	0-50	11	9	2	45,000	0,004
	>50%	11	1	10		
Need biologics	yes	17	10	7	0,412	0,999
	no	5	0	5		
Damage extension	<9	10	7	3	7,000	0,043
	≥9	12	3	9		
Refractoriness	yes	14	10	4	0,286	0,999
	no	8	0	8		
		Multivariate analysis - stepwise				
GNA reactivity	--	--	--	--	--	0,036



IIM patients	N	Poor prognosis	Good prognosis	p-value	Senstivity	Specificity	+PV	-PV
Low GNA	11	1	10	0.004	87.5	71.4	90.9	63.6
high GNA	11	7	4					

J

		N	Good prognosis	Poor prognosis	OR (95%)	p-value
GNA reactivity	0-50	11	10	1	17,500	0,004
	>50%	11	4	7		
Need biologics	no	17	14	3	2,677	0,999
	yes	5	0	5		
Damage extension	<9	10	9	1	12,600	0.036
	≥9	12	5	7		
Flares	0 or 1	10	10	0	3,000	0,999
	>1	12	4	8		
		Multivariate analysis - stepwise				
GNA reactivity	--	--	--	--	--	0.004

Figure 1. Altered *N*-glycosylation profile of muscle biopsies at diagnosis associates with disease course and response to therapy in patients with IIM

(A) Schematic representation of the *N*-glycosylation pathway from high-mannose *N*-glycans (recognized by GNA lectin) toward more complex *N*-glycans, with β1,6-branching antennae (detected by L-PHA lectin).

(B) Representative images of skeletal muscle from IIM and HC, low L-PHA, and high GNA reactivity in IIM biopsies (scale bar = 100 μm).

Figure 1. *Continued*
(C) Qualitative score of GNA and L-PHA staining intensity from total tissue from IIM and HC biopsies. Analysis performed for 22 IIM and 11 HC cases. Unpaired one-tailed Mann-Whitney tests (*p value<0.05, **p value<0.01).
(D) Distribution of IIM disease cases in high (≥50%) and low (<50%) GNA reactivity, at each cellular component (muscular or stromal). Unpaired one-tailed Mann-Whitney tests (*p value<0.05, **p value<0.01).
(E) Quantitative analysis of staining intensity in each cellular compartment (muscular or stromal) of IIM compared with HC. Unpaired one-tailed Mann-Whitney tests (*p value<0.05, **p value<0.01).
(F) *MGAT1* and *MAN2A1* mRNA expression levels on total muscle FFPE biopsies from patients with IIM and HC individuals. Each dot represents one biological sample. Relative quantification (RQ) of mRNA levels is expressed as mean ± SD (Mann-Whitney t-test: *p value<0.05, **p value<0.01).
(G) ROC curve for the GNA reactivity from patients with IIM with poor disease course (more than 1 flare) and its respective AUC and p value.
(H) The predictive capacity of high GNA reactivity and other clinic-pathological parameters to distinguish poor disease prognosis (that have more than 1 flare).
(I) GNA reactivity ROC curve, respective AUC and p value, for IIM patients' refractory to treatment.
(J) The predictive capacity of high GNA reactivity and other clinic-pathological parameters to distinguish non-responder patients (that do not respond to therapy). The error bars represent the standard deviation of the data.

impairment of *N*-glycan branching at the renal cell surface contributes to the pathogenesis of lupus and serves as a potential prognostic biomarker in lupus nephritis.[23]

In the present work, we aimed to characterize the clinical and biological impact of muscle glycome in the immunopathogenesis of IIM, in particular how glycosylation changes at peripheral immune cells regulate the systemic immune response and IIM prognosis. We demonstrated for the first time a clear truncation of the branching *N*-glycosylation pathway in muscle tissue of patients with IIM, which was associated with a poor prognosis. Importantly, we observed that this glycosylation alteration could be detected in the periphery, where circulating $CD4^+$ T cells exhibit deficient *N*-glycosylation associated with IL-6 production. This pathogenic immunophenotype was reversed by metabolic supplementation with glycans from fresh muscle biopsies.

This study contributes to the pathophysiological knowledge of IIM, focusing on epigenetic alterations that may contribute to the pathogenesis evolution and prognosis of this group of rare and heterogeneous diseases. Targeted and more effective therapy relies on a deeper knowledge of the pathogenesis implied in etiology, evolution, and expected prognosis.

RESULTS

IIM muscle biopsies display an altered *N*-glycosylation profile associated with poor disease course and non-response to therapy

Taking in consideration the natural progression of *N*-glycans from high-mannose precursors toward more complex and branched structures (Figure 1A), we aimed to obtain a simple glycosignature from the IIM skeletal muscle based on the levels of these two distant *N*-glycan traits. To do so, histochemistry of GNA and L-PHA lectins were used, to assess the relative levels of high-mannose and β1,6-branched *N*-glycans (respectively) in a cohort of 22 IIM muscle tissue (IIM) and 11 healthy muscle tissue (HC). The demographic and clinical features of patients with IIM are included in the Table S1. The results showed an overall increased reactivity of GNA in muscle biopsies from patients with IIM, compared with HC, together with a decreased L-PHA staining (Figure 1B), despite not statistically significant for L-PHA (Figure 1C). Interestingly, we observed that the major GNA reactivity was detected in the stromal component of the tissue, with less reactivity in the muscle fibers (Figures 1D and 1E). This tissue glycoprofile was further confirmed at transcriptional level, by determining the expression of glycogenes involved in the *N*-glycosylation pathway, such as *MGAT1* and *MAN2A1* (Figure 1A). IIM biopsy cells seem to be significantly deficient in the expression of *MGAT1* (Figure 1F), a gene that encodes for *N*-acetylglucosaminyltransferase-I (GnT-I), a key enzyme that adds the first GlcNAc antenna, converting high-mannose into hybrid *N*-glycan structure. Surprisingly, *MAN2A1*, the gene that encodes for α-mannosidase (an enzyme that acts immediately into the GnT-I product) was increased (Figure 1F), which may be explained by a compensatory mechanism to recover the progression in the *N*-glycosylation pathway and maintain the homeostatic glycan repertoire.

Given our previous knowledge that alterations in the tissues glycome might be associated with pathogenesis and prognosis of the disease,[23,24] we explored, at a longitudinal level, the predictive capacity of GNA reactivity

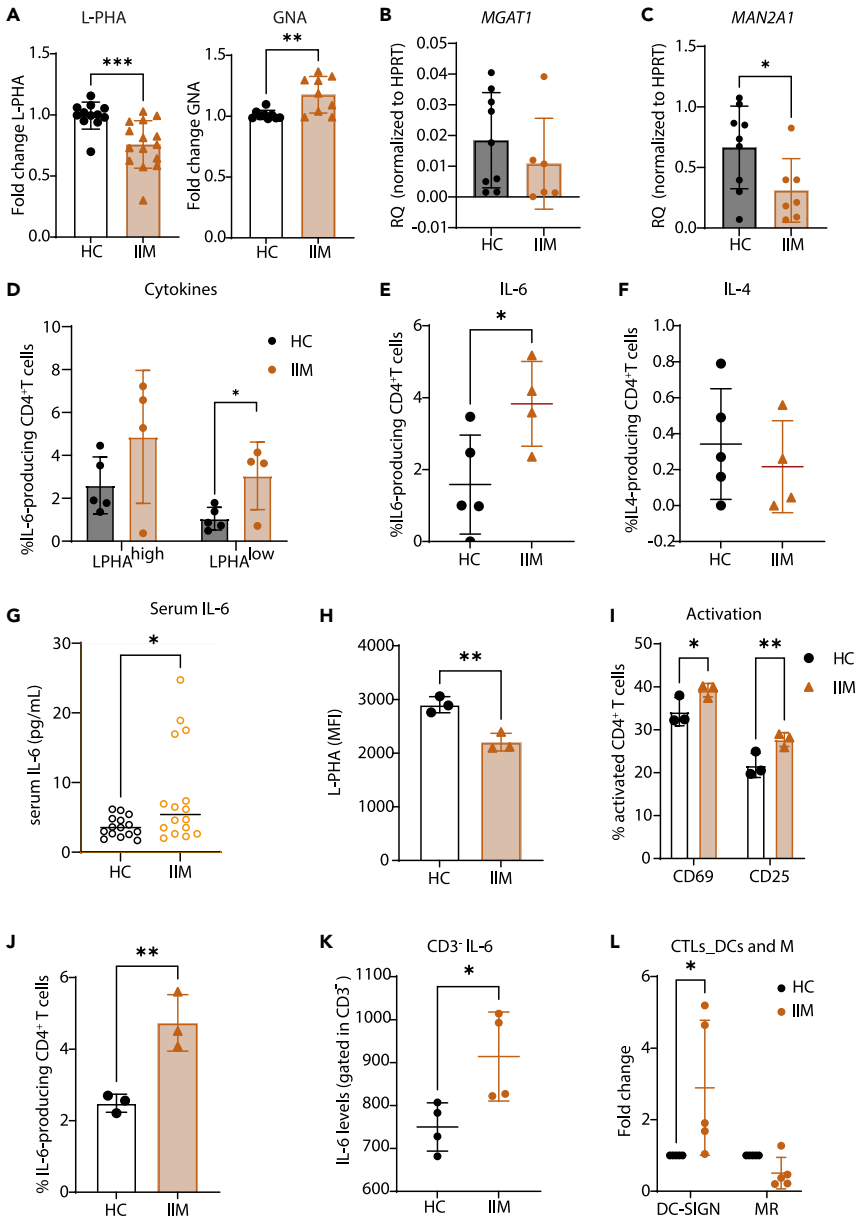


Figure 2. Altered *N*-glycosylation profile of CD4+ T cells associates with increased activation and cytokine production in patients with IIM

(A) Cell surface β1,6-branching *N*-glycans (L-PHA) and high-mannose (GNA) *N*-glycans of CD4$^+$ T cells from IIM and HC peripheral blood, analyzed by flow cytometry. Levels of median fluorescence intensity (MFI) from patients with IIM were normalized for the average of healthy control MFI. Fold change ratio is represented as mean ± SD. Unpaired one-tailed Mann-Whitney tests (*p value<0.05, **p value<0.01).

(B) *MGAT1* and (C) *MAN2A1* mRNA expression levels on peripheral CD3$^+$ T cells from patients with IIM and HC individuals. Each dot represents one biological sample. Relative quantification (RQ) of mRNA levels is expressed as mean ± SD (Mann-Whitney t-test: *p value<0.05,**p value<0.01).

(D) Percentage of IL-6-producing CD4$^+$ T cells in peripheral blood, gated on CD4$^+$ T cells with high vs. low levels of L-PHA staining, from patients with IIM and HC individuals. Percentage of cells are expressed as mean ± SD. Unpaired one-tailed Mann-Whitney test (*p value<0.05, **p value<0.01).

(E) Levels of total IL-6-producing CD4$^+$ T cells, analyzed by flow cytometry. Percentage of cells are expressed as mean ± SD. Unpaired one-tailed Mann-Whitney tests (*p value<0.05, **p value<0.01).

(F) Levels of total IL-4-producing CD4$^+$ T cells, analyzed by flow cytometry. Percentage of cells are expressed as mean ± SD. Unpaired one-tailed Mann-Whitney tests.

Figure 2. *Continued*

(G) Serum IL-6 quantification by ELISA from IIM and HC. Levels are expressed as mean ± SD. Unpaired one-tailed Mann-Whitney test (*p value<0.05, **p value<0.01).

(H) Cell surface β1,6-branching *N*-glycans (L-PHA) of $CD4^+$ T cells from IIM and HC peripheral blood after 24 h stimulated with 0.5 μg/mL anti-CD3, analyzed by flow cytometry. Levels are expressed as mean ± SD. Unpaired one-tailed Mann-Whitney tests (*p value<0.05, **p value<0.01).

(I) Percentage of activated (CD25 or CD69) $CD4^+$ T cells from IIM and HC peripheral blood after 24 h stimulated with 0.5 μg/mL anti-CD3, analyzed by flow cytometry. Percentages are expressed as mean ± SD. Unpaired one-tailed Mann-Whitney test (*p value<0.05, **p value<0.01).

(J) Percentage of IL-6-producing $CD4^+$ T cells from IIM and HC peripheral blood after 24 h stimulated with 0.5 μg/mL anti-CD3, analyzed by flow cytometry. Percentages are expressed as mean ± SD. Unpaired one-tailed Mann-Whitney test (*p value<0.05, **p value<0.01).

(K) Levels of IL-6 produced by $CD3^-$ cells, analyzed by flow cytometry. Percentages are expressed as mean ± SD. Unpaired one-tailed Mann-Whitney tests (*p value<0.05, **p value<0.01).

(L) Median fluorescence intensity (MFI) of DC-SIGN and mannose receptor (MR) in DCs and macrophages from IIM and HC. Levels are expressed as mean ± SD. Unpaired one-tailed Mann-Whitney test (*p value<0.05, **p value<0.01). The error bars represent the standard deviation of the data.

at diagnosis to the disease outcome. We have observed that high GNA reactivity in the stromal compartment (staining for more than 50% of the cells) at diagnosis was able to stratify patients according to the risk of having more than 1 flare (poor prognosis), with a specificity of 90% and sensitivity of 83.3% (Figure 1G). The univariate analysis also demonstrated that high GNA reactivity at diagnosis increased 45-times the odds (p value = 0.004) of patients with IIM to have more than 1 flare at 3 years of follow-up. Interestingly, multivariate analysis revealed that among other clinical parameters used to monitor the disease progression, high GNA reactivity detected at muscle biopsy was the only variable that predicts the development of more than 1 flare in an independent way (Figure 1H). Moreover, high GNA reactivity was also able to identify refractory patients (defined as those that do not respond to 2 immunosuppressive drugs given, concomitantly or subsequently, for at least 3 months) with a specificity of 71.4% and sensitivity of 87.5% (Figure 1I). The univariate analysis showed that high GNA reactivity at diagnosis increased 18-times the odds (p value = 0.004) of patients with IIM to be non-responders at 3 years of follow-up. Multivariate analysis revealed that among other clinical parameters used to monitor the disease, high GNA reactivity at muscle biopsy was the only independent variable that predicts the non-response to treatment (Figure 1J).

Deficiency in complex type *N*-glycans at the surface of peripheral immune cells is associated with pro-inflammatory systemic profile in patients with IIM

Taking in consideration that stromal glycoprofile has shown to be more significantly affected with glycosylation alterations (Figure 1E), we further analyzed the glycoprofile of immune cell population at the periphery, exploring a possible less invasive *proxy* of the *in situ* glycobiomarker. We have found that $CD4^+$ T cells display significantly lower levels of surface β1,6-branched *N*-glycans, concomitantly with significantly increased levels of high-mannose *N*-glycans, detected by L-PHA and GNA reactivity, respectively (Figures 2A and S1A). The transcriptional profile of isolated peripheral $CD3^+$ T cells showed no differences in *MGAT1* gene expression (Figure 2B), but revealed a deficient expression of the gene *MAN2A1*, which may result in the lower hydrolysis of one of the mannose branches precluding the addition of a second GlcNAc antennae and thus leading to the accumulation of less complex *N*-glycans (Figure 2C). Given the previous evidence showing that loss of β1,6-branched *N*-glycans in $CD4^+$ T cells has an impact on the TCR signaling and T cell function,[16,18,21] we analyzed the impact of this glycosylation alteration in $CD4^+$ T cell-producing cytokines, namely IL-6 (one of the key cytokines in the etiopathogenesis of IIM). To do so, we stratified $CD4^+$ T cells into L-PHA high vs. L-PHA low. Interestingly, we have observed that L-PHA low subpopulation showed a significant increase in these IL-6-producing cells in IIM (Figures 2D and S1B). Overall, and as expected, $CD4^+$ T cells from patients with IIM are more prone to produce IL-6, but no differences were found for other cytokines, such as IL-4 (Figures 2E and 2F). Accordingly, serum IL-6 levels from our cohort were shown to be significantly increased in active patients with IIM compared to controls (Figure 2G). Moreover, and to show the dependency of TCR glycosylation in defining the threshold for activation, we have cultured IIM and HC peripheral blood mononuclear cells in the presence of anti-CD3 antibody and checked for the activation and cytokine production of $CD4^+$ T cells. As expected, $CD4^+$ T cells from patients with IIM, displaying lower levels of β1,6-branched *N*-glycans (Figure 2H), revealed increased levels of TCR-dependent activation (Figure 2I) and increased IL-6 production (Figure 2J).

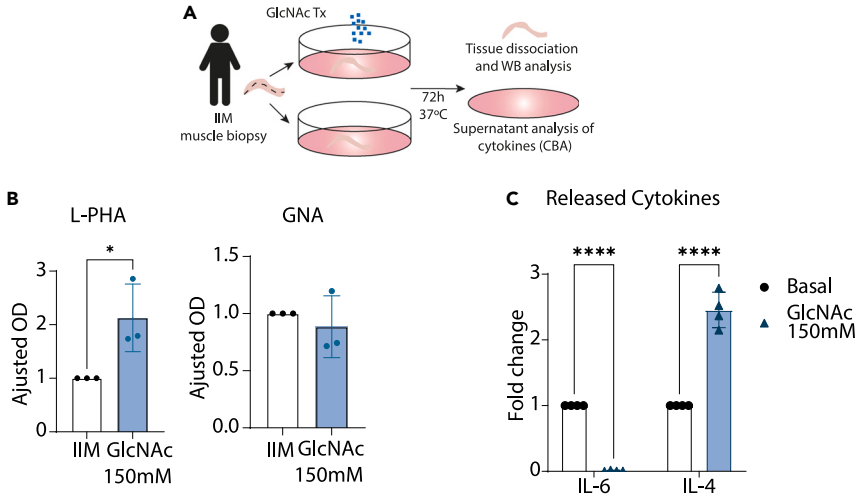


Figure 3. GlcNAc supplementation of fresh muscle biopsies from IIM patients attenuates pro-inflammatory immune response

(A) Experimental outline of 150 mM *N*-acetylglucosamine (GlcNAc) supplementation of IIM biopsies for 72 h. Each patient biopsy was longitudinally cut into 2 similar portions, one for the supplementation (GlcNAc 150 mM) and other for control without supplementation (IIM). After incubation, cells were lysed for protein extraction and lectin blot analysis, together supernatant which was collected and released cytokines were analyzed by cytokine bead assay (CBA).

(B) Adjusted values of intensity from L-PHA and GNA lectin blot of whole tissue cell lysate with or without GlcNAc supplementation. Normalized for loading control (actin). Levels are expressed as mean ± SD. Mann-Whitney tests (*p value<0.05, **p value<0.01).

(C) Fold change of cytokines secreted to the supernatant by IIM biopsies supplemented with 150 mM GlcNAc for 72 h, normalized by the respective basal condition. Levels of released cytokine were analyzed by cytokine bead assay (CBA) One-way ANOVA followed by Tukey's post test.*p < 0.05, **p < 0.01, ***p < 0.001. Levels are expressed as mean ± SD.

Furthermore, we also observed that non-T cells ($CD3^{-}$) from patients with IIM may also contribute for the elevated levels of IL-6 in these patients (Figure 2K), which may include dendritic cells and monocytes, among others. Interestingly, dendritic cells from patients with IIM seem to express higher levels of DC-SIGN, a glycan receptor that recognizes high-mannose *N*-glycans (Figure 2L), implicating a role for innate immunity in the hyperactive environment of IIM.

Ex vivo glycosylation reprograming of muscle tissue with glycans supplementation recovers branching *N*-glycans composition and hampers IL-6 production *in situ*

In order to repair the deficiency in the *N*-glycosylation pathway observed in Figure 1, we have promoted the progression toward branching *N*-glycosylation and thus restoring the levels of branching *N*-glycans. Fresh muscle biopsies from patients with IIM were collected and supplemented *ex vivo* with GlcNAc (Figure 3A). This increases the substrate availability to GnTs and consequently promote the expression of β1,6-branching *N*-glycans on tissue, as previously done by us.[21,25] We have observed a significant increase in the overall levels of β1,6-branching *N*-glycans in tissue glycoproteins (Figures 3B and S2A) which was not significant for high-mannose *N*-glycans (Figures 3C and S2A). We showed that in fact, muscle T cells are a target of glycosylation modification, such as complex branched *N*-glycans (Figures S2B and S2C). Importantly, the increase in β1,6-branching *N*-glycans resulted in a significant reduction of IL-6 production together with an increase of IL-4 cytokine (Figure 3C).

DISCUSSION

Muscle cells surface is enriched in glycans and glycoproteins (the glycocalyx) which role in muscle homeostasis and function remains largely unexplored. In fact, changes in glycosylation are a hallmark among many diseases including cancer, chronic inflammation, and autoimmune diseases.[15,17,23,26] However, whether and how the muscle glycome has a clinical and biological impact in the immunopathogenesis of IIM remains completely unknown. Here, we demonstrated that in fact muscle biopsies from patients with IIM exhibit an abnormal glycosylation profile characterized by a decreased expression of

complex branching *N*-glycans along with an increased expression of mannose-enriched glycoproteins, in comparison with normal controls. These alterations in glycosylation signature appear to be, predominantly observed, in the stroma compartment and inflammatory infiltrate. However, and in order to precisely analyze the structural glycosylation profile of infiltrating muscle T cells, mass spectrometry analysis should be performed in future allowing a better structural insight of the muscle glycome and correlation with the pathogenesis of muscle diseases. Importantly from the clinical standpoint, we showed that this specific glycosignature was found to have prognostic value in patients with IIM. We demonstrated that levels of high-mannose *N*-glycan content in stroma of muscle biopsies, detected at diagnosis, are correlated with a poor disease course (more flares) and non-response to therapy. High GNA reactivity in muscle biopsy, at diagnosis, proved to be an independent variable in predicting the development of more than 1 flare, as well as in identification of refractory patients, with a good sensitivity and specificity, when compared with currently available tools/markers. These tissue-specific glycosylation alterations have been previously reported in other autoimmune diseases, namely in kidney from lupus nephritis,[23,25] synovial fibroblasts from rheumatoid arthritis,[27] as well as fucosylation in IBDs[28] and sialylation in GNE myopathy.[29]

At the periphery, our findings in IIM revealed that $CD4^+$ T cells from peripheral blood of patients with IIM display the same truncation of the *N*-glycosylation pathway as observed in the tissue, suggesting that $CD4^+$ T glycoprofile can be a proxy for what is happening at the muscle tissue, potentially constituting a promising non-invasive biomarker in IIM. Interestingly, we found that peripheral $CD4^+$ T cells with the lower levels of L-PHA (from patients with IIM) seem to present lower TCR thresholds, with increased activation markers and IL-6 production. Accordingly, in 2009, Okayama and colleagues published an elegant study refereeing to the critical role of IL-6 in a murine model of myositis.[5] They found increased IL-6 mRNA levels in the mice muscles (particularly in macrophages) which correlated with the histological severity of myositis. IL-6 was proved to be essential for the development of myositis, in this model, and its blockage suppressed the incidence and severity of myositis. No further studies developing this mechanism were published yet, but IL-6 blockade has been effectively used in some patients with IIM.[30,31] Further studies, using larger cohorts, are needed to understand if this peripheral $CD4^+$ T cell-specific glycosignature may be associated with clinical variables that allow a better monitorization of the patients with less invasive techniques.

The origin for this glycan alteration in the immune compartment of affected muscle in patients with IIM still needs further investigation. However, some mechanisms have been proposed by others and us to explain the link between glycosylation changes and T cells hyperactivity and a pro-inflammatory immune response.[16] In fact, β1,6-branched *N*-glycans were shown to be essential regulator of TCR thresholds in $CD4^+$ T cells in both IBD[19] and multiple sclerosis.[18] In IBD, we showed that a deficiency in branched *N*-glycans in mucosa T cells from patients with ulcerative colitis was associated with disease severity and T cell hyperactivity.[21]

Additionally, we also found that innate immune cells from patients with IIM display an increased capacity to recognize tissue glycosylation alterations as we have observed an increased expression of DC-SIGN in innate immune cells that specifically recognize mannose structures. This observation may underlie the impact of muscle glycosylation alteration in innate immune recognition and consequent potentiation of the pro-inflammatory immune response, as we have observed in lupus,[25] an issue that deserves further investigation.

Therapeutic strategies in immune-mediated systemic diseases, historically and presently, rely on immunosuppression, to reduce the hyperreactivity underlying clinical symptoms and organ dysfunction. Side effects, particularly infections (the main cause of flares in IIM), are the dark face of this approach. Theoretically, restoring immune homeostasis is much more appealing and a more natural way to treat the fundamental problem of these patients, but much harder to achieve. Based in our findings, we propose the glycosylation reprogramming of the muscle as an attractive strategy to recover a homeostatic glycome toward the natural progression of branching *N*-glycosylation with consequences in immune homeostasis by hampering IL-6 production. Overall, glycosylation remodeling by metabolic supplementation with glycans may constitute an appealing therapeutic strategy to attenuate the pro-inflammatory immune response in IIM and might represent a novel target for directed therapy, eventually associated with immunosuppression, in a subset of patients with a hazardous evolution and altered glycosignature.

Limitations of the study

Despite the promising impact of this study in identifying a mechanism that underlies IIM immunopathogenesis, one of the limitations is the retrospective nature of the analysis of the clinical parameters defining poor prognosis. A prospective cohort to validate the findings will be worth. The number of muscle samples from controls was low (comparing to patients), although enough for statistical analysis. The limited sample size of patients with IIM and subgroups precluded some statistical significance supporting the need to validate the mechanism in a larger and prospective cohort of IIM samples and controls.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107172.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. **Study conception and design:** I.A., A.C., C.V., S.S.P. **Acquisition of data:** I.A., A.C., B.S-P., M.M.P., R.N. **Analysis and interpretation of data:** I.A., A.C., B.S-P., R.N., C.V., S.S.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
FVD APC-eFluor780	eBioscience	Cat#65-0865-14
L-PHA-fluorescein, FITC	Vector Laboratories	Cat#FL-1111
GNA biotinylated	Vector Laboratories	Cat#B-1245
Streptavidin PE-Cy7	eBioscience	Cat#25-4317-82; RRID:AB_10116480
BV421 anti-human CD14 (clone 63D3)	Biolegend	Cat#367144; RRID:AB_2810580
PE-Cy7 anti-human CD206 (clone 15-2)	Biolegend	Cat#321123; RRID:AB_10900995
eFluorTM 450 anti-human CD4 (clone RPA-T4)	eBioscience	Cat#48-0049-41; RRID:AB_1272127
APC anti-human CD11c (clone BU15)	eBioscience	Cat#17-0128-42; RRID:AB_11151141
PE anti-human TCRα/β (clone BW242/412)	Miltenyi	Cat#130-098-887; RRID:AB_2661264
BV510 anti-human CD3 (clone OKT3)	BD Biosciences	Cat#69-0037-42; RRID: AB_2848508
Polyclonal DC-SIGN rabbit IgG	Biorad	Cat#AHP627; RRID:AB_323683
Polyclonal swine anti-rabbit IgG (FITC)	DAKO	Cat#E0354
Pacific Blue IL-6 (clone MQ2-13A5)	Biolegend	Cat# 501113; RRID: AB_2561425
APC IL-4 (clone MP4-25D2)	Biolegend	Cat# 500811; RRID: AB_315130
L-PHA biotinylated	Vector Laboratories	Cat#B-1115
Mouse IgG anti-actin (C4)	Santa Cruz	Cat#SC-47778; RRID: AB_626632
anti-CD4 antibody [EPR6855]	Abcam	Cat# ab133616; RRID: AB_2750883
GNA-fluorescein	Vector Laboratories	Cat# FL-1241-2
PE Donkey anti-rabbit IgG	Biolegnd	Cat#406421 RRID: AB_2563484
Anti-human TCRβ polyclonal antibody	Santa Cruz Biotechnology	Cat#sc-9101 RRID: AB_661720
Biological samples		
FFPE Muscle biopsies	Neuropathology Department of Centro Hospitalar Universitário do Porto	https://www.chporto.pt/v0C0B0G0K/neuroporto
Human PBMCs	Clinical Immunology Unit, Porto University Hospital Centre, Porto, Portugal	https://www.chporto.pt/v0C0B0E0M/unidade-multidisciplinar-de-imunologia-clinica
Chemicals, peptides, and recombinant proteins		
Lymphoprep™	Stemcell Technologies	Cat#07801
FBS Premium Heat Inactivated	VWR	Cat#S181H
Mouse serum	Jackson Immunoresearch	Cat# 015-000-120
Phorbol 12-myristate 13-acetate, PMA	Sigma	Cat#P8139
Ionomycin	Sigma	Cat#I0634
Brefeldin A	Sigma	Cat#B7651
Xylene	Fisher Chemical	Cat#1330-20-7
Alcohol 99%	Enzymatic	Cat#64-17-5
30% Hydrogen Peroxide	Fisher Chemical	Cat#7722-84-1
3,3′-diaminobenzidine, DAB	Sigma	Cat#D4293
Haematoxylin	Epredia	Cat#720804
Entellan® new	Sigma	Cat#107961

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
SuperScript IV Reverse Transcriptase	Invitrogen	Cat# 18090050
Random hexamer primers	Invitrogen	Cat#48190-011
RPMI 1640 Medium, GlutaMAX™ supplement, HEPES	Gibco™	Cat#72400-054
Penicillin-Streptomycin (10000 U/mL)	Gibco™	Cat#15140122
N-Acetyl-D-glucosamine, GlcNAc	Sigma	Cat#A8625
Trizma® base	Sigma	Cat#T6066
Hydrochloric Acid, HCl	Merck	Cat#109057
NaCl	Sigma	Cat#S9888
EDTA	Sigma	Cat#E5134
Nonidet P-40	Sigma	Cat#I3021
PMSF	Roche	Cat#10837091001
Sodium ortovanadate	Sigma	Cat#S6508
Complete Protease Inhibitor Cocktail	Roche	Cat#50-100-3301
TMB (3,3′,5,5′-tetramethylbenzidine) chromogen	Invitrogen	Cat#88-7314-88
2N H_2SO_4	Merck	Cat#100731
10% bis-acrylamide	Serva	Cat#10688
0.45 μm nitrocellulose membranes	Amersham	Cat#10600002
Tween-20	Sigma	Cat#1379
Bovine Serum Albumin, BSA	Sigma	Cat#A7906
ECL	Amersham	Cat#RPN2106
Rabbit serum	DAKO	Cat#X0902
Critical commercial assays		
Foxp3/Transcription factor staining buffer Set	eBioscience	Cat#00-5523-00
Vectastain ABC kit	Vector Laboratories	Cat#PK-6100
RecoverAll™ Total Nucleic Acid Isolation Kit	Invitrogen	Cat#AM1975
Mouse ELISA Ready-SET-Go! Kits	eBioscience	Cat#88706488
BD Cytometric Bead Array Human Th1/Th2/Th17 Kit	BD Bioscience	Cat# 551811
Protein G Sepharose™ 4 Fast Flow	Cytiva	Cat#17061801
Oligonucleotides		
MAN2A1-FAM TaqMan probe	Applied Biosystems	Cat# Hs01123597
MGAT1-FAM TaqMan probe	Applied Biosystems	Cat# Hs00159121
GAPDH-FAM TaqMan probe	Applied Biosystems	Cat# Hs02758991
Software and algorithms		
BD Diva	Becton Dickinson	N/A
FlowJo v10	Becton Dickinson (Tree Star Inc)	N/A
Prism 9	GraphPad software	N/A
Image Lab Software	BIO-RAD	N/A
Statistical software SPSS v25	IBM Corp	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Salomé S. Pinho (salomep@ipatimup.pt).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new datasets.

This study does not report original code.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

To characterise the glycoprofile of human skeletal muscle in IIM, 22 formalin-fixed and paraffin-embedded (FFPE) muscle biopsies from IIM patients were selected from the neuropathology department of the Centro Hospitalar Universitário do Santo António (CHUSA) for lectin histochemistry and gene expression analysis. The study population reflects the nature of this group of diseases, which themselves are rare and heterogeneous. Muscle biopsies were selected taking into account availability of samples (as not all patients with suspected myositis undergo muscle biopsy for various reasons), origin of samples (more recent samples were preferred due to their better preservation) and quality of samples (definitive histologic myositis changes were preferred over non-specific changes or samples with low quantity or poor tissue quality) (Table S1). Blood samples were collected from patients who currently had active disease, regardless of treatment.

In addition, 11 FFPE muscle biopsies from healthy controls were used. All samples were collected from patients undergoing a scheduled muscle biopsy (2019–2021) at CHUSA. Biopsies were performed at diagnosis without treatment, all were surgical biopsies, and analyses were performed in 2 steps: fresh samples for immunohistochemistry and haematoxylin-eosin, along with genetic analyses, in preserved samples for each and all patients.

All participants gave informed consent and the procedures were approved by the CHUSA Ethics Committee (2017-155 (132- DEFI 124-CES)).

Poor prognosis was classified as patients who were refractory (defined as patients who did not respond to 2 concurrent or subsequent immunosuppressive drugs for at least 3 months[32] or required biologic treatment, or > had 1 relapse in the last 4 years.

Human PBMCs isolation

Human peripheral blood mononuclear cells (PBMCs) from IIM patients and healthy donors were isolated by gradient centrifugation using LymphoprepTM (Stemcell Technologies). The upper phase, containing the serum, was collected and stored at −80°C. PBMCs (interphase) were collected, washed twice with PBS and incubated with fixable viability dye (FVD)-APC-eFluor780 (eBioscience) for 30 min. Cells were washed with PBS and resuspended in FACS buffer (PBS 2%FBS).

Flow cytometry staining

For lectin staining, cells were incubated with conjugated lectins (Vector Laboratories): Phaseolus Vulgaris leucoagglutinin (L-PHA fluorescein, FITC), Galanthus Nivalis lectin (GNA-biotinylated) for 15 min. Subsequently, non-specific binding was prevented by blocking with 2% mouse serum in FACS for 10 min and the biotinylated lectin was incubated with streptavidin-PE-Cy7 (eBioscience) for 30 min. For surface marker staining, cells were stained for 30 min on ice and protected from light with the following antibodies: BV421 anti-human CD14 (clone 63D3), PE -Cy7 anti-human CD206 (clone 15-2) from Biolegend; eFluorTM 450 anti-human CD4 (clone RPA-T4), APC anti-human CD11c (clone BU15), from eBioscience; PE anti-human TCRα/β (clone BW242/412) from Miltenyi; BV510 anti-human CD3 (clone OKT3) from BD Biosciences; for DC-SIGN staining, cells were incubated with polyclonal DC-SIGN rabbit IgG (Biorad), followed by incubation with polyclonal porcine anti-rabbit IgG (FITC; Dako) for 30 min on ice. Cells were resuspended in FACS buffer prior to analysis. For intracellular staining, cells were previously incubated with 200 ng/mL phorbol myristate acetate (PMA, Sigma), 20 ng/mL ionomycin (Merck) and 100 ng/mL Brefeldin A (Sigma) for 5 h. Fixation and permeabilization was performed using the Foxp3/transcription factor staining buffer set (eBioscience) according to the manufacturer's instructions. Finally, cells were incubated with Pacific Blue IL -6 (MQ2-13A5, Biolegend) and APC IL-4 (MP4-25D2, Biolegend) for 30 min. Data were obtained using a BD FACS Canto II instrument (Becton Dickinson) and analysed using FlowJo v10.0 (Tree Star Inc.).

Lectin histochemistry

FFPE muscle tissue sections (3 μm) from IIM patients (n=22) and healthy controls (n=11) were analysed by lectin histochemistry. For glycan profile determination, sections were deparaffinised, rehydrated and endogenous peroxidase activity blocked (3% H_2O_2). Sections were then incubated with the biotinylated Phaseolus Vulgaris Leucoagglutinin (L-PHA) lectin, which recognises β1,6GlcNAc-branched *N*-glycans, or with the biotinylated Galanthus Nivalis agglutinin (GNA) lectin, which is used for the detection of terminal α-1,3 mannose residues. The avidin-biotin-peroxidase complex was detected using the Vectastain ABC kit and the colour was developed using 3,3'-diaminobenzidine (DAB, Thermo Scientific, DE, USA). L-PHA, GNA and the Vectastain ABC kit were obtained from Vector Laboratories, Burlingame, CA, USA. Finally, all sections were counterstained with haematoxylin, dehydrated and preserved in appropriate embedding medium (Entellan® new, Merck Millipore).

Immunofluorescence staining

FFPE muscle tissue sections (3 μm) were double stained for CD4 receptor and high-mannose (GNA). Firstly, sections were deparaffinized and rehydrated, followed by antigen retrieval in citrate buffer for 40 min. After blocking with rabbit serum (x0902, DAKO) in BSA 10% (1:5) for 30 min, samples were incubated with anti-CD4 antibody (1:500, EPR6855, Abcam) and GNA-FITC conjugated lectin (1:100, Vector Laboratories) for 2 h in the dark. Then, the samples were washed 3 times for 10 min with PBST 0.01%, followed by incubation with donkey anti-rabbit PE-conjugated secondary antibody (ref 406421, Biolegend) in BSA 10% (1:200) for 1 h. Lastly, cell nuclei were stained with DAPI in PBS (1:100) for 5 min at RT and images were recorded in Zeiss Axio Imager Z1.

mRNA expression

Total RNA was isolated from 20 μm sections of formalin-fixed, paraffin-embedded (FFPE) tissue samples from muscle IIM patients (n=22) using the RecoverAll™ Total Nucleic Acid Isolation Kit (Invitrogen) according to the manufacturer's protocol. Total RNA was quantified using the Nanodrop 1000 system and 100 μg was transcribed into single-stranded cDNA using SuperScript II Reverse Transcriptase and Random Hexamer Primers (Invitrogen, Oregon, USA) according to the manufacturer's recommendations. Quantitative real-time PCR (qRT-PCR) was performed in duplicate for the following target genes: *MAN2A1*-FAM (Hs01123597) and *MGAT1*-FAM (Hs00159121), both TaqMan probes from Applied Biosystems, and the housekeeping gene *GAPDH*-FAM (Hs02758991). Reactions were performed using a 7500 Fast Real-time PCR System (Applied Biosystems™) and mRNA data were analysed using the comparative 2(-ΔΔCT) method, normalised to housekeeping gene mRNA expression.

Human IIM muscle explant supplementation

A fragment of each IIM muscle biopsy was cut longitudinally into two similar sections. One of the sections was incubated in 200 μL of complete RPMI supplemented with 150 mM GlcNAc at 37°C and 5% CO_2 for 72 h. The other part of the biopsy was incubated in the same medium. The other section of the biopsy was incubated under the same conditions but without the addition of GlcNAc. At the end of the incubation period, the supernatants were collected for cytokine analysis and the biopsy parts were weighed. Protein lysates were obtained by dissociating the tissue with a pestle in lysis buffer (50 mM TrisHCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) supplemented with protease and phosphatase inhibitors, 100 mm PMSF, 100 mM Sodium ortovanadate and Complete Protease Inhibitor Cocktail (Roche).

ELISA and CBA

IL-6 concentration in sera from IIM and healthy donors (mouse ELISA Ready- SET -Go! kits from eBioscience) was measured by ELISA according to the manufacturer's protocol. TMB (3,3',5,5'-tetramethylbenzidine) chromogen solution (eBioscience) was used as substrate and 2N H_2SO_4 as stop solution. Absorbance was measured at 450 nm and 570 nm using a microplate reader (Biotek Instruments).

For the supplemented human muscle biopsies, cytokine concentrations were analysed by flow cytometry using cytometric bead arrays: the BD Cytometric Bead Array Human Th1/Th2/Th17 Kit (BD Bioscience), according to the manufacturer's instructions. Samples were measured on the BD Accuri C6 instrument (BD Biosciences, US) using a specific template provided by BD Biosciences.

Lectin blot

For analysis of high mannose compared to complex *N*-glycans on the surface of the IIM biopsy cell population, SDS-PAGE -dissolved proteins (10% bis-acrylamide) were transferred to 0.45 μm nitrocellulose membranes (Amersham) and incubated in blocking solution (PBS 0.05% Tween-20 and 5% BSA) ON at 4°C. Membranes were then incubated with 3.5 μg/mL biotinylated L-PHA for 1 h at room temperature, washed 3 times with PBS 0.05% Tween 20 and incubated with ECL (Amersham) for 30 min prior to development with the Vectastain ABC kit. Mouse IgG anti-actin (C4, Santa Cruz) was used for loading control analysis.

Immunoprecipitation and Western-blot

For the immunoprecipitation (IP) of the TCRαβ, protein was obtained from total cell lysates of muscle biopsies treated with GlcNAc or non-treated from IIM patients. Equal amounts (30 μg of protein) of protein were pre-cleared 60 min, at 4°C with Protein G Sepharose™ 4 Fast Flow beads (Cytiva, Sweden) and the supernatant was incubated with polyclonal rabbit anti-human TCRβ antibody (H-197; Santa Cruz Biotechnology) overnight, at 4°C. The immune complex was incubated with Protein G-Sepharose beads, for 2 h, at 4°C under rotation. Then, it was boiled at 96.5°C for 5 min to release the immune complex. The samples were separated by 10% SDS–PAGE electrophoresis and transferred onto nitrocellulose membranes (Amersham). For the TCRβ, the membrane was blocked with 5% non-fat milk and then probed with the primary anti-human TCRβ polyclonal antibody (1:100) and revealed with secondary antibody goat anti-rabbit IgG-HRP (1:2000; GE Healthcare, Life Sciences). For the β1,6 GlcNAc branched glycans, the membrane was blocked with 4% Bovine Serum Albumin (BSA, Sigma) and then incubated with the biotinylated L-PHA (10 μg/mL) lectin and revealed with the VECTASTAIN® ABC kit peroxidase (Vector Labs, Burlingame, CA, USA) kit. The detection was performed by the ECL reagent (Amersham). Quantitative analyses were performed by densitometric scanning of bands (GS-900 Calibrated Densitometer) and analyzed in Image Lab software (BIO-RAD).

In vitro TCR stimulation

Human peripheral blood mononuclear cells were isolated as stated above. After washing, 200 000 cells were cultured for 24h, at 37 ºC in 96-well round bottom plates coated with anti-CD3 monoclonal antibody (mAb clone OKT3, 0.5 μg/mL eBioscience™) in 200 μL of Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-streptomycin (Pen-Strep). For the analysis of TCR-dependent IL-6 production, 100 ng/mL of Brefaldin A (Sigma) were added to the wells.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data visualisation and statistical analyses (non-parametric Mann-Whitney t-test) were performed using GraphPad Prism 9 software.

The predictive power of GNA reactivity to discriminate patients with poor disease progression from those with good disease progression was determined by plotting receiver operating characteristic curves (ROC) and calculating the area under the curve (AUC). Adaptability was assessed using the Hosmer-Lemeshow statistic and test. Results are presented in the form of odds ratios (ORs) for each category compared to a predefined reference category and their respective 95% confidence intervals (CIs). Odds ratios above one or below one are indicative of a higher or lower probability, respectively, of developing a poor disease outcome compared to a reference category.

Datapoints were tested for outliers using ROUT testing and excluded when identified.

Statistical analysis was performed using Statistical Software version 25 (IBM Corp., IBM SPSS Statistics for Windows, version 25.0, Armonk, NY; published 2017). The threshold for statistical significance was $p\text{-value} < 0.05$.

CHAPTER IV

Discussion

1. What have we learned from Unidade de Imunologia Clínica' Idiopathic Inflammatory Myopathies cohort characterisation and analysis?

The Unidade de Imunologia Clínica (UIC)' cohort of IIM describes the first fully characterised cohort of Portuguese myositis patients and one of the longest follow-up time span reported in the literature. Time is of critical importance in chronic diseases as it allows us to get a picture of natural and conditional (therapy-related) evolution and, most importantly, to quantify and qualify the damage accrual.

The EULAR/ACR classification criteria applied to our patients confirmed the moderate probability of IIM and mean maximum score, as previously identified (91), and this is consistent with other cohorts (18-20, 92, 93). In some patients, the clinical diagnosis was completely different from the results of the classification criteria, particularly in relation to IMNM, aSyS and OM, almost all of which were classified as PM. This confirms the increasingly accepted subclassification of IIM patients according to clinical phenotype and positive MSAs, which is also referred to in other cohorts (15-17).

IIM phenotypes of all patients were reviewed based on clinical and serological features and supplemented by histological results when available, according to Anthony Amato's proposed criteria and approved by European Neuromuscular Centre (ENMC) workshop (43, 94). This resulted in a more diverse but also more distributed class of IIM subtypes, decreasing the proportion of DM patients but, most importantly, almost eliminating the PM diagnosis, with most of them diagnosed in the IMNM, OM and aSyS subgroups. We believe this reflects a more real panorama of IIM distribution in clinics.

Muscle biopsy, once considered the gold standard, was found to be less accurate for diagnosis in our cohort, which has also been recently described in other cohorts, especially without concomitant electromyographic examination (95-97). IIM is a patchy muscle disease, which makes it difficult to determine the pathological location of immune-mediated changes at a specific site corresponding to the site where the biopsy is performed. On the other hand, according to the EULAR/ ACR criteria, only some very specific changes are considered significant for myositis, so histological findings of IMNM, for example, are not considered. Finally, muscle biopsy is preferably performed on the upper limbs, but diagnostic accuracy is higher for lower limb samples (95). Nevertheless, it remains today an indispensable tool essentially for differential diagnosis, especially in refractory cases, when diagnosis must be revisited.

Diagnosis occurred in our cohort with an average delay of over 2 years after the first symptoms. This is justified by several reasons, but mainly because of the rarity and heterogeneity of this disease group, which is hardly known to most general practitioners. The other, no less important aspect is the nature of the presentation in a considerable number of patients, such as isolated ILD or arthritis. Knowledge of the different clinical features with which these diseases can present is crucial for timely diagnosis and initiation of treatment. Delayed diagnosis also leads to accumulation of potential damage by the time of diagnosis and implementation of therapy, contributing to disability and poor quality of life, and may also contribute to refractoriness (~38% in our cohort).

Survival of IIM patients has increased in recent decades, as confirmed in our and some other cohorts (98-100), although reports vary widely by cohort. Heterogeneity in subgroup classification, disease involvements staging, inclusion or non-inclusion of myositis associated with malignancy are some of the reasons that make comparison between different studies difficult. Worldwide, reported mortality rates vary between 2-45% (102). In our cohort, mortality was 16.8%, which is higher than in the general population and in other systemic AID, except for SLE with nephritis (103-105). In our study, we chose to include cancer-associated myositis because the relative number of these patients was small. The causes of mortality in our cohort are comparable to those described in other cohorts, including patients with paraneoplastic myositis. Of note, active myositis contributes to few deaths, with the leading cause after malignancy being cardiovascular disease, as described by others (100, 106, 107), which is a direct consequence of the accumulation of cardiovascular risk factors, as shown by our analysis of comorbidities.

Therapeutic strategies are based on the clinical assessment of the patient's condition. In this context, the distinction between activity and damage is crucial in order to neither under-treat, leading to disease progression, nor over-treat, which may lead to severe morbidity.

The damage accrual was enormous in our cohort, affecting more than 90% of patients. Similar figures are reported by others (61, 108). Muscle, gastrointestinal and lung damage, the most common, are mainly a direct consequence of IIM disease. However, more than 60% of patients had endocrine (essentially diabetes and dyslipidaemia) and cardiovascular damage, which is a consequence of corticosteroid therapy and a major contributor to death. Indeed, recent studies show an increased incidence of coronary heart disease in myositis patients (105, 109-111), among other cardiovascular events, as reported in a meta-analysis of 22 observational studies published by Xiong A *et al* in 2021 (112). Dermatomyositis patients appear to have the greatest cardiovascular risk. Some studies have shown that the risk is similar to that of diabetes, and parallel to SLE (113). All

of this is strong evidence of the importance of cardiovascular monitoring, prevention and treatment in patients with IIM.

The Charlson Comorbidity Index (CCI) is the most widely used comorbidity assessment index in the world (114). However, it has several limitations (115) and does not fully reflect the heterogeneity, life-threatening and quality-of-life-impairing comorbidities in IIM patients. Chronic lung disease and psychiatric disorders are examples. Cardiovascular risk factors in CCI are limited to diabetes, not including other factors (hypertension and dyslipidaemia) and especially their combination as a metabolic syndrome, which carries a different and higher risk of end-organ damage. Despite these limitations, the evaluation in our cohort of living IIM patients yielded an estimated 10-year survival rate of just over 50%, taking into account the comorbidities present and highlights their importance. Nevertheless, it differs from the calculated survival rates of our and other cohorts, confirming the low accuracy for morbidity evaluation in this context.

Given the limitations of CCI, we decided to analyse all important comorbidities in IIM patients. The overall picture shows a high prevalence of comorbidities (> 90% of patients) which add to IIM itself. Although comparison with other cohorts is limited as this analysis is not presented as a whole in most series, other studies report similar figures (61, 62, 116). Cardiovascular risk factors top the list, with circa 80% of patients having at least one factor and almost 15% having a metabolic syndrome. This is followed by end-organ consequences, with structural cardiopathy and chronic kidney disease being the most important consequences, directly contributing to death and morbidity. Our and other observations are clear as to the cause of these serious comorbidities (61, 113): a cumulative high dose of steroids, with a contribution from on-going active disease associated with accelerated atherosclerosis. The accumulation of comorbidities places an enormous burden on patients' daily lives. It not only shortens life expectancy, but also complicates the management of basal autoimmune disease. Polypharmacy and drug-drug interactions are main problems, as well as restrictions on the use of some immunosuppressive drugs when thinking of kidney and/or liver failure, heart problems and infection risks, if diabetes is also present for example. The number of tablets took daily increases with the accumulation of comorbidities, which lead to additional side effects and intolerances and affect the patient's psyche.

Depression is very common in patients with IIM (62, 117), which was also confirmed in our study, although it is rarely addressed in most series. Furthermore, mood alterations are not included in myositis damage index (MDI), which makes it more difficult to capture in routine care. Depression contributes to deterioration in functioning and a prolongation of

absenteeism, and affects patients' adherence to medication and non-pharmacological interventions, such as physical rehabilitation programmes. In our cohort, depression was only considered if it required treatment, which means additional medication as well as drug interactions and side effects.

Health Assessment Questionnaire - Disability Index (HAQ-DI) is one of the most used scores to assess quality of life related to health and is included in International Myositis Assessment & Clinical Studies Group (IMACS) Core Set of Domains and Measures (118), although it is not fully validated for IIM patients (119). HAQ-DI was very high in our cohort of myositis patients (median 2.09), describing a low quality of life, comparing with other cohorts (120). The prevalence of depression and the proportion of patients still with active disease (~40%), at the time of evaluation, might have influenced the results. HAQ was higher in sIBM, due to muscle damage and dysphagia, and aSyS, mainly driven by lung involvement and arthritis. Positive correlation was found with other outcome measures of morbidity, except for refractoriness, but low quality of life appears to be affected in an independent way by comorbidities accumulation, highlighting their importance.

Mortality, on the other hand, is influenced in an independent way by damage severity, but not by other morbidity measures. Curiously, delay in diagnosis was inversely correlated with mortality, which is contrary to findings by others (102), with the exception of Bronner *et al* (121), who also describe a lower interval between symptoms initiation and diagnosis as a predictor of death. We believe this is due to the severity of disease at presentation in some patients, particularly in aSyS and CADM with rapidly progressive ILD, which ultimately leads to death when it is refractory to treatment induction.

Analysis among IIM subgroups in our cohort revealed aSyS, after paraneoplastic myositis, globally as the most ominous sub-entity, with highest scores in all morbidities outcome measures, particularly concerning damage and comorbidities, and the second most deadly after cancer associated myositis. Although it is usually therapy responsive, attention should be paid to aSyS, as control of disease activity often implies long-term heavy immunosuppressive therapy (121), which carries pharmacological side effects. With respect to lung involvement in aSS, most times, therapy only serves to avoid/ slow down progression, which actually means slower but progressive damage accrual, with consequent lost in quality of life and higher mortality.

Our study is one of the first to concentrate information on global morbimortality, correlating different outcomes with mortality and quality of life, in the same cohort of IIM. Different parameters correlate with death and, in those who survive, with quality of life. In order to

deliver the best care for myositis patients, all these issues need to be accounted for, concerning diagnosis and management, in routine daily practice. Comprehensive care of IIM patients, as for other systemic autoimmune disease, goes far beyond controlling inflammation and disease activity. Damage, in all its dimensions, and comorbidities, in all its extension, are as or even more important. A specific morbidity index for myositis patients, able to be implemented in routine clinical practice, and prospectively validated, may pave the way for a more rational, homogeneous and effective way of caring for IIM patients, as well as facilitating significant clinical trials design.

2. How can glycans function as immunomodulators in Idiopathic Inflammatory Myopathies?

The surface of muscle cells is rich in glycans and glycoproteins (the glycocalyx), who's role in muscle homeostasis and function remains largely unexplored. Indeed, alterations in glycosylation are a hallmark of many diseases, including cancer, chronic inflammation and autoimmune diseases (67, 69, 73, 80, 87). However, whether and how muscle glycome has a clinical and biological impact on the immunopathogenesis of IIM is still not completely understood.

In our study, we have shown that muscle biopsies from IIM patients do indeed exhibit an abnormal glycosylation profile, characterised by decreased expression of complex branched N-glycans and increased expression of mannose-enriched glycoproteins, in comparison with normal controls. These changes in glycosylation signature appear to be seen primarily in the stromal compartment and inflammatory infiltrate. However, and in order to precisely analyse the structural glycosylation profile of infiltrating muscle T cells, mass spectrometry analysis should be performed in future allowing a better structural characterisation of the muscle glycome and correlation with the pathogenesis of muscle diseases.

From a clinical perspective, it is important that we have shown that this specific glycosignature has prognostic value in IIM patients. We have demonstrated that levels of high-mannose N-glycans content in the stroma of muscle biopsies detected at diagnosis correlate with poor disease course (more disease relapses) and non-response to therapy. High *Galanthus nivallis* agglutinin (GNA) reactivity in muscle biopsy was found to be an independent variable in predicting the development of more than one disease relapse as well as in identification of refractory patients, with good sensitivity and specificity, when

compared to currently available tools/markers. These results highlight the identification of a potential new prognostic biomarker in IIM disease. However, validation in different cohorts, higher sample size and preferentially multicentre and longitudinal cohorts is needed to confirm these results importance in the pathophysiology of a subset of IIM patients. These tissue-specific glycosylation alterations have been previously reported in other autoimmune diseases, namely in kidney from lupus nephritis (80), synovial fibroblasts from rheumatoid arthritis (78), as well as fucosylation in IBD (122) and sialylation in GNE myopathy (84).

In clinical practise, there is an urgent need for disease markers in IIM that are better suited to be reassessed over time in a less invasive way than histology.

At the periphery, our findings in IIM revealed that $CD4^+$ T cells from peripheral blood of patients with IIM display the same truncation of the *N*-glycosylation pathway as observed in the tissue, suggesting that $CD4^+$ T glycoprofile can be a proxy for what is happening at the muscle tissue, potentially constituting a promising non-invasive glycobiomarker in IIM, namely in identifying patients at higher risk of developing long-term damage. Interestingly, we found that peripheral $CD4^+$ T cells with the lower levels of *Phaseolus vulgaris* leucoagglutinin (L-PHA) (from patients with IIM) seem to present lower TCR thresholds, with increased activation markers and IL-6 production. Accordingly, in 2009, Okayama and colleagues published an elegant study refereeing to the critical role of IL-6 in a murine model of myositis (123). They found increased IL-6 mRNA levels in the mice muscles (particularly in macrophages) which correlated with the histological severity of myositis. IL-6 was proved to be essential for the development of myositis, in this model, and its blockage suppressed the incidence and severity of myositis. No further studies developing this mechanism were published yet, but IL-6 blockade has been effectively used in some patients with IIM (124, 125). In this study, we present a possible mechanism supporting the role of IL-6 in the pathogenesis of IIM. We propose the N-glycosylation profile of peripheral $CD4^+$ T cells as a potential factor of systemic inflammation associated with by IL-6 production.

Further studies, using larger cohorts, are needed to understand if this peripheral $CD4^+$ T cell-specific glycosignature may be associated with clinical variables and/ or IL-6 circulatory levels that allow a better monitorization of the patients with less invasive techniques.

The origin of this glycan change in the immune compartment of the affected muscle in IIM patients needs further investigation. However, some mechanisms have been proposed by others and us to explain the association between glycosylation changes and T-cell hyperactivity and a pro-inflammatory immune response (70). Indeed, β1,6-branched *N*-

glycans have been shown to be essential regulators of TCR thresholds in $CD4^+$T cells in both IBD (75) and multiple sclerosis (74). In IBD, we showed that deficiency of branched *N*-glycans in mucosal T cells from ulcerative colitis patients was associated with disease severity and T cell hyperactivity (79).

In addition, we found that innate immune cells from IIM patients have an increased ability to recognise tissue glycosylation changes, as we observed increased expression of DC-SIGN in innate immune cells that specifically recognise mannose structures. This observation could explain the influence of altered muscle glycosylation on recognition by the innate immune system and the resulting potentiation of the proinflammatory immune response as we have observed in lupus nephritis (82). Furthermore, we propose that innate immunity deregulation, through an altered glycan signature, among other changes in the milieu of muscle, is the most probable culprit for local inflammation, which then propagates systemically (adaptive immunity), causing IIM.

Therapeutic strategies for immune-mediated systemic diseases have historically and currently relied on immunosuppression to reduce the hyperreactivity underlying clinical symptoms and organ dysfunction. Side effects, especially infections (the main cause of relapses in IIM) are the downside of this approach. In theory, restoring immune homeostasis is much more attractive and a more natural way to treat the underlying problem in these patients, but much more difficult to achieve. Based on our findings, we propose the reprogramming of muscle glycosylation as an attractive strategy to restore a homeostatic glycogen towards the natural course of branched *N*-glycosylation, which affects immune homeostasis (126), e.g. by hindering the production of IL-6. Overall, remodelling glycosylation through metabolic supplementation with glycans may represent an attractive therapeutic strategy to attenuate the proinflammatory immune response in IIM and may be a new target for targeted therapy, possibly in combination with immunosuppression, in a subset of patients with a hazardous progression and altered glycosignature.

CHAPTER V

Conclusions and Future Perspectives

IIM are difficult diseases to diagnose, follow-up and treat. Heterogeneity and rarity have been obstacles in disease classification and therapeutic guidelines. Survival has improved in recent years, but patients continue to have poor quality of life, with an important impact of adverse events of the therapies currently used. Pathophysiology involves a complex interplay between immune (innate and adaptive) and non-immune mechanisms, which most probably are dynamic according to disease stage, and which may contribute to the different phenotypes, disease severity and response to treatment.

Cumulating evidence suggests a prominent role of glycans in the regulatory circuits that govern immune responses, supporting the impact of complete branched *N*-glycans in the maintenance of self-tolerance and homeostasis. The impairment or perturbation of N-glycan branching may therefore contribute to the pathogenesis of various autoimmune diseases, including IIM, through epitope modification or (glyco)neo-antigen formation that triggers the activation of the immune response. An altered glycosylation (particularly a decreased expression of branched *N*-glycans) has been related with break of immune tolerance and an enhanced susceptibility to autoimmune disorders and was also shown to be associated with disease severity in this setting.

We and others have demonstrated these effects from the first beginning and priming of immune cells (thymus differentiation) until local (tissue) immune responses, where glycans changes may be the link between innate and adaptive immune responses and susceptibility.

This glycosignature, besides contributing to a deeper understanding in pathophysiology, has potential to be used as a biomarker for prognosis stratification and may be a target for directed therapy, and as such operate as real change in IIM and other autoimmune disease panorama.

After deeper research into IIM, we were left with more and further questions, which are starting points for next steps.

Questions that remain open:

What is the culprit for disease initiation and immune-deregulation perpetuation? Innate immunity? Adaptive immunity? Both? Interdependent and how? Or not? What is the contribution and relation with non-immune mechanisms?

Why do “apparent” phenotypically similar patients evolve in such distinct directions/ prognostic pathways?

Can we identify the different contributions in a specific patient (e.g., a glyco-immune pattern) and therefore contribute to phenotype IIM patients and aiming to a personalized follow-up and treatment?

How can we operate into a practical approach in order to reproduce these results into a non-invasive and easily reproducible method to apply in clinical practice, to improve patients' clinical management and therapy?

Aims to the future:

Reproduce the specific glycosignature in a validation cohort of IIM patients from other service/country, and/or through a multicentre and longitudinal study.

Perform a prospective cohort for prognosis according to clinical phenotype, including damage, refractoriness and mortality, and glycosignature (histological/ serological).

Validate through a non-invasive way (e.g. blood) to identify patients in which this pathophysiological process (glyco-immune pattern) has meaning in clinical terms and may be used as therapy guidance.

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APPENDIX I

Supplemental material of ‘‘Idiopathic inflammatory myopathies – The burden of disease: Cohort analysis focusing on damage and comorbidities’

Autoimmunity reviews, volume 22

Supplemental material of 'Idiopathic inflammatory myopathies – The burden of disease: Cohort analysis focusing on damage and comorbidities'

Campar A, Campar A, Alves I, Martins da Silva A, Farinha F, Vasconcelos C.

Supplementary data 1

HAQ correlations with other morbidity outcomes.

	N	Low HAQ	High HAQ	OR (95% CI)	p-value
Comorbidities (< 6)	37	28	9	61.185	0
Comorbidities (> 6)	62	3	59		
Damage extension (< 5)	31	28	18	25,926	0
Damage extension (> 5)	68	3	50		
Damage Severity (< 25)	31	28	21	20,089	0
Damage Severity (> 25)	68	3	47		
Refractoriness (no)	61	19	12	0,98	0,964
Refractoriness (yes)	38	12	26		
Multivariate analysis – stepwise					
Comorbidities (> 6)				**33,493**	**<0.0001**

HAQ - Health Assessment Questionnaire Disability Index; OR – odds ratio; CI – confidence interval.

Supplementary data 2

Mortality correlations with other morbidity outcomes.

	N	No mortality	Mortality	OR (95% CI)	p-value
Comorbidities (< 6)	45	36	9	1,107	0,826
Comorbidities (> 6)	79	63	16		
Damage extension (< 5)	55	46	9	0,349	1,543
Damage extension (> 5)	69	53	16		
Damage severity (< 25)	55	49	6	**3,103**	**0,026**
Damage severity (> 25)	69	50	19		
Refractoriness (no)	77	61	16	0,903	0,826
Refractoriness (yes)	47	38	9		
Diagnosis delay (< 12)	79	58	21	**0,269**	**0,024**
Diagnosis delay (> 12)	45	41	4		
Multivariate analysis – stepwise					
Damage severity (> 25)				**10,152**	**0,015**
Diagnosis delay (> 12)				**0,279**	**0,035**

OR – odds ratio; CI – confidence interval.

APPENDIX II

Supplemental material of ‘Muscle glycome in idiopathic inflammatory myopathies: Impact in IL-6 production and disease prognosis’

iScience, Volume *26*

Supplemental information

Muscle glycome in idiopathic inflammatory myopathies: Impact in IL-6 production and disease prognosis

Ana Campar, Inês Alves, Beatriz Santos-Pereira, Rafaela Nogueira, Miguel Mendonça Pinto, Carlos Vasconcelos, and Salomé S. Pinho

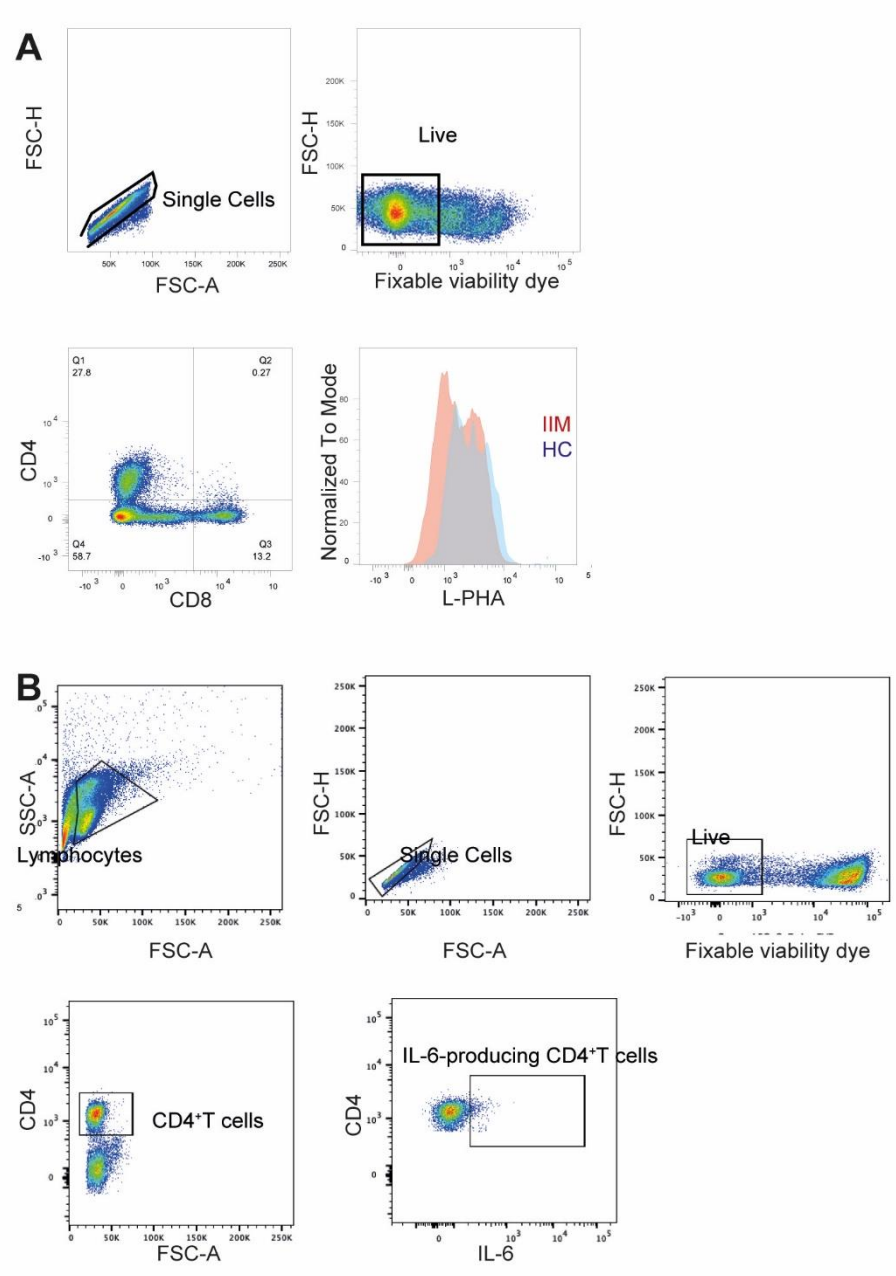
Supplemental table 1: Demographic and clinical characteristics of patients with IIM (related to STAR methods).

Demographic characteristics		N (%)
Gender	19♀; 3♂	-
Age at diagnosis (mean)	48 years-old	-
Clinical characteristics		
Diagnosis	Dermatomyositis	10 (45.4)
	Polymyositis	3 (13.6)
	IMNM	3 (13.6)
	Anti-synthetase syndrome	3 (13.6)
	Inclusion Body Myositis	2 (9.1)
	Overlap myositis	1 (4.5)
ACR/EULAR criteria – IIM probability (mean)	86.3	-
Years of disease (Dec/ 2021) (mean)	6.4	-
Organic involvements	Muscle	22 (100)
	Skin	13 (59.1)
	Bulbar	13 (59.1)
	Lung	6 (27.3)
	Articular	6 (27.3)
	Other	6 (27.3)
Autoantibodies	Specific	17 (77.3)
	Associated	9 (40.9)
Refractoriness	Yes	8 (36.4)
Flares, last 4 years (mean)	2.5	-
Damage (MDI, mean)	MDI extension	9.14
	MDI severity	45.23
Treatments	Corticosteroids	21 (95.4)
	Methotrexate	9 (40.9)
	Azathioprine	10 (45.4)
	Mycophenolate mofetil	3 (13.6)
	Tacrolimus	4 (18.2)
	IV Immunoglobulin	15 (68.2)
	Cyclophosphamide	4 (18.2)

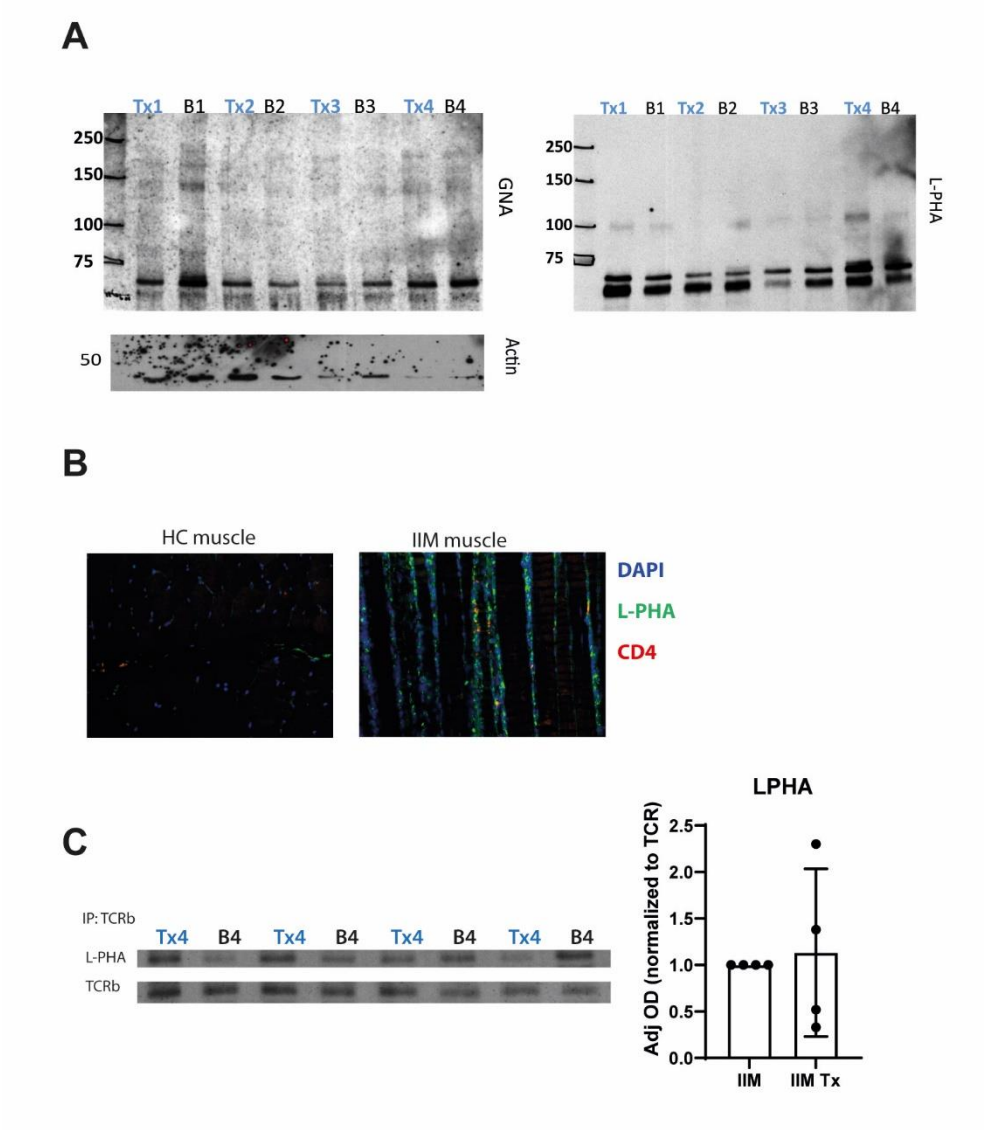
	JAK inhibitors Biological (Rituximab)	1 (4.5) 4 (18.2)
Overlap with other AI diseases	Yes	6 (27.3)
Mortality	-	3 (13.6)
Prognosis	Good Poor	10 (45.5) 12 (54.5)

IIM – Idiopathic Inflammatory Myopathy; IMNM - Immune Mediated Necrotizing Myopathy; MDI – Myositis Damage Index; IV – intravenous; JAK – Janus Kinase; AI – autoimmune.

Supplemental Figure 1 – Flow cytometric analysis (related to figure 2). (A) Gating Strategy of peripheral blood mononuclear cells analysis by flow cytometry. **(B)** Gating Strategy of peripheral blood mononuclear cells analysis for IL-6-producing $CD4^{+}$T cells analysis by flow cytometry.



Supplemental Figure 2 – Glycoprofile of IIM muscle lesions (related to figure 3). **(A)** Lectin blot for L-PHA (β1,6-branhcing *N*-glycans) and GNA (high-mannose *N*-glycans) reactivity of whole tissue cell lysate with (Tx) or without (B) GlcNAc supplementation. Loading control (actin) of respective samples. **(B)** Immunofluorescence images representing the co-localization of CD4 (red) and L-PHA (green) at the stromal compartment of muscle biopsies from healthy controls (HC) and myositis (IIM). **(C)** Immunoprecipitation (IP) of TCRβ from IIM-derived muscle biopsies, treated and non-treated with GlcNAc. IP was stained with L-PHA lectin and anti-TCRβ antibody for the normalization of L-PHA reactivity. On the right is represented the quantification of the bands. Adjusted optical density (Adj. OD). The error bars represent the standard deviation of the data.



APPENDIX III

Glycans as Key Checkpoints of T Cell Activity and Function

frontiers in Immunology

REVIEW
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Glycans as Key Checkpoints of T Cell Activity and Function

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The immune system is highly controlled and fine-tuned by glycosylation, through the addition of a diversity of carbohydrates structures (glycans) to virtually all immune cell receptors. Despite a relative backlog in understanding the importance of glycans in the immune system, due to its inherent complexity, remarkable findings have been highlighting the essential contributions of glycosylation in the regulation of both innate and adaptive immune responses with important implications in the pathogenesis of major diseases such as autoimmunity and cancer. Glycans are implicated in fundamental cellular and molecular processes that regulate both stimulatory and inhibitory immune pathways. Besides being actively involved in pathogen recognition through interaction with glycan-binding proteins (such as C-type lectins), glycans have been also shown to regulate key pathophysiological steps within T cell biology such as T cell development and thymocyte selection; T cell activity and signaling as well as T cell differentiation and proliferation. These effects of glycans in T cells functions highlight their importance as determinants of either self-tolerance or T cell hyper-responsiveness which ultimately might be implicated in the creation of tolerogenic pathways in cancer or loss of immunological tolerance in autoimmunity. This review discusses how specific glycans (with a focus on *N*-linked glycans) act as regulators of T cell biology and their implications in disease.

Keywords: *N*-glycosylation, glycans, T cells, immune response, autoimmunity, self-tolerance

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INTRODUCTION

The immune system is highly regulated by a series of stimulatory and inhibitory pathways that are crucial to maintain a healthy and balanced system. Disruption of the control of this immunological balance can result in abnormal stimulatory signals associated with the loss of immune tolerance in autoimmunity or in the creation of aberrant immunosuppressive networks that occur in cancer. Accumulating evidences have been demonstrating the importance of glycans and glycans binding proteins [including galectins (1, 2), C-type lectins (3), and sialic acid-binding immunoglobulin-type lectins (siglecs) (4, 5)] in the regulation of both innate and adaptive immune responses. In fact, all cells are covered with a dense coat of glycans that constitute a major molecular interface between cells and their environment. The diversity of glycans presentation at cell surface is enormous, encoding a myriad of important biological information that remains to be fully characterized. Glycosylation is the enzymatic process responsible for the attachment of glycans (carbohydrates) to

proteins or lipids (predominantly via nitrogen (*N*) and oxygen (*O*) linkages), a process that occurs in the Endoplasmic Reticulum/Golgi compartment of essentially all cells being mediated by the coordinated action of a portfolio of different glycosyltransferases and glycosidases enzymes (6). The proper development and function of the immune system relies both on the dynamic regulation of the expression of glycan-structures and glycan-binding proteins, and the interactions between them (7). This review discusses the role of glycans (with a focus on *N*-linked glycans) on T cells biology and function, including T cell development, activation, differentiation, and signaling. This dynamic interplay between glycans and T cells activity controlling both auto-reactivity and self-tolerance will be presented and discussed (**Figure 1**).

GLYCANS IN T CELL DEVELOPMENT AND THYMUS SELECTION

T cells are developed in the thymus where a microenvironment is set, which enables the selection of T cell receptors (TCRs) to generate a diverse repertoire of potential antigen recognition (8). Lymphoid progenitors from the bone marrow enter into the cortical tissue of the thymus, where they start to expand and develop (9, 10). Despite the fact that the role of glycosylation in T cell development and thymus selections still remains to be fully understood, some important findings highlight the relevance of glycans in this process (**Figure 2**).

Role of Glycans in Thymus Seeding and T Cell Lineage Commitment

The initial step of T cell development, the trafficking of thymus-seeding progenitors (TSPs) to the thymus, is an active process that relies on the expression of P-selectin in the thymic epithelium and its partner, P-selectin glycoprotein ligand-1 (PSGL-1), expressed by circulating TSPs-derived from the bone marrow (11). The expression and post-translational modifications of PSGL-1 are regulated in bone marrow progenitors. The deficiency of α1,3 fucosylation on PSGL-1, required for its binding to P-selectin, was shown to be associated with the impairment of TSPs homing into the thymus (12). Once TSPs enter the thymus, they develop into early thymocyte progenitors (ETPs), a subset of the $CD4^{-}CD8^{-}$ double negative 1 (DN1) population, which give rise to multiple lymphoid lineages (8). The conserved Notch signaling pathway is responsible for the commitment of DN1 thymocytes to the T cell lineage (13). The glycosylation profile of Notch receptors (and ligands) was shown to regulate Notch-dependent intracellular signal transduction. The lunatic, manic, and radical Fringe are the glycosyltransferases that modify Notch receptors by transferring *N*-acetylglucosamine (GlcNAc) to *O*-linked fucose glycans of epidermal growth factor-like (EGF-like) repeats, present in the extracellular domain of Notch, and described to regulate its cell-surface signaling and function (14, 15). Loss of the three Fringe glycosyltransferases leads to a reduced binding of Notch to Delta-like ligands (DLL), namely DLL4, altering the frequencies of several T cell subsets in the thymus (16). The first indication that Fringe-mediated Notch glycosylation was involved in T cell development was shown when the lunatic Fringe gene, *Lfng*, was misexpressed under a *lck*-proximal promoter (17). This alteration of the Notch glycosylation profile (lack of GlcNAc in the EGF-like repeats) resulted in a large B cell population developed from lymphoid progenitors in the thymus. In fact, further work showed that *Lfng* is poorly expressed in $CD4^{+}CD8^{+}$ double positive (DP) thymocytes, but when ectopically expressed in that population (under *lck*-proximal promoter), led to an increased binding of Notch to its ligands on stromal cells, blocking DN development, and enabling B cell differentiation (18). These studies also revealed that changes in the glycosylation of Notch across T cell development also impacts on its signaling pathway. At DN stages, the reactions that drive development are dependent on Notch interactions with DLLs, which exist at functionally limiting concentrations. The high levels of *Lfng* expression in DNs facilitate Notch interactions with DLLs and the dramatic downregulation of *Lfng* in DPs coincides with Notch-independent reactions of T cell development. The final commitment to the T cell lineage occurs at the DN3 stage, where a recombination-activating genes (RAG)-mediated productive rearrangement of the *Tcrb* leads to the expression of the ß chain of the TCR (TCRß) and the formation of a pre-TCR signaling complex (13, 19).

Role of Glycans in Thymocyte ß Selection

Together with Notch and Interleukin (IL)-7, the pre-TCR signaling initiates ß-selection, by inducing the downregulation of the RAG complex expression (*Rag1* and *Rag2*) in quiescent DN3 (DN3a), becoming large cycling DN3 thymocytes (DN3b), which differentiate into DN4 cells. A deficient pre-TCR signaling in *lck*-null cells is rescued by *Lfng* overexpression, but not in a $Rag2^{-/-}$ background, indicating a pre-TCR dependency for development (20). Upon ß-selection, it was recently demonstrated that DN4 cells upregulate glucose and glutamine metabolites that enter into the hexosamine pathway, increasing the production of UDP-GlcNAc, which is needed to undergo clonal expansion (8, 21). The UDP-GlcNAc is also the substrate of the *O*-GlcNAc transferase (OGT) in the process of *O*-GlcNAcylation of intracellular proteins on serine and threonine residues (22). Recent evidences showed that *O*-GlcNAcylation regulates the process of T cell development (23). Using a conditional knockout mouse model of OGT in the DN stage, it was shown a reduced population of DPs, indicating either a deficiency on ß-selection or in clonal expansion of DN4s. The absence of OGT appeared not to impact self-renewal of DNs, or their differentiation into DPs, but to promote the failure of the clonal expansion of DN4, in response to Notch ligands. A feedback mechanism was proposed in which the metabolic changes (the shift to glycolysis) that support the DN-to-DP stage of thymocyte differentiation, controlled by Notch, induces c-Myc expression, which in turn controls the rate of T cell nutrient uptake as well as the expression of OGT and consequently the abundance of *O*-GlcNAc (15). The *O*-GlcNAcylation of c-Myc was also shown to increase its stability (24), further contributing to the feedback loop.

In the stage of post-β selected DN4 thymocytes, it was seen a 10-fold increase in expression of ST6 β-Galactoside

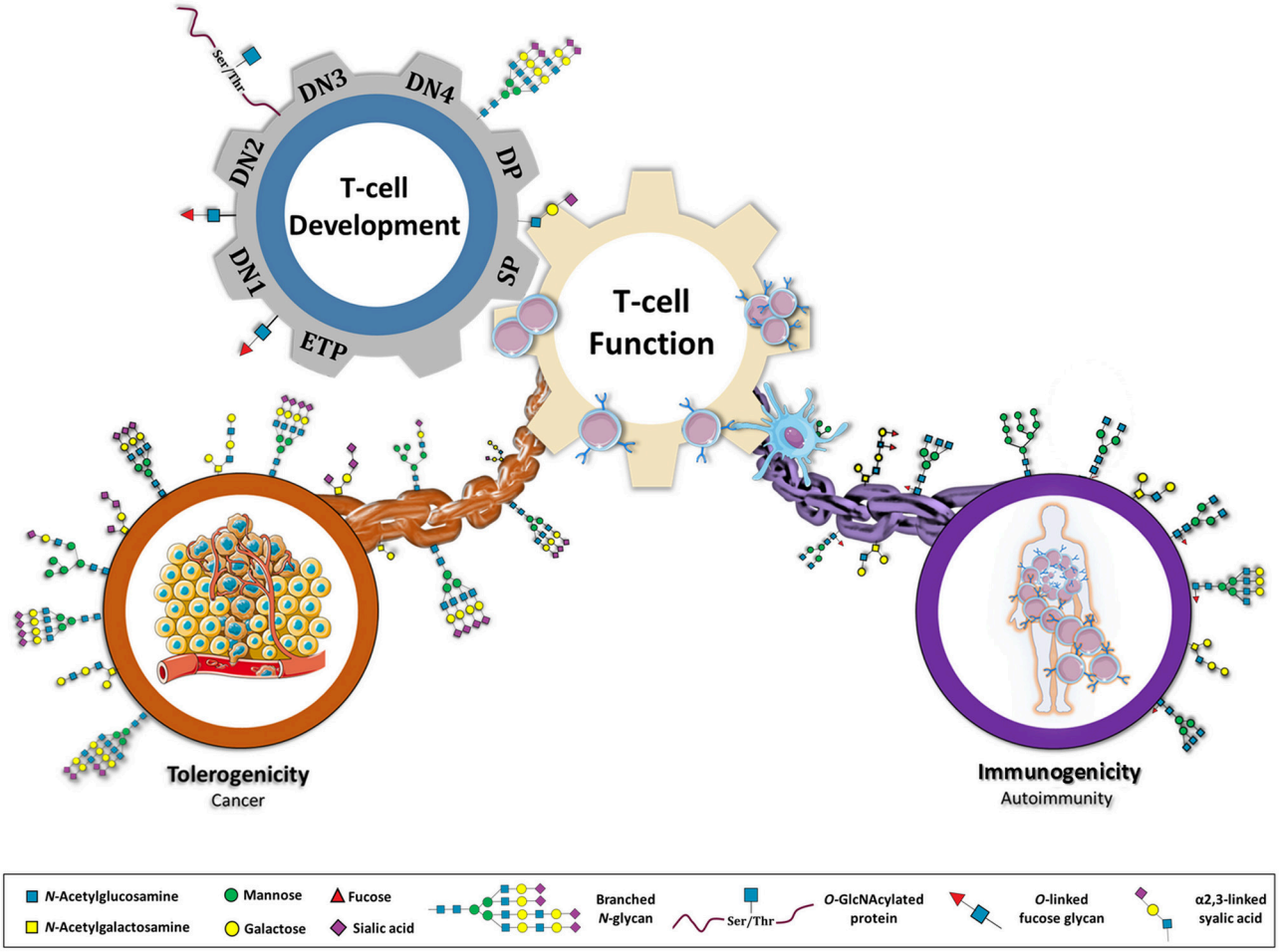

FIGURE 1 | Glycans as a major connective chain that controls T cell response in either a tolerogenic or immunostimulatory scenario. Glycosylation appears to be central in regulating several steps of a T cell's life. During T cell development, different population of T cells (ETP, early thymocyte progenitor; DN1, 2, 3, and 4, double negative; DP, double positive; SP, single positive) display specific glycosylation patterns. The normal glycosylation of SP population results in an educated T cell function. However, by genetic, environmental or metabolic constrains, T cell glycosylation can be compromised re-directing immune system toward an immunostimulatory or tolerogenic response. Glycans are proposed here as key players in immune-unbalanced diseases, such as autoimmunity and cancer.

α2,6-Sialyltransferase 1 (ST6Gal I) when comparing to the DN3 population, which resulted in an increase in α2,6-linked sialic acid (25). Accordingly, in *ST6Gal1* deficient mice, the DN populations were decreased, beginning at the DN1 subset. Microarray data showed a downregulation of CD96 (receptor molecule of nectin-1, that plays a putative role in cell migration) in the DN2 and DN3 populations in the *ST6Gal1* deficiency background, and a disruption of thymopoiesis in these mice was proposed. Moreover, ST3 β-Galactoside α2,3-Sialyltransferase 1 (ST3Gal I) expression is decreased in most DN and in all DP, only increasing in single-positive (SP) thymocytes (26). In *ST3Gal1*$^{-/-}$ mice, the TCR repertoire was significantly altered, indicating a role for sialylation in thymocyte selection (27).

Role of Glycans in Positive and Negative Selection in the Thymus

The ß-selected DN4 cells undergo rapid self-renewal, giving rise to a clonally expanded population, that differentiate into DP CD4^{+}CD8^{+} thymocytes (8). In this developmental stage, mature TCRαß receptors are formed (28) and the expression of the co-receptors CD4 and CD8 confer a MHC class II and class I restriction of TCR activation, respectively. The newly formed mature TCRs are then screened by thymic epithelial cells (TECs) by the specificity and binding strength for the MHC ligands presented. The next developmental process is named positive selection, where the DP population is enriched for cells that express an immunocompetent TCR (8, 29). The selected DPs then commit to the SP CD4^{+} or CD8^{+} lineage and go through a process called negative selection, which eliminates autoreactive T cells (8, 29). The affinity of the correctly assembled TCRαβ for the MHC-antigen complexes determines cell survival and differentiation. Glycosylation modifications of the TCR may provide an alternative mechanism to control positive and negative selection by directly affecting the TCR-MHC-antigen binding, TCR interaction with its co-receptors and the threshold of activation (30), an issue that is far from being fully elucidated.

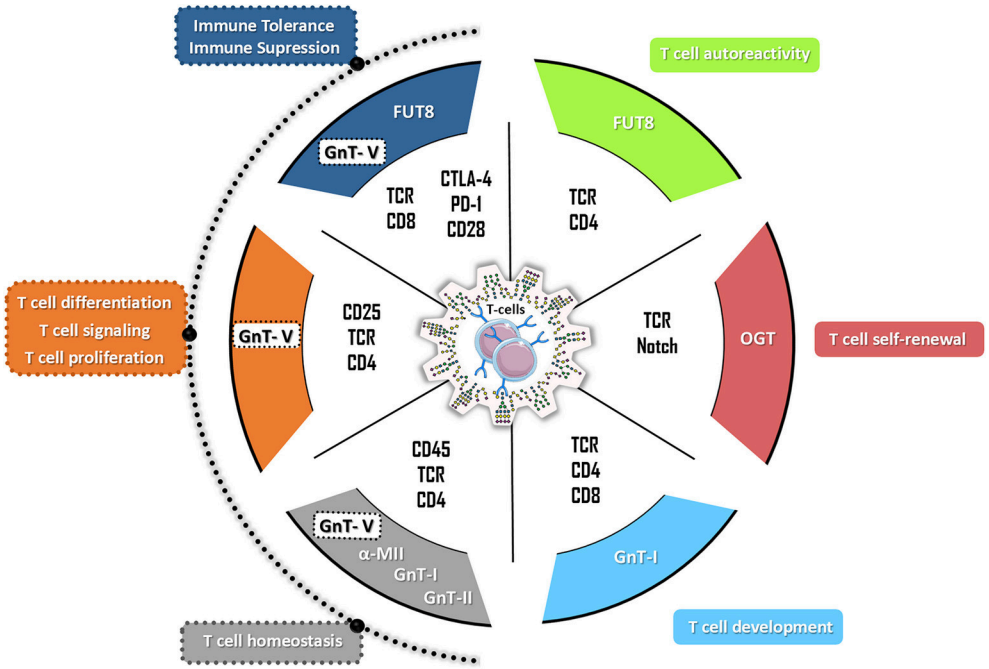

FIGURE 2 | The hallmarks of glycans in T cell biology. *N*-glycans have a broad effect on the multiple T cell functions with impact both in autoreactivity and in immune tolerance. Particularly, the complex branched *N*-glycans catalyzed by beta 1,6-*N*-acetylglucosaminyltransferase V (GnT-V) (encoded by *MGAT5* gene) have been demonstrated to control different T cells functions by targeting different T cells receptors (such as TCR, CD25, and CD4) and therefore regulating T cell proliferation, T cell differentiation, T cell signaling as well as the production of inflammatory cytokines. Alterations on GnT-V activity but also in alpha-mannosidase II (α-MII) as well as in *N*-acetylglucosaminyltransferase I (GnT-I, *MGAT1* gene) and II (GnT-II, *MGAT2* gene) activity were shown to compromise T cell homeostasis being associated with the development of several autoimmune disorders in humans and mouse models (such as EAE, IBD, SLE, TID). The FUT8-mediated core fucosylation of TCR was associated with hyperactivation of $CD4^+$ T cells (T cells autoreactivity) whereas the modification of the co-inhibitory receptors (CTLA-4 and PD-1) by FUT8-mediated core fucose results in immune tolerance. The T cell development and T cell self-renewal are controlled by GnT-I-mediated glycosylation and by *O*-GlcNAcylation through OGT (*O*-GlcNAc transferase), respectively.

The subunits of the TCRαβ contain at least 7 potential sites for *N*-linked glycosylation and the TCR-CD3 complex is estimated to have 12 *N*-glycan addition sites that contribute to TCR folding and functions (31, 32). Indeed, selective removal of conserved *N*-glycosylation sites of the constant regions of the TCR, enhanced its functional avidity (the sensitivity of the T cell response to other cell which carries the respective MHC-peptide) (32). However, whether *N*-glycosylation in the variable regions of the TCR affect its selection remains to be addressed. Moreover, low levels of sialylation in DPs are associated with binding to Major Histocompatibility Complex (MHC) class I (common to all nucleated cells) and the increased expression of sialic-acid linkages on differentiated SP $CD8^+$ thymic T cells was shown to decrease the binding avidity of CD8 for MHC class I molecules, which acts as a regulation for a TCR affinity dependent negative selection (33).

Furthermore the deficiency of the *Mgat5* gene, that encodes for a Golgi branching enzyme *N*-acetylglucosaminyltransferase V (GnT-V) was shown to markedly increases TCR clustering and signaling at the immune synapse, resulting in a lower T cell activation threshold and increased incidence of autoimmune disease *in vivo* and in human (30). In a model of positive selection, it was demonstrated that branching *N*-glycosylation dynamically expands the affinity spectrum of positive selection by differentially controlling both the lower and upper limits of positively selected TCR-MHC-antigen interactions (34). The intracellular domains of CD4 and CD8 co-receptors bind Lck, enhancing TCR responses to low-affinity MHC-antigen complexes when coupled to the TCR (35). Both co-receptors have *N*-glycosylation sites and it was shown that the branching deficiency in $Mgat1^{f/f}$Lck-Cre^+ T cells resulted in decreased surface expression of CD4 and CD8 receptors (34). The lack of branched *N*-glycans in the same genetic background also decreased TCR threshold signaling (30). These evidences supported that branching *N*-glycans display an important role in the maturation of DN cells and/or TCR selection.

Changes in the expression of *O*-linked glycans also impact T cell development by modulating galectin binding. Galectin-1 was shown to induce apoptosis of immature thymocytes through binding to core 2 *O*-glycans expressed in CD43 and CD45 (36). In contrast, CD45 on mature thymocytes bears core 1 *O*-glycans as well as *N*-glycans capped with α2,6-linked sialic acid, which inhibits galectin-1 binding (36).

Overall, glycosylation appears to play a critical role in the different stages of thymocyte development and in the generation of an efficient immune system. Nevertheless, further research is

needed in order to understand how glycans control each stage of thymocytes development, differentiation and selection, which might reveal novels insights on the influence of the glycome in major diseases, such as autoimmunity and cancer.

GLYCANS IN THE REGULATION OF T CELL ACTIVITY AND FUNCTIONS

The proper function of T lymphocytes is highly dependent on their surface receptors, which in turn are highly mediated by glycosylation. Although *O*-glycan structures have been shown to play important roles on immune-associated molecules (37), the prominent role of *N*-linked glycans is emphasized in this section (**Figure 2**).

As previously mentioned, MHC I is expressed by almost all nucleated cells and interacts with TCRs on $CD8^+$ T cells; in turn, MHC II is expressed by professional antigen presenting cells (APCs) (dendritic cells - DC, macrophages, B cells and TECs) and is recognized by $CD4^+$ T cells (7, 38). More than 3 decades ago, it was demonstrated that blocking MHC1a *N*-glycosylation, through acceptor site mutation, results in significant increases in intracellular misfolded protein along with decreases in cell surface expression (39). MHC II is assembled by two glycoproteins, α and β chains. The α chain contains *N*-linked high-mannose and complex glycans whereas the β chain is only constituted by complex *N*-glycans (40). In contrast to the role of MHC I, MHC II glycosylation was shown to have a particular impact on the effective antigen binding, as well as in the presentation of microbial carbohydrate antigens, which consequently influences downstream T cell responses. This was demonstrated by the depletion of the *Mgat2* gene, which compromises *N*-glycan branching, decreasing carbohydrate antigen presentation by MHC class II and leading to loss of T cell stimulatory activity (41).

During TCR signal transduction, glycans play a key role in stabilizing individual molecules in the complexes at the immunological synapse and by protecting them from the action of proteases during T cell engagement (31). Additionally, glycans can also restrict nonspecific protein-protein interactions, like aggregation of TCRs on the membrane, helping to orient the interactions of the proteins in the central clusters (31). Demetriou et al. demonstrated that β1,6-GlcNAc branched *N*-glycans structures (catalyzed by GnT-V) regulate T cell activity, namely in $CD4^+$ T cells by increasing the threshold of T cell activation, suppressing T cell growth and signaling (30, 42). Moreover, core-fucosylation, which refers to fucose attached to the innermost *N*-acetylglucosamine of *N*-linked glycans, catalyzed by α1-6 fucosyltransferase (FUT8), was also shown to affect T cell activity in immune mediated disorders (42, 43).

The T cell activity is also dependent on glycosylation of co-receptors, such as the complex formation between TCR and CD45. Galectin-3 is a key mediator of this complex, by establishing a molecular lattice through binding to polylactosamine structures in branched *N*-glycans. Consequently, CD45 phosphatase activity induces downregulation of T cell signaling, preventing T cell activation (44). Furthermore, CD45 is alternatively spliced into five different isoforms on human leukocytes (CD45ABC, CD45AB, CD45BC, CD45B, and CD45RO) (45–47), all decorated with up to 11 *N*-glycans in the membrane proximal region. Importantly, all isoforms present different glycosylation profiles (48, 49), that change during T cell differentiation and activation (50, 51), as reviewed in (36). CD28 is another T cell surface glycoprotein acting as a secondary signaling molecule of T cell activation. Interestingly, nearly 50% of the molecular mass of CD28 is constituted by *N*-glycans (52). Previous studies reported that *N*-glycosylation of human CD28 can negatively regulate CD28-mediated T cell adhesion and co-stimulation, namely the interaction between CD28/CD80. Mutation of all potential *N*-linked glycosylation sites of CD28 as well as treatment of Jurkat cells with inhibitors of *N*-glycosylation pathway resulted in a defective CD28 glycosylation with enhancement of the binding to CD80 expressed on APCs (52). The branching *N*-glycosylation of CD25 receptor also modulates its cell surface retention controlling T differentiation with impact in immune tolerance. Recently, it was demonstrated that a decreased UDP-GlcNAc and complex branching *N*-glycosylation induces a decreased cell surface retention of CD25 and IL-2 signaling, promoting a T helper (T_H) 17 over induced regulatory T cell (iTreg) differentiation (53) (**Figure 2**).

Importantly, the co-inhibitory receptors are likewise modulated by *N*-glycosylation. One of the major negative regulators of T cell response is the cytotoxic T-lymphocyte protein 4 (CTLA-4), that comprises two *N*-glycosylation sites described to modulate its cell surface retention on T cells and thereby its affinity for CD80/CD28 on APCs (54–56). The impact of *N*-glycosylation in the modulation of the inhibitory functions of CTLA-4 and programmed cell death protein-1 (PD-1) is discussed in more detail in section "Glycans in tolerogenic/immunosuppressive responses". Nonetheless, other co-inhibitory receptors like Lymphocyte-activation gene 3 (Lag-3), mucin-domain-containing molecule-3 (Tim-3), and T cell immunoreceptor with Ig and ITIM domains (TIGIT) may also undergo glycans-mediated regulation, as they exhibit *N*-glycan-binding sites, however the role of glycans on these molecules remains to be explored (57).

Taken together, *N*-glycosylation plays an instrumental role in the regulation of T cell activation and functions by targeting not only TCR but also its co- receptors (**Figure 2**).

GLYCANS AS MODULATORS OF HYPER-REACTIVE/AUTOIMMUNE RESPONSES

Autoimmunity is characterized by the loss of self-tolerance and development of an autoreactive immune response toward the individual's own organism. Glycan motifs play a crucial role in the determination of self/non-self antigens. Specific glycan structures, expressed by microbial pathogens, are commonly responsible for the primary activation of the innate immune system; however, the mechanisms involved in the self/non-self discrimination, mediated by glycans are far from being fully

elucidated. Abnormal levels of branched *N*-glycans have been associated with exacerbated immune responses in murine models (58). Particularly, the dysregulation of the *N*-glycosylation pathway has been associated with autoimmune-like phenotypes. The inability to synthetize β1,6-GlcNAc antennae, in *Mgat5*$^{-/-}$ mice has been associated with an increased susceptibility to immune-mediated disorders such as an increased delayed-type hypersensitivity responses, as well as increased susceptibility to develop experimental autoimmune encephalomyelitis (EAE) (30, 59) and severe forms of colitis (60). The lack of β1,6 branching *N*-glycans favors TCR clustering, leading to a decrease of the TCR threshold and consequently increased T cell activation (30) associated with the hyperimmune response observed in these mice (**Figure 2**). This hyperimmune phenotype is also due to an abnormal formation of lattices between TCR-branched glycosylation and galectins (61, 62). Accordingly, β3 GnT2-deficient mice show T cell hypersensitivity due to the reduction of polylactosamine on the *N*-glycans (ligands of galectins), similarly to what is observed in *Mgat5* deficient mice (30, 61). Furthermore, absence of α-mannosidase II (which catalyses the last hydrolysis of the α-mannose), was shown to result in signs of glomerulonephritis, deposits of glomerular IgM immunocomplexes and complement component 3 as well as high levels of anti-nuclear antibodies (63, 64), which is consistent with a Lupus-like syndrome (**Figure 2**). Taken together, these evidences support the role of *N*-glycosylation in the perspective of T cell biology.

The role of *N*-glycans in antigen presentation and recognition is still elusive, and in fact abnormal glycoantigen presentation might also impact T cell activity. Abnormal accumulation of high-mannose, paucimannose, and agalactosyl bi-antennary glycans, have been detected in kidney tissue from MRL-lpr mouse (a well-stablished murine model of SLE) (65). Moreover, evidences have been showing that *Mgat1*$^{f/f}$Syn1-Cre mice, with *Mgat1* deletion at the Synapsin I-*expressing cells* (abundant in neural tissues), presented neurological defects, with high levels of neuronal apoptosis and caspase 3 activation (66). These high levels of apoptosis are observed in several autoimmune diseases, which results in activation of immune system (67) (**Figure 2**). Although highly unexplored, rare autoimmune diseases are also associated with *N*-glycosylation dysfunctions. As example, idiopathic inflammatory myopathies (IIM) are a group of rare diseases of autoimmune nature, whose etiopathogenesis is far from being totally understood (68). Muscle cells surface is enriched with glycoproteins and several lines of evidence provide support for a fundamental role of glycosylation in muscle homeostasis and function (69, 70). Glucosamine (UDP-*N*-Acetyl)-2-Epimerase/*N*-Acetylmannosamine Kinase (GNE) genetic mutations (a gene that encodes *N*-acetylmannosamine (ManNAc) kinase enzyme, responsible for the biosynthesis of *N*-acetylneuraminic acid) results in hypo-sialylation of muscle glycoproteins; the prophylactic supplementation with sialic acid precursor (ManNAc) was shown to prevent the muscle phenotype in mice with gene mutations that cause hereditary inclusion-body myositis (hIBM), a muscle phenotype that resembles one type of IIM (71). Altogether, these findings highlight the importance of further studies addressing the role of *N*-glycosylation in the perspective of *neoautoantigens*, since autoantigens contain a significant amount of glycoantigens due to the increased number of *N*-glycosylation sites comparing with other proteins (72).

The Glycan binding proteins (GBPs) are expressed in the APCs being characterized by a carbohydrate recognition domain which specifically recognizes glycan structures present at the cell surface receptors. This glycan-GBPs engagement results in either an anti- or pro-inflammatory response (73). C-type lectins, siglecs, and galectins are examples of GBPs, that are instructors of immune responses (5, 73). As example, SIGN1R (expressed by APCs and the analogous of the human dendritic cell-specific ICAM-grabbing non-integrin - DC-SIGN) signaling was shown to result in the expansion of IL-10-secreting Treg cells, preventing the development of autoimmune diseases such as EAE and type 1 diabetes (T1D) (74). Galectin-1 also plays an important immune-regulatory role in EAE (75) as mice deficient in galectin-1 (*Lgals1*$^{-/-}$) have increased T_H1 and T_H17 responses being more susceptible to EAE when compared with wild type mice (76). More recently, Galectin-1 was shown to modulate the cytolytic activity of $CD8^+$ T cell. The interaction of Galectin-1 and Fas ligand seems to be responsible for the retention of this glycoprotein at the surface of cytotoxic T lymphocytes hampering the cytolytic ability of these cells (77). Overall, GBPs-glycoprotein interaction is essential to instruct a T cell—mediated immune response.

Notably, one of the first evidences addressing the relationship between the dysregulation of *N*-glycosylation and human autoimmunity was observed in multiple sclerosis (MS) patients. During active, relapse or in very early stages of remission, peripheral blood mononuclear cells from MS patients display a significant decrease of the enzymatic activity of Golgi β1,6 *N*-acetylglucosaminyltransferase (core 2 GlcNAc-T), compared to healthy subjects (78). Moreover, *MGAT5* polymorphisms were associated with MS severity (79) together with *MGAT1*, *IL2R,* and *IL7R* Single Nucleotide Polymorphisms (80–82). Additionally, in Inflammatory Bowel Disease (IBD), it was also demonstrated that *lamina propria* T lymphocytes from ulcerative colitis (UC) patients exhibited a deficiency in β1,6-GlcNAc branching *N*-glycans due to decreased levels of *MGAT5* gene expression (83). Importantly, low levels of branched *N*-glycans in lamina propria early at diagnosis were shown to predict UC patients that will fail the response to standard therapy, thus displaying a bad disease course (84). The supplementation of intestinal T cells from UC patients and mouse models with colitis with GlcNAc promoted the enhancement of β1,6 branching *N*-glycans on T cells, suppressing TCR signaling and reducing the production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ). Pre-clinical studies both in IBD and MS demonstrated the immunomodulatory properties of *N*-glycans in the control of T cell-mediated immune response (60, 85), paving the way for the development of human clinical trials, that are currently on going (53, 60). Less explored but of utmost importance is the study of *N*-glycosylation profile in rare autoimmune disorders, since its etiopathogenesis is still very elusive. Glycosylation changes in muscle-associated human disease have focused in muscular dystrophies (86) and congenital

disorders of glycosylation (87). Recent studies have shown that muscle cell surface glycosylation is finely regulated and subjected to alterations under inflammatory conditions (88), pointing to a possible interaction between muscle glycocalyx and the extracellular milieu, which is particularly enriched in immune cells and antibodies in IIM patients (89).

Overall, glycans are critical determinants in autoreactive responses both by directly regulating T cell activity and also through the creation of abnormal glycoantigens that may unleash an autoreactive immune response.

GLYCANS IN TOLEROGENIC/IMMUNOSUPPRESSIVE RESPONSES

Recent studies have been highlighted that alterations on the glycosylation pattern of T cells' receptors, as well as the alterations of the glycosylation profile of tumor cells (tumor glyco-code), are implicated in the modulation of the immune response leading to immunosuppressive pathways, known to occur in the tumor microenvironment associated with tumor immunoescape (90).

Role of Glycans in the Modulation of Inhibitory T Cell Receptors

PD-1, as already introduced, is a cell surface inhibitory T cell receptor responsible for immune-inhibitory responses associated with the so-called "T cell exhaustion" (91). The expression of this cell surface receptor, as well as Tim-3, was described to be positively regulated by the core fucosylation pathway, catalyzed by FUT8 enzyme (92). The inhibition of core fucosylation in PD-1 was demonstrated to lead to an anti-tumor immune response mediated by T cells activation, being a new attractive target for enhancing anti-tumor immunity in future clinical settings (**Figure 2**). This was a pioneer study that supported the importance of PD-1 post-translational modifications by glycosylation on T cell-mediated immunosuppression (92). Additionally, the glycosylation of programmed death ligand-1 (PD-L1), a PD-1 ligand, was described to have an important role in its cellular stabilization. The interaction of non-glycosylated PD-L1 with glycogen synthase kinase 3β (GSK3β), a key enzyme on glycogenesis, leads to the degradation of this molecule (93). In triple-negative breast cancer cells, it was further shown that the β1,3-*N*-acetylglucosaminyl transferase (B3GNT3), involved in the biosynthesis of poly-*N*-acetyllactosamine chains, is important for the interaction between PD-1 and its ligand PD-L1 (94). The use of an antibody targeting the glycosylated form of PD-L1 resulted in its degradation and internalization, with the blockage of PD-L1/PD-1 interaction and consequently the inducement of anti-tumor activity in triple-negative breast cancer *in vitro* and *in vivo* models (94). In accordance, Tregs from healthy humans and mice were shown to display an increased variability on its *N*-glycosylation pattern when compared with $CD4^+$ T cells. The levels of the complex branched *N*-glycans were shown to be correlated with the expression of proteins involved in Treg suppressive functions, including PD-1, PD-L1, and also other negative regulators of T cell response, namely CTLA-4 (95). In fact, the CTLA-4 protein, comprises multiple *N*- and *O*-glycosylation sites known to modulate its retention at T cell surface and consequently affecting its function (56). The TCR activation is associated with an increased β1,6-GlcNAc branched *N*-glycosylation of CTLA-4, which enhances CTLA-4 retention at the T cell surface and thereby suppresses T cell activation promoting immune tolerance (96) (**Figure 2**). Accordingly, the presence of Thr17Ala polymorphism in human *CTLA-4* was shown to result in the reduction of the *N*-glycosylation sites from one to two sites, which limited CTLA-4 retention at T cell surface (80). Supplementations with GlcNAc and Vitamin D promoted an enhancement of *N*-glycans branching expression, increasing the cell surface retention of CTLA-4, culminating in immunosuppression (80).

Glycans as Instructors of Immunosuppressive Responses

Tumor cells aberrantly express different types of glycans structures when compared with normal counterparts, such as an increased sialylation, an expression of truncated glycans and an overexpression of branched *N*-glycans (97). This alteration in the cellular glycosylation profile governs several steps of tumor development and progression, such as tumor cell dissociation, proliferation, invasion, metastasis, angiogenesis, with recent evidences pointing toward its effects in tumor immunoediting and immunosurveillance (98). GBPs expressed on immune cells are able to recognize altered glycan structures expressed at tumor cell surfaces instructing either immunostimulatory or immunoinhibitory responses.

The expression of sialylated glycans, such as Tn antigen and Lewis antigens, aberrantly expressed in tumor cells, were described to be recognized by DC-SIGN, expressed by macrophages and immature DCs, which lead to immunosuppression (99). The fucose residues present in Lewis structures (Lewis x and Lewis y), attached to carcinoembryonic antigen (CEA) (100), were described to trigger the upregulation of the anti-inflammatory cytokines IL-10 and IL-27 by APCs and the induction of T_H2, follicular (T_Hf), and Treg immune responses (101, 102). Besides, antigen-containing liposomes modified with DC-SIGN-binding Lewis b and x resulted in glycans recognition and internalization through DCs with consequent activation of $CD4^+$ and $CD8^+$ T cells (103). Furthermore, macrophage galactose binding lectin (MGL) was found to be able to recognize Tn antigen and *N*-acetylgalactosamine (GalNAc) residues, resulting in an increased recognition by Toll-like receptor 2, ultimately resulting in the secretion of cytokines (IL-10 and TNF-α). (104). Its interaction with terminal GalNAc residues on CD45 glycoprotein negatively regulates TCR signaling, with consequent decrease of T cell proliferation and increased T cell death (105). Moreover, by blocking the tumor-infiltrated macrophages (responsible for the high levels of IL-10), it was observed an effective $CD8^+$ T cells response, highlighting the importance of combining anti-tumor immune therapy with conventional chemotherapy (106). Furthermore, it was recently demonstrated in chronic infection that IL-10 induces the upregulation of the *Mgat5*

gene increasing branched *N*-glycans on $CD8^+$ T cells, which in turn decreases T cell activity and allows viral persistence (107). Despite the different context in which this hypothesis was studied, *Mgat5*-mediated branching glycosylation can constitute a potential mechanism by which IL-10 is suppressing $CD8^+$T cells in cancer.

In addition, sialylated glycans also play a role in immunosuppression, mediated by siglecs, a family of lectin receptors that predominantly exhibit immune-inhibitory functions. In *in vitro* and *in vivo* studies, the binding to sialylated antigens by siglec-E expressed on DCs promoted an increase of antigen-specific Treg response and a reduced numbers of antigen-specific Teff cell response, associated with tumor growth (108, 109). Indeed, the sialylated tumor antigens, such as Sialyl-Tn (sTn) and Sialyl-T (sT) expressed in mucins, namely MUC1, were associated with tumor immune tolerance. The recognition of MUC1-ST by siglec-9 on tumor-infiltrating macrophages was shown to initiate inhibitory immune pathways mediated by MEK-ERK signaling (110). Moreover, siglec-binding to sTn-expressing mucins, led to the maturation of DCs and DC-mediated induction of $FOXP3^+$ Treg cells and reduced INFγ-producing T cells (111, 112). A recent study also demonstrates that siglec-9 expressed by $CD8^+$ tumor infiltrating lymphocytes (TILs) in non-small cell lung cancer (NSCLC) patients was associated with reduced survival. Accordingly, siglec-9 polymorphisms were associated with the risk of developing lung and colorectal cancer. Additionally, the characterization of siglec-9^+ $CD8^+$ TILs revealed that these cells concomitantly express several inhibitory receptors, including PD-1, TIM-3, Lag3, and others. In addition, the same study further reveals that lack of sialic acid-containing glycans in tumor cells led to a delay of tumor growth and an increased infiltration of $CD3^+$ and $CD8^+$ T cells (113).

Another important GBP that have been pointed out as a crucial checkpoint in T cell viability and activity are galectins. Galectin-1, 3, and 9 were predominantly described in T cell immunosuppression. Galectin-1, was demonstrated to be expressed by tolerogenic DCs (75) and $CD4^+CD25^+$ T cells (114), triggering T cell apoptosis through binding to *N*-glycans and *O*-glycans on CD45, CD43, and CD7 or by sensitizing resting T cells to FAS-induced death (115, 116). The T_H1 and T_H17 activated cells are susceptible to galectin-1-induced cell death once these cells express the repertoire of glycans required for galectin-1 binding, while T_H2 cells are protected via α2,6-sialylation on cell surface glycoproteins, which was described to preclude galectin-1 recognition and binding (76). In addition, several tumors have the capacity to secrete galectin-1 in order to promote immunosuppression, through a mechanism that involves a bias toward a T_H2 cytokine profile and activation of tolerogenic circuits mediated by IL-27-producing DCs and IL-10-producing type 1 Treg cells (117). On other hand, galectin-3 has an ambiguous role in T cell viability: when it is localized at intracellular level, this protein presents a protective role through a cell death inhibition pathway that involves B-cell lymphoma 2 (Bcl-2) (118), whereas extracellular galectin-3 induces cell death in activated T cells, by binding to glycosylated receptors of T cells through a distinct way than galectin-1 (115). Moreover, galectin-3 has the capacity to bind to *N*-glycans on CTLA-4 prolonging the inhibitory signals (119), as well as to Lag-3 on the surface of $CD8^+$ T cells, suppressing its function (120). Finally, galectin-9 abrogates T_H1, T_H17, and $CD8^+$ T cells through glycosylation-dependent binding to Tim-3 (121–123), whereas may regulate pro-inflammatory cytokine production by binding with other receptors (124).

Altogether, these findings support the relevance of glycans on T cells-mediated immunosuppressive/tolerogenic pathways which have relevant implications in tumor progression. Targeting the abnormal glycosylation pattern of cancer cells constitutes a promising strategy to instruct an effective anti-tumor immune response, an issue that needs to be further explored.

GLYCANS AS METABOLIC REGULATORS OF T CELL FUNCTION

The impact of glycosylation on T cell development and functions is enormous, as revealed by the critical roles of glycans in the development and progression of major diseases such as auto-immunity and cancer, as described herein. In order to accompany the bioenergetic and biosynthetic demands required for T cell proliferation and activation, a shift in the T cell metabolism is required. While naïve T cells are in a metabolic quiescent state, mainly using oxidative phosphorylation to maximize ATP production, T cells under clonal expansion or under differentiation, reprogram their metabolic status to aerobic glycolysis and glutaminolysis in order to increase the availability of glycolytic precursors for the biosynthesis of nucleotides, amino acids and lipids (125–127). During T cell activation, the hexosamine biosynthetic pathway (HBP—a branch of the glucose metabolism) is upregulated in order to generate the nucleotide sugar-donor substrate UDP-GlcNAc, required for *N*-glycosylation, O-GlcNAcylation, and glycosaminoglycans production that are needed for a proper T cell function (128).

Mediators from the glycolytic pathway such as glucose (Glc), glutamine (Gln), acetyl CoA are known to interfere with the availability of the UDP-GlcNAc in the cell (129–131). Together Glc and Gln were shown to increase UDP-GlcNAc in nutrient-starved T cells. In the same setup, the supplementation of both Glc and glucosamine (GlcN–a metabolite of the HBP) further increased the UDP-GlcNAc cellular content, demonstrating the sensitivity of the HBP to nutrients that enter directly (GlcN) or through a precursor pathway (Glc in glycolysis) (130). Despite the general use of the UDP-GlcNAc as a substrate donor of HBP, there are some glycosyltransferases that are more susceptible to nutrient changing than others, such as the case of OGT (132). In fact, the supply with Glc and Gln are crucial for protein O-GlcNAcylation, that is important during T cell development, being associated with T cell malignant transformation (23). Among the *N*-acetylglucosaminyltransferases (GnTs) that participate in the HBP, the less sensitive to nutrient changing (and thus substrate availability) are GnT1, GnT2, and GnT3, due to lower *Michaelis Constant* (K_m) levels, meaning that these enzymes require low

levels of the substrate to synthetize the specific glycans. In contrast, GnT4 and GnT5 present higher K_m and therefore their activity is highly dependent on the availability of the UDP-GlcNAc substrate (119, 133). Therefore, these two enzymes are sensitive to alterations in glucose and HBP metabolism (as the GlcN or *N*-acetyl glucosamine–GlcNAc) (62), which ultimately will interfere in the *N*-glycan branching biosynthesis on T cells with impact in their activity, as detailed in section "Glycans in the regulation of T cell activity and functions" (60). In fact, supplementation with Glc, Gln, and GlcNAc increases branching *N*-glycans on Jurkat cells and resting T cells from mice (85, 119, 130). Moreover, $CD4^+$ T cells from $MGAT5^{+/+}$ or $MGAT5^{+/--}$ mice supplemented with oral GlcNAc also results in up to 40% increase of branching *N*-glycans, detected by L-PHA (130). This enhancement of branching *N*-glycosylation upon GlcNAc supplementation was shown to functionally impact on T cells activity by reducing T cell activation, decreasing T_H1 differentiation, and increasing retention of the growth inhibitory receptor CTLA-4 at T cell surface (85, 130).

Importantly, evidences suggest that the glycolysis and glutaminolysis compete with HBP pathway for the same metabolites. Recently, Araujo et al showed that, during T_H17 differentiation the existence of common mediators shared between HBP, glycolysis (fructose-6-phosphate) and glutaminolysis (Gln) results in a starvation of the HBP mediators, translated in a reduction of *N*-glycan branching due to the limitation on the UDP-GlcNAc availability (53). Fueling HBP with GlcNAc switched the cell fate from T_H17 to iTreg differentiation, through stimulation of IL2-Rα signaling (53). This interplay between metabolic pathways was further demonstrated by the increase on Glc, Gln, fatty-acids uptake, and lipid storage upon stimulation of the HBP with GlcNAc supplementation, suggesting a reprogramming of the cellular metabolism upon GlcNAc flux (53, 134).

The impact of glycans as metabolic regulators of T cells is also testified by its effects in *ex vivo* and *in vivo* models of autoimmune diseases. The metabolic supplementation with GlcNAc in *ex vivo* human colonic T cells from IBD patients resulted in an enhancement of the branching *N*-glycosylation pathway that was accompanied by a significant reduction of T cell proliferation, supression of T_H1/T_H17 immune response (through decreased production of IFN-γ and IL-17A pro-inflammatory cytokines) and decreased TCR signaling (60). Accordingly, the GlcNAc supplementation of mice models with auto-immune diseases such as EAE, TID, and IBD results in inhibition of T_H1, T_H17 immune response concomitantly with a significant improvement of the clinical symptoms (60, 85). Treatment with GlcNAc after disease onset also demonstrate inhibitory effects on the development of the EAE, by reducing the secretion of INF-γ, TNF-α, IL-17, and IL-22 (85). Interestingly, a dual role of GlcN (the precursor of GlcNAc) on the progression of autoimmune disorders was shown, by demonstrating its impact in preventing T_H1-mediated Type I diabetes (through the reduction of IFN-γ producing $CD4^+$ T cells), but also the GlcN effects in exacerbating T_H1/T_H17–mediated EAE symptoms (trough stimulation of T_H17 response) (135). In contrast, another study showed that GlcN suppresses acute EAE through the blockage of T_H1 and induction of T_H2 response (136). GlcN supplementation was further shown to mediate T cell activation by decreasing the *N*-glycosylation of CD25 (IL-2Rα) from $CD4^+$ T cell (135). This down-regulation of *N*-glycosylation might be explained by the competition between GlcN and Glc for the same glucose transporter which might impact in the reduction of the GlcNAc concentration.

Altogether, alterations on the glucose metabolism and partially changes in the metabolic flux of HBP have a direct impact on T cells *N*-glycosylation profile with major consequences in their function and activity. Ultimately, the modulation of the HBP constitutes an important metabolic target able to control both autoreactive and immunosuppressive responses known to occur, respectively, in autoimmunity and cancer.

CONCLUDING REMARKS

The contribution of the glycome as a major regulator of the immune system is clear. Glycans actively participate in the cellular and molecular mechanisms underlying the genesis of the loss of immunological tolerance associated with (auto)immunity, from one hand, participating also in the creation of tolerogenic pathways associated with cancer progression, from the other. The importance of glycans in immune response spans from its role in the modulation the T cell development; their importance as a source of glycoantigens presentation; as well as their role as fine tuners of T cell response. In this context, glycans can exert a dual role, acting either as immune inhibitory checkpoints or as immune stimulatory signals. Understanding in depth the influence of glycans in the immune regulatory circuits that mediate the pathophysiology of autoimmunity and cancer will generate a platform with extraordinary potential to illuminate the identification of novel biomarkers and targets for the development of efficient immunomodulatory strategies with applications in the clinical setting.

AUTHOR CONTRIBUTIONS

All the authors wrote the manuscript. AD and NP created the figures. SP performed the critical review of the manuscript.

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APPENDIX IV

The Role of Glycosylation in Inflammatory Diseases

The Role of Glycosylation in Inflammatory Diseases

13

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Abstract

The diversity of glycan presentation in a cell, tissue and organism is enormous, which reflects the huge amount of important biological information encoded by the glycome which has not been fully understood. A compelling body of evidence has been highlighting the fundamental role of glycans in immunity, such as in development, and in major inflammatory processes such as inflammatory bowel disease, systemic lupus erythematosus and other autoimmune disorders. Glycans play an instrumental role in the immune response, integrating the canonical circuits that regulate innate and adaptive immune responses. The relevance of glycosylation in immunity is demonstrated by the role of glycans as important danger-associated molecular patterns and pathogen-associated molecular patterns associated with the discrimination between self and non-self; also as important regulators of the threshold of T cell activation, modulating receptors signalling and the activity of both T and other immune cells. In addition, glycans are important determinants that regulate the dynamic crosstalk between the microbiome and immune response. In this chapter, the essential role of glycans in the immunopathogenesis of inflammatory disorders will be presented and its potential clinical applications (diagnosis, prognosis and therapeutics) will be highlighted.

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Inflammatory diseases · Glycosylation · T-cells · IBD · Glomerulonephritis · Myositis

13.1 Role of Glycans in Adaptive Immune Development

Protein glycosylation has the potential to take part in the majority of cellular processes and to regulate cell-fate decisions, activity, function, for instance. Adaptive immune cells play a central role in the orchestration of an inflammatory response, as well as in its resolution. One of the factors contributing to inflammation is the recognition of non-self or altered biological material. T and B cells acquire the potential to recognize such signals during their early stages of development and glycans have been shown to play an essential role in these processes. The next section discusses the work on the influence of glycans in developmental checkpoints.

13.1.1 Glycans in Early T Cell Development

T cell development is one of the major physiological processes that occur in complex organisms, which ensures the formation of a proper repertoire of T cell receptors (TCRs), fundamental in immune responses (Koch and Radtke 2011). T lymphocytes develop in the thymus from a multistep program characterized by the sequential rearrangement of the *Tcrb* and *Tcra* loci in combination with lineage and control steps (Takaba and Takayanagi 2017). As the several events of development are dependent on both cell-intrinsic and -extrinsic factors, the study of glycans constitutes a major developmental feature of knowledge (Marth and Grewal 2008; Pereira et al. 2018a, b).

The initial step of T cell development occurs in the bone marrow, where lymphoid progenitors exit to the bloodstream, then to enter into the corticomedullar tissue of the thymus, in which they start to expand and develop. The trafficking of thymus seeding progenitors (TSPs) requires the expression of P-selectin glycoprotein ligand-1 (PSGL-1) by those progenitor cells and its partner, P-selectin, by the thymic epithelium (Rossi et al. 2005). The glycosylation profile of PSGL-1, namely its α1,3 fucosylation, was shown to be required for its binding to P-selectin, and its absence, by the genetic deletion of the Fut4 and Fut7 fucosyltransferases, led to the impairment of TSPs homing into the thymus (Sultana et al. 2012). In the sialyltransferase *St8Sia-IV*$^{-/-}$ mouse model, a deficient thymic seeding was also observed, caused by inefficient progenitor bone marrow egression (Drake et al. 2009). Once TSPs enter the thymus, they develop into early thymocyte progenitors (ETPs), a subset of the $CD4^{-}CD8^{-}$double negative 1 (DN1) population, which can give rise to multiple lymphoid lineages (Takaba and Takayanagi 2017). The major determinant responsible for the commitment of DN1 thymocytes to the T cell lineage is the presence of Notch signalling (Shah and Zúñiga-Pflücker 2014). The glycoprofile of Notch receptors (and ligands) was shown to regulate Notch-dependent intracellular signal transduction. These proteins are modified by the lunatic, manic and radical Fringe glycosyltransferases, which transfer *N*-acetylglucosamine (GlcNAc) to *O*-linked fucose glycans, in the repeats of the consensus epidermal growth factor-like (EGF-like) sequences present in the extracellular domain of Notch (Rampal et al. 2005). The presence of these glycans regulates the trafficking of the Notch receptors (Takeuchi et al. 2017) and its differential affinity for Notch-ligands (Rampal et al. 2005). It was shown that the mouse model of triple fringe deficiency had reduced the binding of Notch to Delta-like ligands (DLL), namely DLL4, altering the frequencies of several T cell subsets in the thymus (Song et al. 2016). The decisive commitment to the T cell lineage occurs at the DN3 stage, where a recombination-activating genes (RAG)-mediated productive rearrangement of the *Tcrb* leads to the expression of the ß chain of the TCR (TCRß) and the formation of a pre-TCR signalling complex (Takaba and Takayanagi 2017). Together with Notch and

IL-7, the pre-TCR signalling initiates ß-selection, by inducing the transition to DN4 cells. These cells then begin a round of multiple divisions, giving rise to the most represented thymocyte population, the $CD4^+CD8^+$double-positive (DP) cells.

The major function of the thymus as an organ is to generate an environment where randomly generated TCRs are probed for their reactivity and selected according to their self-reactive potential (Miller 2020). These biological processes occur in the DP stage, after the Tcra locus suffers rearrangements by the RAG complex, leading to the expression of a mature TCR (Shih et al. 2011). The new mature TCRs are then screened by thymic epithelial cells (TECs) by the specificity and binding strength for the presented MHC ligands. The initial process is named positive selection, where the DP population is enriched for cells that express an immunocompetent TCR. Afterwards, cells that display high levels of activation, which indicates self-reactive potential, are targeted for apoptosis, a process called negative selection. Finally, cells that go through both selections develop into $CD4^+CD8^-$ or $CD4^-CD8^+$single positive (SP) cells. One of the major contributions of glycans is represented by the ones of the CD4 and CD8 co-receptors. It was shown that chemical desialylation of CD8 mature cells increases CD8/MHC-I interactions (Daniels et al. 2001), and in fact, *ST3Gal1*$^{-/-}$ mice show a significantly altered TCR repertoire, indicating a role for sialylation in thymocyte selection (Moody et al. 2001). Furthermore, it was demonstrated that branching *N*-glycosylation expands the range of TCR signalling of positive selection by differentially controlling both the lower and upper limits of positively selected TCR–MHC–antigen interactions. This was pointed out to be due to decreased surface expression of CD4 and CD8 receptors, in *Mgat1* and *Mgat2* DP-conditional knockout models (Zhou et al. 2014).

Surface glycans also modulate galectin (gal-) binding, which in turn influence cellular functions (Rabinovich and Toscano 2009). Histological analysis of thymus from mice revealed differential galectin spatial distribution, suggesting functions in distinct developmental stages (Nio-Kobayashi 2018). Perillo et al. showed that gal-1 was able to induce apoptosis of human thymocytes in vitro, with high effect in combination with anti-CD3 stimulation, suggesting a role in negative selection (Perillo et al. 1997). Later on, Galvan et al. demonstrated that the downregulation of galectin-binding glycans occurs in positively selected DP thymocytes, which are resistant to gal-1-induced apoptosis (Galvan et al. 2000). Gal-3 was shown to regulate thymocyte-epithelial cell interaction, influencing developmental transitions. In fact, *Lgals3*$^{-/-}$ mice show decreased absolute numbers of thymocytes, with lower levels of proliferation (Oliveira-de-Abreu et al. 2018).

13.1.2 Glycans in Early B Cell Development

B cell lymphocytes are key players in the adaptive immune response, being essential regulators of immunity through the secretion of antibodies, soluble proteins with antigen specificity. B cell development is a highly regulated process that takes place in the bone marrow and the spleen (Hardy and Hayakawa 2001). Much like T cells, B cells undergo somatic gene rearrangement in the immunoglobulin loci, and are selected to ensure a self-tolerant repertoire of antibodies. Its development is crucial to ensure proper immune function throughout the life of the organism.

B cells are generated from common lymphocyte progenitors (CLPs) that remained in the bone marrow. As in DN1 thymocytes T cell lineage commitment, the CLP commitment to the B cell lineage is influenced by Notch signalling. CLPs require absent Notch signalling to develop into progenitor B cells (pro-B). Unlike the role of this pathway in T cell development (promoting effect), Notch has to be absent for B cell development to occur (Stanley and Guidos 2009). Glycosylation profiles of the Notch receptor and ligands regulate affinity and surface expression. Interestingly, conditional knockout of Lfng in thymocytes led to B cell development in the thymus (Koch et al. 2001).

When pro-B cells successfully rearrange the Igh locus, mediated by the RAG complex, they develop into precursor B cells (pre-B). The cells in this developmental stage express a pre-BCR complex, with no antigen specificity, which triggers a signalling cascade, driving proliferation. The pre-BCR receptor is composed by the rearranged immunoglobulin heavy chain that assembles with a surrogate chain, to ensure surface expression (Burrows et al. 2002). It was seen that the *N*-glycans of the rearranged immunoglobulin heavy chain influence this assembly and are specifically required for pre-BCR function (Übelhart et al. 2010). Moreover, core fucosylation was also demonstrated to play a role in pre-BCR formation (Li et al. 2012). Stromal gal-1 was shown to be a ligand of the pre-BCR, triggering its signalling pathway and enabling further B cell development (Gauthier et al. 2002). In fact, B cells in this stage are found in the stromal niches where gal-1 is enriched (Mourcin et al. 2011) and their development is compromised in the absence of gal-1(Espeli et al. 2009). The signalling activation of the pre-BCR cascade leads to the rearrangement of the immunoglobulin light chain, which results in the expression of a mature BCR, and development into the immature B cell stage.

Immature B cells are then screened for their autoreactive potential in the bone marrow. Cells reacting with low or high affinity suffer receptor editing, with a secondary rearrangement of their immunoglobulin light chain allele (Schatz and Ji 2011). Afterwards, BCR with affinity for self-peptides are negatively selected, and cells that express a tolerant BCR are positively selected, by which central B-cell tolerance is achieved (Nemazee 2017). Recently, it was shown that branched *N*-glycans are required for B cell selection (Mortales et al. 2020). By the conditional knockout of *Mgat1* in the B cell lineage, it was observed that branched *N*-glycan deficiency decreased the surface expression of the BCR co-receptor CD19, which inhibited positive selection. Moreover, the nerve growth factor IB (Nur77) was shown to be upregulated in immature B cells in the absence of branched *N*-glycans, indicating a role of threshold establishment, similarly to T cells (Mortales et al. 2020).

B cells that go through central selection migrate to the spleen and commit to either the marginal zone (MZ) or follicular cell fates, according to the strength of their BCR signal (Pillai and Cariappa 2009). Interestingly, mice deficient for CD22, a B cell siglec that binds α2,6-sialic acids, which inhibits BCR signalling, show decreased cellularity on the MZ B cell compartment (Samardzic et al. 2002). B cell homing was shown to be compromised in a *Cosmc*$^{-/-}$ genetic background, demonstrating the role of elongated *O*-glycans in this process (Zeng et al. 2020).

When resting B cells encounter an antigen, one of the major features of the humoral response takes place: the antibody diversification and maturation. This process occurs in germinal centres (GC) where the selection of B cells according to their antigen affinity. Cell clones which display high antigenic affinity, proliferate and differentiate into antibody-secreting plasma cells and memory B cells (Mesin et al. 2016). Galectins have been implicated in the regulation of BCR signalling, influencing therefore B cell germinal centre selection. Interestingly, loss of gal-3 resulted in increased B cell activation and spontaneous GC formation (Beccaria et al. 2018). It was also shown that both gal-1 and gal-8 promote plasma cell differentiation (Tsai et al. 2011). In fact, it was demonstrated that binding of gal-8 endorses antigen recognition by B cells, promoting the formation of the immunological synapse (Obino et al. 2018).

13.2 Role of Glycans in the Inflammatory Process

13.2.1 Glycans in Innate Immune Responses

Innate immune cells are the first line of defence against pathogens and play a major role in the inflammatory response. This immune cellular group comprises mast cells, phagocytes (dendritic cells (DCs), macrophages and neutrophils), NK cells and innate lymphoid cells (ILC). Their glycosylation profile should be taken into consid-

eration to understand their functions in an inflammatory environment or during an immune response. Trafficking and recruitment of innate cells to the sites of tissue injury are controlled through glycosylation-mediated recognition between leukocytes and endothelial cells. Moreover, the migration of these cells to the inflammation sites allows the accumulation of specific cytokines and chemokine cocktails which are also responsible for altering the glycosylation of surrounding cells. Moreover, this interplay between innate immune cells and the inflammatory environment is also mediated by specific glycan-recognizing receptors. It is clear that protein glycosylation plays a fundamental role in each major step of the inflammatory process—initiation, propagation and abrogation of inflammation—and the next section details the contribution of glycans in each one.

13.2.1.1 Glycans in Immune Cell Trafficking and Recruitment

Upon infection or tissue damage, endothelial cells exhibit cell surface changes in order to promote the migration and extravasation of immune cells to the site of injury. Roll, arrest and adherence steps are controlled through the interaction between endothelial selectins (E-Selectin and P-Selectin) and ligands present in leukocytes (Zarbock et al. 2011). Selectins are C-type lectins (calcium-dependent glycan-binding proteins) characterized by their ability to recognize and bind specific carbohydrates structures, such as Sialyl Lewis X (sLeX) (Schnaar 2016). Changes in cellular glycosylation affect selectin-binding, such as PSGL-1 (P-Selectin) or ESL-1 (E-Selectin), modulating the process of recruitment and homing of immune cells (Sperandio 2006). In fact, it was described that mice lacking *ST6Gal1* gene, which codes the sialyltransferase responsible for the addition of terminal α2,6-sialic acids, showed an impaired migration of immune cells toward draining lymph nodes (Zarbock et al. 2011). The hyaluronic acid receptor CD44 is also an important leukocyte ligand for endothelial E-Selection. CD44-E-Selectin interaction is highly dependent on the surface sialylation and fucosylation of its *N*-glycans (Kansas 1996). Thus, the activity of glycosyltransferases during this process of recruitment is vital to mount a correct in situ inflammatory response.

13.2.1.2 Glycans as Recognition Moieties (PAMPs and DAMPs)

After cellular migration to the injury site, the recognition of pathogens and/or injured host cells is an important step in the inflammatory process. Innate immune cells express a variety of cell surface receptors with the specific capacity of recognize 'danger' structures, known as pathogen-associated molecular pattern (PAMP) or damage-associated molecular pattern (DAMP). PAMPs and DAMPs are often glycosylated biomolecules present in the surface of the pathogens or damaged cells, or even released to the extracellular space (Ablasser and Chen 2019). One of the mechanisms by which these molecules are recognized by innate immune cells is through carbohydrate-recognizing receptors, such as C-type lectins (CTLs). Antigen-presenting cells, such as macrophages and DCs, bear a diverse and robust collection of several C-type lectins that enable them to recognize several danger glycosignals (Brown et al. 2018). The Dectin-1 CTL recognizes β-linked glucose polymers, a major constituent of pathogen cell walls, and is able to trigger cellular activation with its intracellular domain, through the NF-κB signalling cascade (Ferwerda et al. 2009). Dectin-2 recognizes mannose residues displayed by pathogens and host cells and is able to interact with FcγRs through its extracellular domain, inducing cellular activation (Hollmig et al. 2009). Interestingly, *Dectin-2*$^{-/-}$mice have shown a decreased susceptibility to house dust mite-induced lung inflammation, highlighting the importance of this first mechanism of defence (Parsons et al. 2014). Another well-known CTL, mostly studied in the context of cancer, is dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN). This CTL is mainly expressed in immature DCs, monocytes and macrophages, and recognizes high-mannose and fucose residues. DC-SIGN assists also in the

antigen recognition leading to the maturation of DCs (RodrÍguez et al. 2018;Brown et al. 2018).

13.2.1.3 Glycans in Innate Immune Cell Function

During an inflammatory process, APCs and monocytes migrate to the tissue where they encounter the inflammatory agent (tumour cell, pathogen, for instance). After recognition, in which specific receptors bind to the surface molecules of the agent, DCs suffer a shift on its phenotype, characterized by increased expression of MHC-II, costimulatory molecules (CD80, CD86) upregulation, as well as increased cytokine and chemokine secretion. This protein shift is accompanied with changes in surface glycosylation that distinguish mature (mDCs) and immature (iDCs) DCs. Maturation of DCs is associated with an increase of elongated poly-*N*-acetyllactosamine chains with terminal α2,3-sialic acid and fucose (Bax et al. 2007). These glycan expression alterations represent an important mechanism that regulates DC functions, such as antigen presentation to T and B cells in the lymph nodes. Higher abundance of terminal sialic acid was shown to enable the *trans* binding of sialoadhesins (CD22), expressed by B cells, supporting a potential DC-B cell interaction during antigen presentation (Varki and Gagneux 2012). Moreover, the increased presence of elongated poly-*N*-acetyllactosamine chains favours galectins binding, which in turn have been shown to regulate cell adhesion, cell activation, chemoattraction, cell growth and apoptosis in DCs (Videira et al. 2008).

Macrophages, characterized by the spectrum between classical M1 and M2 phenotype, also bear glycan-binding receptors, such as mannose receptor (MR) and DC-SIGN. Interestingly, the role of these CTLs in macrophages is still very broad, strongly relying on the context of inflammation. For instance, in the context of germinal centre formation, mannosylated IgM B cell receptor seems to promote the activation of B cell receptor (Amin et al. 2015). On the other hand, in a context of *Mycobacterium tuberculosis* infection, DC-SIGN-mediated recognition of bacterial glycans seems to induce an anti-inflammatory polarization in macrophages (Lugo-Villarino et al. 2018).

13.2.2 Role of Glycans in Adaptive Immune Cells Functions

Initially, the adaptive immune response relies on the ability to differentiate self-antigens derived from pathogens/damage host cells. This process is orchestrated by antigen-presenting cells (APCs), which display antigens attached to major histocompatibility complexes (MHCs) that are presented to T cells (den Haan et al. 2014; Lee et al. 2020). The MHC is a family of structurally and genetically related glycoproteins that are able to control immune response through T cell activation (Neefjes et al. 2011). It encompasses two major classes of MHCs: the MHC class I (MHC-I), which can be expressed by all nucleated cells and reacts to intracellular bacteria, viral infections and cellular transformation (Hewitt 2003; Comber and Philip 2014); and the MHC class II (MHC-II), which responds to exogenous proteins (Storni and Bachmann 2004). MHC-II expression is limited to professional APCs, such as DCs, macrophages and B cells (Roche and Furuta 2015).

The glycosylation of proteins and receptors that participate in adaptive immune response, such as MHC molecules and TCRs, is essential for correct protein folding (Trombetta and Helenius 1998) to protect protein backbone from proteolysis (Wang et al. 2001) and to ensure a suitable distance between receptors and other molecules at the cell surface, in order to facilitate interactions (Grigorian et al. 2009). Indeed, it was already described that the blockade of MHC1a *N*-glycosylation leads to a severe increase in intracellular misfolded protein content and an impairment in cell surface expression and peptide presentation (Barbosa et al. 1987).

MHC-II molecules are constituted by two α and two β chains, each of which contains a transmembrane domain, contrasting with MHC-I molecules, which display only one β chain. The glycan composition of both chains within MHC-II also differs, with the α chain displaying

predominantly *N*-linked high-mannose and complex *N*-glycans and the β chain being composed by complex *N*-glycans (Unanue et al. 2016). MHC-I exhibits one single conserved site for *N*-glycosylation at Asn86, whereas MHC-II has three highly conserved *N*-glycosylation sites, two on the α chain (Asn78 and Asn118) and one on the β chain (Asn19) (Gauthier et al. 1998; Ryan and Cobb 2012). The *N*-glycan site on MHC-I is important for antigen binding to occur due to its role in recruiting chaperones that are involved in peptide loading (68). Glycoprotein modifications of MHC molecules are able to mediate immune responses. Ostankovitch and colleagues have described that the presentation of an MHC-I restricted epitope, derived from the membrane protein tyrosinase, requires retrotranslocation of glycosylated molecules from the endoplasmic reticulum to the cytosol. In particular, they have shown that proteasomes degrade tyrosinase molecules that are glycosylated and generate intermediate molecules that are not found in degradation of non-glycosylated molecules. In this context, the authors suggest that the glycosylation of these intermediate molecules influences their processing by the proteasome, stating a relevant role for glycosylation for the presentation of an MHC-I-restricted epitope derived from tyrosinase (Ostankovitch et al. 2009).

Besides presentation of glycan antigens to T cells, the glycosylation of MHC-II is intricately associated with T cell response also by modulating antigen binding. Accordingly, in APCs deficient for *Mgat2* glycogene it was demonstrated that the lack of complex *N*-glycans was detrimental for glycan antigen presentation by MHC-II and led to loss of T-cell activity (Ryan et al. 2011).

As mentioned above, T cell development, growth and differentiation are regulated by *N*-glycans and *O*-glycans (see Sect.13.1.1). The role of glycans in these cells has been demonstrated in several studies on different autoimmune disorders and also murine models of inflammation-associated diseases (Mkhikian et al. 2011; Dias et al. 2018a; Verhelst et al. 2020). Foremost, over the past 20 years, we have witnessed how critical are *N*-glycosylation alterations in regulating adaptive immunity, namely by mediating the T cell function, not only through the regulation of its primordial receptor, the TCR, but also its partner receptors (namely CTLA-4, CD45, CD25, CD28) (Pereira et al. 2018a, b).

An altered glycosylation of the TCR, namely reduced expression of complex branched *N*-glycans, has been described to lower the threshold for T cell activation, further modulating the surface expression of important growth inhibitory receptors such as cytotoxic T lymphocyte antigen-4 (CTLA-4) (Demetriou et al. 2001; Morgan et al. 2004; Grigorian et al. 2007). In fact, one decade ago it was demonstrated that TCR *N*-glycosylation is regulated by TCR signalling. More precisely, it upregulates *N*-acetylglucosaminyltransferase V (GnT-V) and Golgi α-mannosidase enzymes at the mRNA level, in a synchronous manner, to enhance complex branching *N*-glycans branching in activated T cells, to avoid hyperactivation (Chen et al. 2009a).

Similarly, an increase of branched *N*-glycans on CD25 has been associated with increased cell surface retention with impact in the regulation of T cell differentiation and immune tolerance. It was demonstrated that by reducing UDP-GlcNAc (substrate for the initiation of branching in *N*-glycans) intracellular availability or the expression of branching glycosyltransferase, there is a decrease of CD25 surface retention and IL-2signalling, which promotes T helper-17 (Th17) over induced regulatory T cell (iTreg) differentiation (Araujo et al. 2017). Additionally, the function of CD28 co-stimulatory receptor has been shown to be mediated by *N*-glycosylation which can negatively regulate the interaction between CD28/CD80. Different approaches inhibiting *N*-glycosylation (in vitro *by* site mutagenesis on the five *N*-glycosylation sites in the extracellular domain and by enzymatic inhibitors of biosynthesis of *N*-linked oligosaccharide structures) resulted in a defective CD28 glycosylation and enhancement of the binding to CD80 expressed on APCs (Ma et al. 2004). Later, other study demonstrates that the high levels of polylactosamine in CD8 are on *N*-glycans, further suggesting polylactosamine structures on CD28 as critical players in regulating T cell activation

(Togayachi et al. 2007). Moreover, in 2011, another study has also shown that IL-2 and IL-7 have distinct effects in resting and activate T cells. Early by lowering branching *N*-glycans and TCR activation thresholds, these cytokines enhance T cell growth. Later, they promote self-tolerance by enhancing branching and CTLA-4 surface retention (Mkhikian et al. 2011).

Additionally, galectins are at the crossroad of tolerance and inflammation (Modenutti et al. 2019). Different members of the galectin family exhibit a 'double-edge sword' effect, acting either as negative or positive mediators of T cell homeostasis. Galectin-1, -2, -3 act as inhibitors of inflammation and T cell activity. One of the major regulations of T cell function by galectins is related to Gal-1's ability to negatively regulate Th1 and Th17 effector cells by inducing cell death. Interestingly, it was also shown that Th2 cells upregulate the expression of terminal α2,6-sialic acids, which inhibit Gal-1 binding, render a Gal-1-induced-apoptosis resistance by these cells (Toscano et al. 2007). Gal-3 also plays a pivotal role in the regulation of T cell activity, as it can constrict TCR clustering, by the binding to complex branched *N*-glycans of these receptors, generating lattice formation, controlling the threshold of T cell activation (Demetriou et al. 2001; Chen et al. 2009b). Moreover, Gal-2 also exhibits a suppressive effect by inducing apoptosis of *lamina propria* T lymphocytes attenuating acute and chronic mouse colitis (Paclik et al. 2008). In contrast, Gal-8 and -4 act as promoters of T cell activation, as it was described that Gal-8 binds to T cells through unique interactions with CD45 and promotes T-cell proliferation (Tribulatti et al. 2009), and that Gal-4 mediates $CD4^+$T cell stimulation, through IL-6 production, leading to exacerbation of T cell-mediated chronic colitis (Hokama et al. 2004).

The dual impact of Gal-9 on inflammation is still controversial since most studies have been conducted only using exogenous Gal-9 and the endogenous counterpart has been overlooked. In fact, exogenous Gal-9-based approaches have suggested it as a immunoregulator of T cell function and a suppressor of immune disease in vivo (Zhu et al. 2005; Madireddi et al. 2014; Wu et al. 2014). However, a very recent study has elucidated the role of endogenous Gal-9, being crucial for Th17 differentiation and T cell proliferation. Moreover, the same work described that high levels of Gal-9 in $CD4^+$ T cells isolated from PBMCs of multiple sclerosis patients are positively correlated with disease severity, highlighting its potential value as biomarker for autoimmune diseases (Chen et al. 2020).

Overall, galectins have a master role in the regulation of inflammation but one of the main questions remains to be elucidated: What are the precise mechanisms involved in the anti-inflammatory and immunoregulatory effects of different members of the galectin family?

13.3 Glycans in Chronic Inflammatory Diseases

13.3.1 Role of Glycans in Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic debilitating disorder from the gastrointestinal tract comprising Crohn's disease (CD) and ulcerative colitis (UC). The etiopathogenesis of IBD is influenced by a complex interaction between environmental and genetics factors, microbiome and/or host immune response (Kaplan and Ng 2017). Its incidence has been increasing worldwide, including in paediatric populations (Ruel et al. 2014; Kaplan and Ng 2017).

Over the past decade, several studies on IBD pathogenesis have unveiled the fundamental role of glycans in the regulation of innate and adaptive immune responses associated with IBD development and progression (as reviewed in detail in (Dias et al. 2018a, b)).

13.3.1.1 Role of Glycans in the Regulation of Innate Immune Response and Gut Microbiome in IBD

The intestinal epithelial barrier represents the largest interface between the internal organs and the environment. Placed at the cell surface of

enterocytes, heavily glycosylated membrane mucins constitute the intestinal glycocalyx, which is the first line of defence against microbial translocation. This dense microbial community exerts a significant impact on intestinal physiology due to their ability to modulate immune development and to inhibit pathogen colonization (Hooper and Gordon 2001). Indeed, it poses an enormous challenge to the immune system, particularly for innate cells, since it needs to properly respond to pathogens without mounting an inflammatory response that may be detrimental to commensal microbes and may trigger the development of spontaneous inflammation.

Although innate immune cells are essential regulators of a healthy intestinal environment, not much is known about how glycosylation alterations dictate innate cell functions. Intestinal macrophages are one of the most represented populations of leukocytes in the intestine, which makes them first-aid players to maintain intestinal homeostasis (Bain and Mowat 2014). As it was discussed above for general inflammatory processes, alterations in glycosylation pathways were described to influence this innate cell population in the context of IBD. Shinzaki et al. have demonstrated, using a transgenic mouse model of GnT-V overexpression, that increased expression of complex branched *N*-glycans leads to increased colitis severity by inducing macrophage dysfunction and subsequently enhancing colorectal tumorigenesis (Shinzaki et al. 2016). Moreover, intestinal epithelial cell–specific deficiency of core 1–derived *O*-glycans in mice is associated with development of spontaneous colitis, inducing exacerbated infiltration by TNF-producing myeloid cells in colon mucosa (Fu et al. 2011; Nakayama et al. 2019).

Innate lymphoid cells (ILCs) display a relevant role in the establishment of intestinal homeostasis, since they are highly responsive to microbial stimulation (Vivier et al. 2018). In particular, group 3 ILCs (ILC3) are considerably abundant in mucosal tissues, being particularly involved in epithelial barrier integrity through the production of IL-17, IL-22 and GM-CSF (Neill and Flynn 2018). ILC3 are also able to modulate the intestinal glycocalyx via production of IL-22, which induces the upregulation of fucosyltransferase 2 (FUT2) mRNA on intestinal epithelial cells that in turn lead a protective effect against pathogens through the stabilization of commensal gut microbiota (Goto et al. 2014).

Glycans can act as major sources of energy for the microbiota (Koropatkin et al. 2012). Besides being able to utilize glycans derived from the diet, several bacteria can degrade *O*-linked glycans present in the epithelial mucus layer or *N*-linked glycans shed by epithelial cells to use them as nutrient source (Tailford et al. 2015; Ravcheev and Thiele 2017). Specific deletion of core 1–derived *O*-glycans on gut epithelial cells, using an IEC conditional mouse model lacking *C1galt1* specifically in intestinal epithelial cells, was shown to induce spontaneous colitis in mice (Fu et al. 2011). To clearly demonstrate that the loss of intestinal epithelial core 1–derived *O*-glycans is at the basis of colitis development in adult mice, the authors crossed *C1galt1*$^{f/f}$ mice with VillinCre-ER^{T2} transgenic mice, creating an inducible model of deficiency of intestinal epithelial *O*-glycans that developed colitis 5 days after induction with tamoxifen (Shinzaki et al. 2016). Indeed, alterations in the *O*-glycosylation profile of mucin-2 are described in UC patients with active disease, with an increment of smaller glycans and a decrease of complex glycans, and it associates with increased intestinal inflammation (Larsson et al. 2011), stating the crucial role of glycosylation alterations in intestinal epithelial barrier function.

13.3.1.2 Role of Glycans in the Regulation of Adaptive Immune Response in IBD

The disruption of gut mucosal barrier leads to a cascade of events starting with innate immune response, which initiates and drives a subsequent adaptive immune response within the colon *lamina propria*. This second line of defence involves mainly the activation of Th1, Th2, Th17 cells and suppression of the activity of Treg cells (Iwasaki and Medzhitov 2010). Nevertheless, in inflamed intestinal mucosa (in CD but not in UC or healthy controls), there is a unique subset of FoxP3^{+} T

cells that produce IL17 (the so-called Treg/Th17 axis) (Hovhannisyan et al. 2011). The origin of this distinct cell population is not fully understood, but it is postulated to be shaped by gut microbiome, despite the precise mechanism(s) underlying it remains unclear (Omenetti and Pizarro 2015).

Several studies have been shown that the T cell differentiation can be influenced by *N*-glycosylation alterations, both in mouse and human cells (Araujo et al. 2017; Dias et al. 2018a, b). Interestingly, in UC patients with active disease and also murine models of IBD (DSS-induced colitis), characterized by highly activated Th1 and Th17 immune response, it was demonstrated that T lymphocytes of *lamina propria* are deficient in complex branched *N*- glycans (codified by *MGAT5*) on the TCR (Dias et al. 2014). However, when this deficient mechanism is repaired by in vitro glycan supplementation of patient-derived colonic T cells, both Th1 and Th17 responses are diminished through reduction of respective pro-inflammatory cytokines production (TNF-α, IFN-γ and IL17A) and transcription factors at mRNA level (T-bet and RORγT). Moreover, in vivo studies showed that disease severity and progression were attenuated upon the restore of TCR branched *N*-glycosylation (Dias et al. 2018a, b). Similar impact was observed in the absence of core fucose *N*-glycans (codified by FUT8) which are highly expressed in T cells from patients with active IBD. Regarding the impact of glycans in Treg population, there are no significant alterations in IBD models (Dias et al. 2018a, b). Accordingly, in mouse and human cells, it was demonstrated that Treg suppressive function correlates with glycan expression levels, as Tregs with high expression of tri/tetra-antennary complex *N*-glycans present an enhanced ability to suppress $CD4^+$ and $CD8^+$ T cell proliferation (Cabral et al. 2017). However, these findings still impose more comprehensive studies about the glycophenotype of Treg subset, using realistic in vivo disease models and in clinical settings.

More recently, in two independent European cohorts of UC patients, specific genetic variants of *MGAT5* were shown to have a functional impact in the modulation of T cell glycosylation and plasma IgG glycome, being associated with worse disease course (Pereira et al. 2020). Briefly, UC patients display *MGAT5* single-nucleotide polymorphisms (SNPs) that are functionally associated with low transcription levels of *MGAT5* glycogene in colonic and circulating T cells and with agalactosylation of IgGs (a pro-inflammatory glycophenotype of IgG, observed in other autoimmune disorders (Ercan et al. 2010; Vučković et al. 2015; Momozawa et al. 2018)). Importantly, despite these evidences suggesting *MGAT5* gene as a common driver that simultaneously regulates the function and activity of both humoral and adaptive components involved in IBD development, it is not clarified yet whether the alterations on IgG glycoprofile are or not a T-cell-dependent mechanism.

Overall, glycans are master regulators of intestinal homeostasis and inflammation as they play a role on driving a fine-tuned dynamic between intestinal epithelial barrier function, the immune system and the gut microbiome. Nevertheless, further studies focused on the glycobiome are needed to fully understand the regulatory mechanisms associated with IBD.

13.3.2 Role of Glycans in Glomerulonephritis

Glomerulonephritis is a general inflammation of a specific portion of the kidney—glomeruli. Glomeruli represent an essential functional unit of the kidney, composed by podocytes organized together to build a filtration barrier. This barrier is highly regulated by the ability of podocytes of contracting and stretching, therefore controlling the passage of water or proteins/compounds (Petrosyan et al. 2019).

Inflammation of glomeruli can occur as an acute event which can be resolved within days, or be prolonged through months/years, precluding in a chronic inflammation with severe damage to the kidney function. The etiopathogenesis of glomerulonephritis is still to be elucidated, however it can be associated with autoimmune disorders (such as SLE or IgA nephropathy) as well as with

an aggravation and extension of a primary acute inflammation, such as drug- or infection-induced (Webster and Pusey 2017). In both scenarios, it is important to account for two different compartments which can be dysregulated: both immune infiltration, which seems to be over-activated and instructing a cytotoxic immune response against podocytes and mesangial cells in the glomeruli, as well as the non-immune compartment, which could be triggering an aberrant immune recruitment. Glycosylation plays a crucial role in both compartment perspectives, giving rise to new hypotheses in the etiopathogenesis of glomerulonephritis.

GnT-V (as mentioned before) is the enzyme responsible for the addition of β1,6-branched GlcNAc, allowing the following extension of poly-*N*-acetyl-lactosamine (Gal-β1,4-GlcNAc) group. This group is a binding motif for Gal-3 lattice, allowing a spatial distance between TCRs, preventing TCR clustering and activation. Hereupon, Demetriou et al. showed that at 12–20 months of age, *Mgat5* null mice appear to exhibit signs of glomerulonephritis (Demetriou et al. 2001). This glomerulonephritis was characterized by immune infiltration in the glomeruli and mesangial area, as well as severe glomeruli destruction; phenotypically most severe cases of glomerulonephritis (32%) showed haematuria, proteinuria and a characteristic crescentic glomerulonephritis with fibrosis in the Bowman capsule. The hyperactivation observed in *Mgat5*$^{-/-}$ could be instructing a stronger immune response in the kidney, as it does in the colon (Demetriou et al. 2001; Dias et al. 2018a, b).

Interestingly, Chui et al. have observed that murine kidney glycoproteins are highly dependent on α-mannosidase II for the correct glycan repertoire, a special feature which was not observed in other tissues, since an alternative form of α-mannosidase II takes its role. This observation reveals the importance of α-mannosidase II enzyme kidney-associated inflammation. Accordingly, these mice develop a severe autoimmune-associated glomerulonephritis at 12 months of age, displaying high levels of autoantibodies, with IgG, IgM, IgA and C3 complement deposition on glomeruli, as well as plasma cells and neutrophil infiltration (Chui et al. 2001).

In both mice models, it is possible to assume that non-immune compartment is also playing a role, since MGAT5 or MAN2A1 are absent in all organism cells/tissues. Given that, an interesting library of C-type lectins has been studied in the scope of immune cell recognition, not only in infection models, but also in inflammatory diseases, since aberrant glycans are being exposed at the surface of supposedly healthy cells. It was interesting to observe that glomerulonephritis was attenuated in mice after treatment with anti-DC-SIGN antibody (Cai et al. 2016). Moreover, mannose-binding lectin (MBL), a soluble c-type lectin which recognizes agalactosylated glycoproteins, was detected in the glomerulonephritis kidney biopsies of lupus patients, with no changes in the serum levels (Lhotta et al. 1999). Altogether, there is a body of evidence that argues a possible role in the aberrant glycoprofile of non-immune compartment for the immune response triggering.

Despite the lack of knowledge regarding the role of glycosylation in SLE, recent findings have been describing a deficiency in complex *N*-glycans at the surface of kidney epithelial compartment (Alves et al. 2020). This altered glycoprofile appears to be associated with the promotion of a chronic inflammatory response, however more studies are needed to validate these observations.

13.3.3 Role of Glycans in Myopathies

Idiopathic inflammatory myopathies (IIM) are a group of rare diseases of autoimmune nature, whose etiopathogenesis is far from being totally understood. Factors implicated in autoimmune diseases in general, namely immune innate and adaptive dysregulations associated with non-immune mechanisms, have been implicated, but how they interplay to give rise to the diverse pathogenic phenotypes remains elusive (Miller et al. 2018). Muscle cells surface is enriched in glycoproteins, either alone or in glycoprotein complexes, and several lines of evidence provide

support for a fundamental role of glycosylation in muscle homeostasis and function (Broccolini et al. 2009; Townsend 2014; McMorran et al. 2016). Hereditary inclusion-body myositis (hIBM) bears a muscle phenotype that resembles in most aspects sporadic inclusion-body myositis (sIBM) (one of the IIM subgroups) and is associated with mutations in the UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE) gene. GNE codes for an enzyme expressed in several tissues and it is critical for the biosynthesis of sialic acid. GNE mutations result in glycosylation changes, namely hyposialylation of muscle glycoproteins and prophylactic supplementation of a sialic acid precursor (*N*-acetylmannosamine (ManNAc) was shown to prevent the muscle phenotype in mice with gene mutations that cause hIBM (Malicdan et al. 2009). Changes in muscle glycosylation in human disease have focused in muscular dystrophies and congenital glycosylation disorders, but recent studies have shown that muscle cell surface glycosylation is finely regulated and subjected to alterations under inflammatory condition (Wiendl et al. 2005), pointing to an interaction between muscle glycocalyx and the extracellular milieu, which is particularly enriched in immune cells and antibodies in IIM patients (Afzali et al. 2018).

Changes in the signature of healthy muscle cells could be indeed associated with immune infiltration and development of a dysregulated response in a tissue-target manner. Understanding the role of *N*-glycans, either at the immune compartment as well as in the epithelial component may contribute to a better knowledge of the etiopathogenesis of inflammatory myopathies.

13.4 Glycans as Potential Targets for Diagnosis, Prognosis and Therapy in Chronic Inflammatory Diseases

Chronic Inflammatory Diseases including IBD, SLE and IIM present a big challenge for the correct diagnosis and are characterized by a large variation in response to treatment and issues in predicting outcomes to conventional therapies (Podolsky 2002; Dias et al. 2018a, b; Verhelst et al. 2020). Early diagnosis and prognosis of these types of diseases are critical to decrease morbidity, improve quality of life and decrease disability of patients, since the delayed initiation of appropriate therapy may contribute to unsuccessful outcomes. Therefore, there is a clinical need to develop better diagnostics, more effective and preventive approaches. The identification of novel biomarkers of disease whose exploitation may represent a promising novel therapeutic strategy for inflammatory diseases will allow the selection of patients according to their proneness to develop aggressive/complicated disease course and consequently an adequate redirection of therapy (Pereira et al. 2018a, b).

Due to their role in cell functions, glycans have recently been appreciated as a crucial factor regulated in pathologic events leading to development of immune-mediated diseases. In fact, the relationship between glycosylation alterations and its functional impact in the etiopathogenesis of many autoimmune diseases is a new and promising field for the development of novel therapies directed to improve individualized therapy and to develop better diagnostic and prognostic approaches (Dias et al. 2018a, b; Hanić et al. 2019; Verhelst et al. 2020). Lower levels of branched *N*-glycans in colon biopsies diagnosis predict patients that do not respond to standard therapy with 75% specificity, whereas patients presenting high levels of branched *N*-glycans display a favourable therapeutic and disease outcome. Interestingly, this glyco-biomarker combined with the analysis of C-reactive protein (CRP), a clinical biomarker used as a predictor of inflammation, constitutes a powerful tool with improved prognostic capacity. IBD patients with low branched *N*-glycans and high CRP levels at diagnosis early require an aggressive and non-conventional therapy (Pereira et al. 2018a, b).

Additionally, recent discoveries on the role of plasma glycoproteins have pushed forward the expanding area of GlycoMedicine to the forefront for many clinical applications (Theodoratou et al. 2014; Hanić et al. 2019; Reily et al. 2019). Highly inflammatory glycosylation signature of plasma immunoglobulin G (IgG) has been asso-

ciated as clinical features of IBD and SLE (Arnold et al. 2007; Šimurina et al. 2018). In fact, a pronounced decreased galactosylation of IgG-Fc is observed in SLE and IBD patients, representing in turn a great indicator of chronic inflammation with significant diagnostic value. Moreover, a decrease of di-galactosylated IgG *N*-glycans in IBD patients compared with healthy controls was already reported (Shinzaki et al. 2008). The role of glycans as diagnostic and prognostic IBD biomarkers was further illustrated by *N*-glycan analysis of total plasma proteins where an increase in glycan branching, a decreased abundance of hybrid and high-mannose structures, higher total sialylation, and lower fucosylation were observed in IBD patients compared with healthy individuals (Clerc et al. 2018). Likewise, some reports have shown that the glycosignature of IgG changes between patients with UC and CD, revealing differences on level of fucosylation, galactosylation, and bisection (Trbojević Akmačić et al. 2015; Šimurina et al. 2018). A higher α2,3-linked sialylation and higher bisection of plasma glycoproteins were detected in CD compared with UC (Clerc et al. 2018). Interestingly, increased agalactosylation (loss of a terminal galactose) of IgGs serum levels displayed by patients with CD is correlated with levels of CRP and associated as well with more extensive and progressive disease (Dubé et al. 1990). Thus, IgG Fc-galactosylation seems to be a relevant biomarker for the prognosis of IBD.

Another glycobiomarker for IBD is glycoprotein acetylation (GlycA). Higher complex *N*-glycans expression in acute-phase glycoproteins (such as haptoglobin, α-1-acid glycoprotein, transferrin and α-1-antichymotrypsin) has been associated with worse disease severity in SLE (Connelly et al. 2017; Dierckx et al. 2019).

The exploration of pathogenic role of glycans variation in IBD is also extended to the expression of glycan receptors. Studies demonstrated that differential expression of glycan receptors, such as galectins, play a major influence on the IBD development and the serum galectins can be used as potential biomarkers of IBD and disease activity (Yu et al. 2020). As already mentioned along the chapter, circulating galectins are usually altered in disease context. L-selectin, a cell adhesion molecule of lymphocytes that recognizes endothelial ligands, was found to be increased in serum samples of UC patients compared with healthy controls (Seidelin et al. 1998). Patients with active IBD showed higher serum levels of galectin-1 and -3 comparatively with healthy individuals (Frol'ová et al. 2009). Galectin-1 discriminated IBD from healthy individuals with 71% sensitivity and 87% specificity (Yu et al. 2020); in turn galectin-3 discriminated IBD from healthy controls with 53% sensitivity and 87% specificity (Yu et al. 2020). Hence, galectins might be useful as a powerful biomarker for the diagnosis of IBD.

It has been reported the contribution of genetic variants of key glycoenzymes has an important role in regulating susceptibility and etiopathogenesis in chronic inflammatory diseases (Podolsky 2002; Dias et al. 2018a, b). Recently, a novel genetic risk locus was identified including intronic SNPs in the glycogene MGAT5 that are functionally correlated with glycosylation alterations on T cells and on plasma IgGs and that presented strong association with clinical severity/complication of the UC disease (Dias et al. 2018a, b; Pereira et al. 2020). The rs3814022 and rs4953911 were found to be significantly correlated with lower levels of Fc domain monogalactosylation of IgG2 and IgG3. The rs4953911 was also found to be associated with agalactosylation of IgG1 of which it is associated with induction of a proinflammatory effector functions. Individuals with genetic variations on FUT2 are known to present increased susceptibility to develop IBD (Rausch et al. 2011; Lewis et al. 2015).

Additional biomarkers used into diagnostic of autoimmune and inflammatory disorders are the serum antibodies against glycans (Zhou et al. 2016). Anti-glycoprotein 2, anti-mannobioside carbohydrate IgG (Li et al. 2008; Bogdanos et al. 2011) and anti-*Saccharomyces cerevisiae* are some of the anti-glycan antibodies (Annese et al. 2004) used to perform the diagnosis of IBD and

differentiate UC from CD with a prognostic value (prediction of aggressive disease course). These observations pinpointed the role of glycosylation patterns, such as serum glycome, as a potential diagnostic and prognostic tool among different inflammatory disorders.

Recent discoveries on the role of glycans as a powerful translational therapy have pushed forward the expanding area of glycotherapy to the forefront of in clinical context.

The application of carbohydrate-recognizing receptor inhibitors as a pharmacological tool to block target-pathogenic processes is an example of new generation of therapeutics. Pharmacological blockade of selectins by anti-selectin monoclonal antibodies has gained special interest as a therapy to IBD. Natalizumab (an α4-integrin antagonist) (Gordon et al. 2001, 2002) and vedolizumab (which selectively blocks trafficking of α4β7-positive lymphocytes to the gut) (Feagan et al. 2013; Sandborn et al. 2013) have been clinically applicated to treat IBD. Moreover, the administration of glycoengineered therapeutic monoclonal antibodies, as deglycosylated antibodies (treated with endoglycosidase S), were found to hamper the formation of immune complexes, reducing pathology of disease in case of SLE (Lood et al. 2012).

Interestingly, the potential of glycans supplementation has been described as a promising adjuvant therapy, namely GlcNAc for patients with UC. The metabolic GlcNAc supplementation of mucosal T cells isolated from patients with active UC improved branched *N*-glycosylation on the T cell receptor, consequently controlling T cell activation and function (Dias et al. 2018a, b). Remarkably, a pilot study of oral supplementation with GlcNAc in paediatric IBD reveals a potential role of GlcNAc as a powerful therapeutic agent. More than half of children under GlcNAc treatment exhibited clinical remission with evidence of histological improvement. Moreover, the properties of GlcNAc as a therapy were already verified in a pilot study of paediatric patients with IBD (Salvatore et al. 2000).

13.5 Concluding Remarks/ Conclusion

The relevance of glycans in the study of inflammatory diseases extends from the intrinsic effects on immune cells as well as the correct recognition of danger glycosylation patterns. From immune cells' correct development through the migration and extravasation to the inflamed tissues, glycosylation plays an important role. Moreover, specific glycan switches seem to contribute to the regulation and/or dysregulation of the immune cell response.

Various advances on the field of glycosylation have been contributing to a paradigm shift in the patients' stratification, enabling a personalized medicine through optimized preventive and improving prognostic accuracy in the clinical management of patients. Moreover, the identification of glycosylation unbalance into inflammatory diseases' pathophysiology constitutes an opportunity to improve the target-specific therapy of patients without side effects and with a low cost.

Compliance with Ethical Standards

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