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Dynamics and functions of *N*-glycans in T cell development

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Dynamics and functions of *N*-glycans in T cell development

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Declaration

The author of this thesis declares that, in accordance with ”nº 2, alínea a) do artigo 31º do Decreto-Lei nº 115/2013 de 7 de Agosto”, afforded a major contribution to the design and technical execution of the work, interpretation of the results and manuscript preparation resulting in the following accepted and submitted articles:

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Review articles

- Fernandes, Â., Azevedo, C. M., Silva, M. C., Faria, G., Dantas, C. S., **Vicente, M. M.**, & Pinho, S. S. (2022). Glycans as shapers of tumour microenvironment: A sweet driver of t-cell-mediated anti-tumour immune response. *Immunology*, *168*(2), 217–232. https://doi.org/10.1111/imm.13494 **[Appendix II]**
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Opinion articles

- **Vicente, M. M.**, Gomes, E. & Pinho, S. S. (2023) Leveraging the dynamics of glycans along T and B cell development in defining basic mechanisms of immunity with clinical applications. *Trends in Immunology* (submitted following invitation) **[Chapter 1.2]**

Book chapters

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Initial stages of modern biomedical research in the 20th century included interest and developments regarding all major classes of biomolecules: nucleic acids, proteins, lipids and carbohydrates (or glycans). The diversity and complexity found in the latter set of molecules, as well as technical hurdles for their characterization, effectively cause the underappreciation of glycans in life sciences. I would like to acknowledge the work of Glycobiologists and Glycoimmunologists that, despite the difficulties, managed to push our understanding forwards highlighting the potential of this field. I truly feel that I stand in the shoulders of giants.

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"The difficult we do right away, the impossible takes a little longer."

- Mario Capecchi

RESUMO

O desenvolvimento de células T assegura a formação de repertórios de recetores de células T, que reconhecem a variedade de antigénios apresentados durante a vida de um organismo. A glicosilação é uma modificação pós-translacional importante, que ocorre em virtualmente em todos os tipos celulares, incluindo em linfócitos, regulando a atividade celular e sendo um elemento crucial na interface com o ambiente extracelular. O conjunto de evidências sobre o papel dos glicanos no desenvolvimento de células T destaca as principais contribuições destas estruturas nos estádios de desenvolvimento. No entanto, acentua o elevado grau de complexidade e a escassez no entendimento deste assunto. Nesta tese de doutoramento, o nosso objectivo era compreender a contribuição dos glicanos na formação de células T maturas. Aqui, descobrimos assinaturas especificas da composição de glicanos nos diferentes estadios do desenvolvimento de células T, em timócitos humanos e de murganhos, bem como alterações dinâmicas ao longo do desenvolvimento. No sentido de abordar o paper dos *N*-glicanos no desenvolvimento de células T, gerámos novos modelos de murganho, recorrendo à glicoengenharia de timócitos em fases iniciais de desenvolvimento, com o bloqueio da via de *N*-glicosilação. Utilizando estas linhas, mostrámos alterações significativas em estadios de controlo do desenvolvimento, incluindo seleção ß, formação de células T reguladoras e desenvolvimento de células T $\gamma\delta$. Verificámos também a associação destas perturbações a uma maior suscetibilidade à inflamação no cólon e nos rins, e também em contexto de infeção. Em conclusão, revelámos que o bloqueio da via de *N*-glicosilação condiciona o desenvolvimento de células T, resultando maior suscetibilidade à inflamação.

Como o trabalho de doutoramento foi realizado entre 2018 e 2023, foi influenciado pela pandemia COVID-19, sendo um produto do seu tempo. Face à necessidade de uma maior compreensão do novo vírus SARS-CoV-2 e das respostas imunitárias à sua infeção, acrescentámos nesta tese o objectivo de melhor compreender o papel dos glicanos nas respostas imunitárias adaptativas à infeção pelo SARS-CoV-2. Enfrentámos esta questão em duas direcções, descrevendo o perfil de glicanos em células T e imunoglobulinas circulantes em doentes com COVID-19, ao diagnóstico, de forma a estudar respostas imediatas à infeção. De facto, observámos perfis diferenciais de glicanos em células T circulantes em doentes assintomáticos quando comparados com não infetados e sintomáticos, impulsionados por fatores serológicos. Finalmente, revelámos o potencial dos *N*-glicanos de imunoglobulinas como preditores da progressão da doença.

Em suma, o trabalho aqui apresentado mostra o potencial dos glicanos na regulação do desenvolvimento e das respostas das células imunitárias.

ABSTRACT

T cell development ensures the formation of diverse repertoires of T cell receptors, that recognize a variety of antigens. Glycosylation is a major post-translational modification present in virtually all cells, including lymphocytes, regulating cellular activity and being a crucial element in the interface with the extra-cellular environment. The body of evidence on the role of glycans in T cell development highlights major contributions of these structures in developmental checkpoints, but also discloses the high degree of complexity and how little is known on this subject. In this thesis, we aimed to understand the contribution of glycans in the formation of mature T cells. Here, we discovered stage-specific glycome compositions along T cell development in human and murine thymocytes, as well as dynamic alterations across developmental progression. In order to specifically address the role of *N*-glycans in T cell development, we generated novel glycoengineered murine models of early developmental stage thymocyte-specific *N*-glycosylation pathway blockade. Using these lines we showed remarkable defects in key developmental checkpoints, including ß-selection, regulatory T cell generation and $\gamma\delta$ T cell development, associated with increased susceptibility to colon and kidney inflammation, and infection. In conclusion, we revealed that *N*-glycosylation blockade renders a dysregulation in T cell development, associated with inflammation susceptibility.

As the doctoral work was performed between 2018 and 2023, it was influenced by the COVID-19 pandemic, actually being of product of its time. To answer the worldwide call for for the understanding of the new SARS-CoV-2 virus and the human immune responses to its infection, we added the aim to contribute to the understanding of the role of glycans in adaptive immune responses to SARS-CoV-2 infection. We tackled this challenge in two directions, describing the glycan profile of circulating T cells and immunoglobulins in COVID-19 patients, at diagnosis, in order to study immediate responses to the infection. In fact, we observed differential glycan profiles in circulating T cells in asymptomatic patients, when compared to non-infected and symptomatic ones, which was driven by serological factors. Finally, we disclosed the potential of immunoglobulin *N*-glycans as disease progression predictors.

Altogether, the work here presented showcases the potential of glycans in the regulation of immune cell development and responses.

ABBREVIATIONS

ACPA – anti-citrullinated protein antibody

APC – antigen-presenting cell

Asn – asparagine

ATAC – assay for transposase accessible chromatin

B-ALL – B cell precursor ALL

B-CLL – B cell chronic lymphocytic leukemia

BCR – B cell receptor

CDG – congenital disorder of glycosylation

CLP – common lymphocyte progenitor

CMAS – CMP-sialic acid synthase

CSR – class switch recombination

DN – double negative

DNA – deoxyribonucleic acid

EGF – epidermal growth factor

ER – edoplasmic reticulum

ETP – early thymocyte progenitor

Fc – crystallizable fragment

FcγR – Fc γ-receptors

Fuc – fucose

FUT4 – fucosyltransferase 4

FUT7 – fucosyltransferase 7

Gal – galactose

Gal-2 – galectin-2

Gal-3 – galectin-3

Gal-8 – galectin-8

GalNAc – *N*-acetylgalactosamine

GBP – glycan-binding protein

GC – germinal center

GFAT1 – glutamine-fructose-6-phosphate transaminase 1

Glc – glucose

GlcNAc – *N*-acetylglucosamine

GnT-I – *N*-acetylglycosaminyltransferase I

GnT-II – *N*-acetylglycosaminyltransferase II

GnT-IV – *N*-acetylglycosaminyltransferase IV

GnT-V – *N*-acetylglycosaminyltransferase V

HSC – hematopoietic stem cell

IBD – inflammatory bowel disease

IgG – immunoglobulin G

IL-10 – interleukin 10

IL-2 – interleukin 2

iTreg – induced regulatory T cell

LacNAc – *N*-acetyllactosamine

Man – mannose

MHC – major histocompatibility complex

MPP – multipotent progenitor

MS – mass spectrometry

NeuAc – *N*-acetylneuraminic acid

OGA – *O*-GlcNAcase

OGT – *O*-linked *N*-acetylglucosamine transferase

PHA – *Phaseolus vulgaris* haemagglutinin

PSGL-1 – P-selectin glycoprotein ligand-1

RAG – recombination-activation genes

RNA – ribonucleic acid

Ser – serine

SHM – somatic hypermutation

Sia – sialic acid

SP – single positive

ST3GAL4 – ST3 ß-galactoside alpha-2,3-sialyltransferase 4

Tconv – conventional T cell

TCR – T cell receptor

Th17 – helper-17 T cell

Thr – threonine

Treg – regulatory T cell

TSP – thymic seeding progenitor

VCAM – vascular cell adhesion molecule

VDG – variable domain glycan

VLA – very late antigen

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CHAPTER 1

GENERAL INTRODUCTION
EXTENDED THESIS SYNOPSIS
AIMS AND THESIS OUTLINE

CHAPTER 1.1

GENERAL INTRODUCTION

Fundamental Principles of Glycobiology

Initial stages of modern biomedical research in the 20th century included interest and developments regarding all major classes of biomolecules: nucleic acids, proteins, lipids and carbohydrates (or glycans). The diversity and complexity found in glycan molecules, as well as technical hurdles for their characterization, effectively cause the underappreciation of glycans in life sciences. Glycans form the outermost structure of cells, being vital for communication interfaces. Notably, the glycome integrates all levels of biological information from genome to metabolome.

From the 1930 Nobel Prize in Physiology and Medicine, awarded to Karl Landseiner for the discovery of human blood groups, to the 2022 Nobel Prize in Chemistry, awarded to Carolyn Bertozzi for the development of bioorthogonal chemistry, the field of Glycobiology, the study of glycans, has proven the major impact in biological systems of such structures.

Glycosylation is a major post-translational mechanism characterized by the enzymatic addition of carbohydrate structures (glycans) to proteins and lipids of essentially all living cells [1]. Glycans play a pivotal role at the molecular level, by regulating protein function and stability, and also at the cellular level, by influencing cellular interactions with the extracellular environment [2]. Being composed of a set of several monosaccharides, glycans can be assembled into structures with different degrees of complexity. These molecules are usually composed of six carbon atoms [3]. Many monosaccharides are derived from glucose (Glc) by single epimerization (a change in the stereochemical configuration of a single carbon atom in a sugar), substitution or addition/loss. Exemplarily, mannose (Man) is derived from the epimerization at the C2 position, while epimerization at C4 position yields galactose (Gal). A substitution of the 2-hydroxyl group of Glc or Gal for an acetylated amino group gives rise to *N*-acetylglucosamine (GlcNAc) or *N*-acetylgalactosamine (GalNAc), respectively, while the loss of the 6-hydrozyl group generates fucose (Fuc). *N*-acetylneuraminic acid (NeuAc), or sialic acid (Sia) is a nine-carbon sugar acid that is usually found in a six-member ring configuration.

Complex structures resulting by different linkages of these monosaccharides generate glycans, which are predominantly linked to proteins via nitrogen (*N*) and oxygen (*O*) linkages, forming *N*- or *O*-glycans, respectively. This process occurs in the Endoplasmic Reticulum (ER)/Golgi compartment of essentially all cells, being mediated by the coordinated action of a portfolio of different glycosyltransferase and glycosidase enzymes [1]. *O*-glycosylation consists in the addition of a sugar molecule to the oxygen atom of serine (Ser) or threonine (Thr) residues in a protein. Several types of *O*-glycosylation exist, depending on the monosaccharides that are first attached to the protein and the type of extension that occurs, being GalNAc and GlcNAc the most common [4]. Interestingly, *O*-GlcNAc is the only glycosylation modification that occurs in intracellular proteins, competing with protein phosphorylation for the Ser and Thr substrates [5].

Focusing on the *N*-glycosylation pathway, glycans are attached to nitrogen atoms in asparagine (Asn) residues, in Asn-X-Ser/Thr consensus sequences (X being any amino acid except proline), and can originate several glycan types: high mannose, hybrid and complex. In this pathway, a pre-assembled glycan composed of GlcNAc, glucose and mannose monosaccharides is transferred into the nascent peptide, in the luminal side of the ER. There, following the attachment of the $Glc_3Man_9GlcNAc_2$ glycan, processing by glucosidase enzymes and transfer to the Golgi occurs. There, terminal mannose residues are removed by mannosidases, and the $Man_5GlcNAc_2$ glycan is formed (high-mannose glycan) [1].

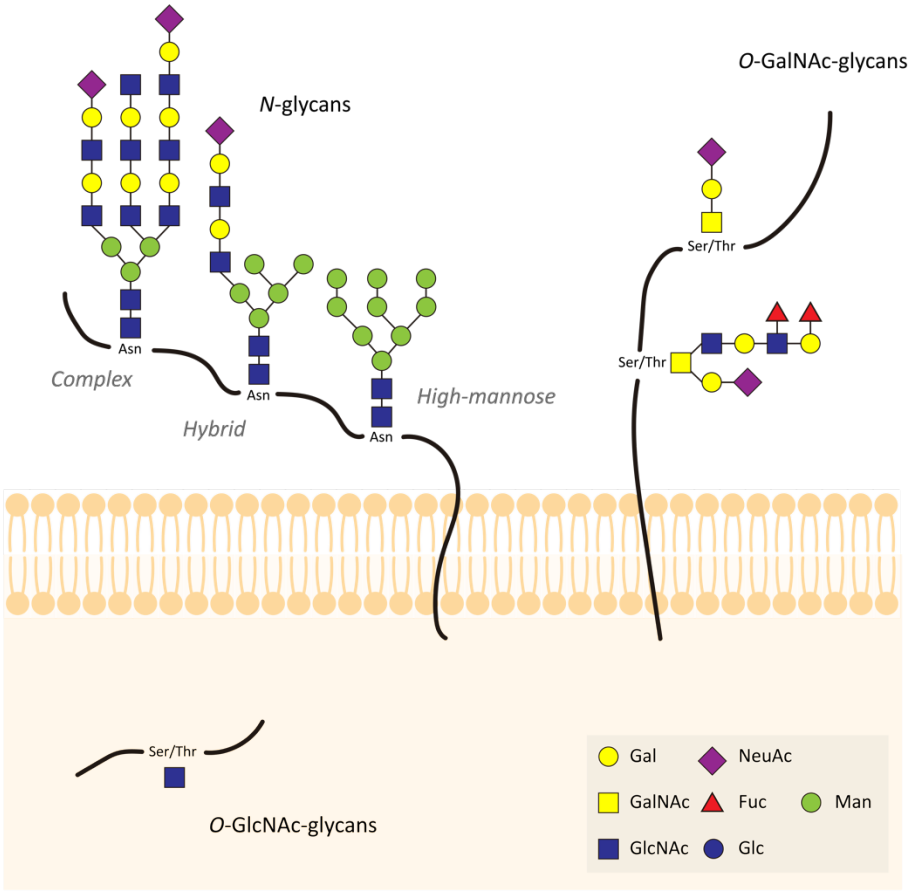


Figure 1: Schematic representation of the major protein glycosylation types. Adapted from [6].

The nomenclature of hybrid *vs*. complex *N*-glycans depends on the number of GlcNAc antennae present in the glycan structure. The first GlcNAc antenna is added by the *N*-acetylglycosaminyltransferase I (GnT-I), encoded by the *Mgat1* gene, which adds a GlcNAc monosaccharide in a ß1,2 linkage to one of the core Man residues. The resulting glycan, with one GlcNAc antenna, is termed hybrid *N*-glycan. Only once this occurs, the glycan structure is further processed into $GlcNAcMan_3GlcNAc_2$ and used by the GnT-II, encoded by the *Mgat2* gene, to generate $GlcNAc_2Man_3GlcNAc_2$. Additional GlcNAc branches may be initiated by GnT-IV and GnT-V, giving rise to complex *N*-glycans. The several GlcNAc branches can be maturated with the addition of galactose residues, forming the "building block" Galß1-4GlcNAc repeat (also termed *N*-acetyllactosamine, or LacNAc). Finally, *N*-glycan elongation may be interrupted by the addition of a sialic acid or fucose monosaccharide. Placed together, the sequential building of *N*-glycans render a vast repertoire of structures, possibly differing in antenna number, elongation and termination composition [1]. These concepts are further discussed in **Chapters 1.2**, **2** and **3**, in the context of immune cell features.

The rise of glycans in modern Immunology

The beginning of the Cellular Immunology era, with the enabling of leukocyte manipulation *in vitro*, was associated with Peter Nowell's landmark work entitled "*Phytohemagglutinin: An Initiator of Mitosis in Cultures of Normal Human Leukocytes*" [7]. Being a cancer cytogeneticist, Nowell was focused on the study of chromosomal aberrations found in malignant cells, having later discovered the Philadelphia chromosome, in chronic myelogenous leukemia patient samples [8]. Related to his research on leukemia, Nowel isolated leukocytes from healthy individuals, in an experiment. Plant extracts (containing lectins, proteins with carbohydrate specificity) were used to agglutinate erythrocytes from blood samples since the 50's [9], to isolate leukocytes. Serendipitously, Peter Nowel added *Phaseolus vulgaris* (red kidney bean) haemagglutinin (PHA) to the healthy control blood samples and placed them in culture, without finishing the protocol (isolation of leukocytes). Surprisingly, three days later Nowel found mitotic cells (later known to be lymphocytes) and suggested PHA to be a mitogen of leukocytes, possibly acting on the cellular surface [7]. Later in the 80's, PHA was shown to bind the human T cell receptor (TCR) [10], CD2 [11], and CD45 receptors [12], all contributing to the clustering of the TCR-associated protein complex, thus inducing T cell activation [13]. Contemporary to these findings was the discovery of the carbohydrate specificity of PHA lectins (L-PHA and E-PHA), to different linkages of GlcNAc monosaccharides [14]. The information of the target receptors combined with the carbohydrate specificity of lectins (PHA and others), revealed the degree of complexity in the mitogenic activity of these plant proteins. Altogether, the "omnipresent" contribution of protein carbohydrates to the birth of modern Immunology is clear.

Glycans in T cell function

The adaptive immune response relies on the ability to differentiate self-antigens from pathogen-derived ones and then present these foreign antigens to T cells. This process is orchestrated by antigen-presenting cells (APCs), which display antigens attached to major histocompatibility complexes (MHCs), before analyzed by T cells. Virtually all cell types display surface glycans, and T cells are no exception [15]. As mentioned above, mitogenic activity of lectins is observed in T cells due to surface glycans in these cells. Over the past two decades, several bodies of work have unequivocally shown how critical are glycome alterations in regulating adaptive immunity and T cells responses (reviewed in Pereira *et al*. 2018) [16]. The cellular glycome signature can be decoded and read by complementary

glycan-binding proteins (GBPs) that translate cell-extrinsic glycan recognition into function. Nowhere is this more evident than in the immune system. Galectins, C-type lectins (including selectins) and Siglecs are the classical examples of GBPs through which glycans control immunity. Specifically, galectins usually bind LacNAc and tolerate α2,3-Sia termination [17], and have been shown to play a key role in the regulation of T cell function. In a pivotal study from Demetriou *et al*. it was shown that ß1,6-GlcNAc branched *N*-glycans (GnT-V driven GlcNAc antenna) present in T cell receptors (TCR) are recognized by galectin-3 (Gal-3). Furthermore, *Mgat5*$^{-/-}$ T cells, where the ß1,6-GlcNAc *N*-glycan antenna is absent, were shown to possess decreased thresholds of TCR-mediated activation, due to increased TCR clustering in the absence of Gal-3-mediated distancing [18]. Decreased levels of ß1,6-GlcNAc branched *N*-glycans have been found in Inflammatory Bowel disease (IBD) patients, which correlates to chronic inflammation phenotypes [19, 20]. In a chronic inflammation context, Smith *et al*. showed a regulatory loop of CD8 T cell activity by interleukin 10 (IL-10), where the *Mgat5* expression was upregulated upon IL-10 signaling, thus controlling cellular activity [21]. Related to the influence of galectin binding in T cell function, Gal-2 exhibits a suppressive effect by inducing apoptosis of gut-resident T cells colitis in murine models [22]. In contrast, Gal-8 can act as a promoter of T cell activation in the immune synapse [23]. It is now clear that with alterations in activity, T cell glycans are involved in the cellular status shift. Further studies, with in-depth glycome characterization and manipulation, in the context of T cell responses, are needed to uncover the degree of complexity of such changes.

The T cell cellular pool contains several subsets. One of the features responsible for cellular identity, although underrecognized, is the glycome. Regarding T cell identity, Toscano *et al*. described differential binding of Gal-1 to Th1, Th2 and Th17 cellular states. In fact, Gal-1 binding to Th1 and Th17 cells led to the induction of apoptosis, whereas Th2 ones were not Gal-1 targets, therefore, Gal-1-mediated apoptosis resistant. It was demonstrated that Th2 cells, and not Th1 and Th17, contained terminating α2,6-Sia, which is known to inhibit galectin binding [24]. Interestingly, Cabral *et al*. highlighted the differential branching *N*-glycan signature of regulatory T cells (Tregs) in comparison with conventional ones (Tconvs). In fact, Tregs display higher levels of surface complex *N*-glycans, when compared to Tconvs [25]. The relationship between cellular identity mechanisms and glycans is far from being understood. One key example is the underappreciation of glycans across T cell development.

Common lymphocyte progenitors (CLPs) are generated from a subset of dividing hematopoietic stem cells (HSCs) in the bone-marrow [26]. These hematopoietic progenitors

egress the bone-marrow into the blood stream and reach the thymus, in order to develop into early thymocyte progenitors (ETPs). In the thymus, a T cell development-favorable microenvironment is set, so thymocytes go through developmental checkpoints with the final goal to generate a mature T cell pool with a diverse TCR repertoire [27]. In **Chapter 1.2**, the mechanisms of T cell development are described, and the current knowledge regarding the role of glycans in each one is discussed. Moreover, in **Chapter 2** the role of *N*-glycans in T cell development is addressed, namely their contribution in the key developmental checkpoints.

As already mentioned, glycans have been shown to play crucial roles in T cell biology, both in homeostasis and disease. In fact, inflammatory bowel disease patients showed decreased levels of ß1,6-GlcNAc *N*-glycan branching in T cells, that was associated with mucosal T cell hyperactivation and disease severity [15]. Interestingly, the increase of branched *N*-glycans on CD25 has been associated with increased cell surface retention with impact in the regulation of T differentiation and immune tolerance. In fact, reduced *N*-glycan branching led to a decrease of CD25 surface retention and IL-2 signaling, which promotes T helper-17 (Th17) over induced regulatory T cell (iTreg) differentiation [28]. In **Chapter 1.2** it is also discussed how the influence of glycans in T cell development mediates disease susceptibility and peripheral T cell function. Finally, in **Chapter 3**, the glycome alterations in circulating T cells from SARS-CoV-2 infected individuals, at diagnosis, is shown.

Glycans in IgG function

B cells also develop from CLPs, but from the progenitors that remained in the bone-marrow. This cell type has a major role in adaptive immunity, specifically in the recognition of foreign antigens and in the humoral immune response [29]. B cell lymphocytes are 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 peripheral immune organs [30]. In **Chapter 1.2**, the contribution of glycans in B cell development is described and, as in T cell development, the fact that the glycome of developing cells accompanies the progression of development checkpoints is discussed.

One of the main features of adaptive immunity is how the immune system can produce molecules that recognize pathogens with high affinity. These proteins, termed antibodies are produced by B cells. Antibodies play a central role in humoral immunity against pathogens, as they are able to mediate immune responses to viral infections, by the

recognition of specific foreign antigens. Immunoglobulin G (IgG) antibodies are the prevalent in circulation, having a major role in systemic immunity, triggering leukocyte activation and inflammation [31]. Being a glycoprotein, human and murine IgGs are comprised of multiple glycoforms, owing to the addition of a diverse type of glycans structures in the IgG Fc (crystallizable fragment) region [32]. In fact, all IgG Fc domains contain a single, highly conserved, glycosylation site in Asn297 that carries a singly complex biantennary *N*-glycan. As it sits in the IgG Fc, a conserved domain responsible for modulating cellular response through interaction with Fc γ-receptors (FcγRs) in effector immune cells, the IgG Fc *N*-glycans mediate Fc receptor binding [33].

As expected, the glycan structures found in IgG Fc are diverse, with over 30 variants found in healthy individuals [34]. Interestingly, the glycan structure attached to the IgG Fc can instruct the opposite pro- and anti-inflammatory responses (binding to effector or inhibitory FcγRs). Increased levels of fucosylation of IgG are known to increase affinity to FcγRIIIa, promotin antibody-dependent cell-mediated cytotoxicity [33, 35]. Terminal Sia also modulates FcγR binding. In fact, α2,6-Sia on the Fc glycan significantly reduces FcγR affinity being associated with anti-inflammatory activity [34].

The IgG Fc *N*-glycome has been shown to be associated to with several diseases, such as rheumatoid arthritis [35], IBD [36] and type 2 diabetes [37]. In **Chapter 4**, the work on the association of circulating IgG *N*-glycome in COVID-19 is presented. Much like in steaty-state and other inflammatory diseases, the signature of IgG *N*-glycans is dynamic and correlates to overall inflammatory status.

The **Chapters 2**, **3** and **4** contain introductory sections to the specific glycosylation aspects of each manuscript, with more details on specific glycans and their functions in adaptive immunity.

In **Chapter 1.2** we provide an extended thesis synopsis on the mechanisms of T and B cell development and how glycans influence these processes. Moreover, we discuss how the influence of glycans in lymphocyte development is translated into disease susceptibility.

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CHAPTER 1.2

[OPINION PAPER]

EXTENDED THESIS SYNOPSIS - LEVERAGING THE DYNAMICS OF GLYCANS ALONG T AND B CELL DEVELOPMENT IN DEFINING BASIC MECHANISMS OF IMMUNITY WITH CLINICAL APPLICATIONS

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Leveraging the dynamics of glycans along T and B cell development in defining basic mechanisms of immunity with clinical applications

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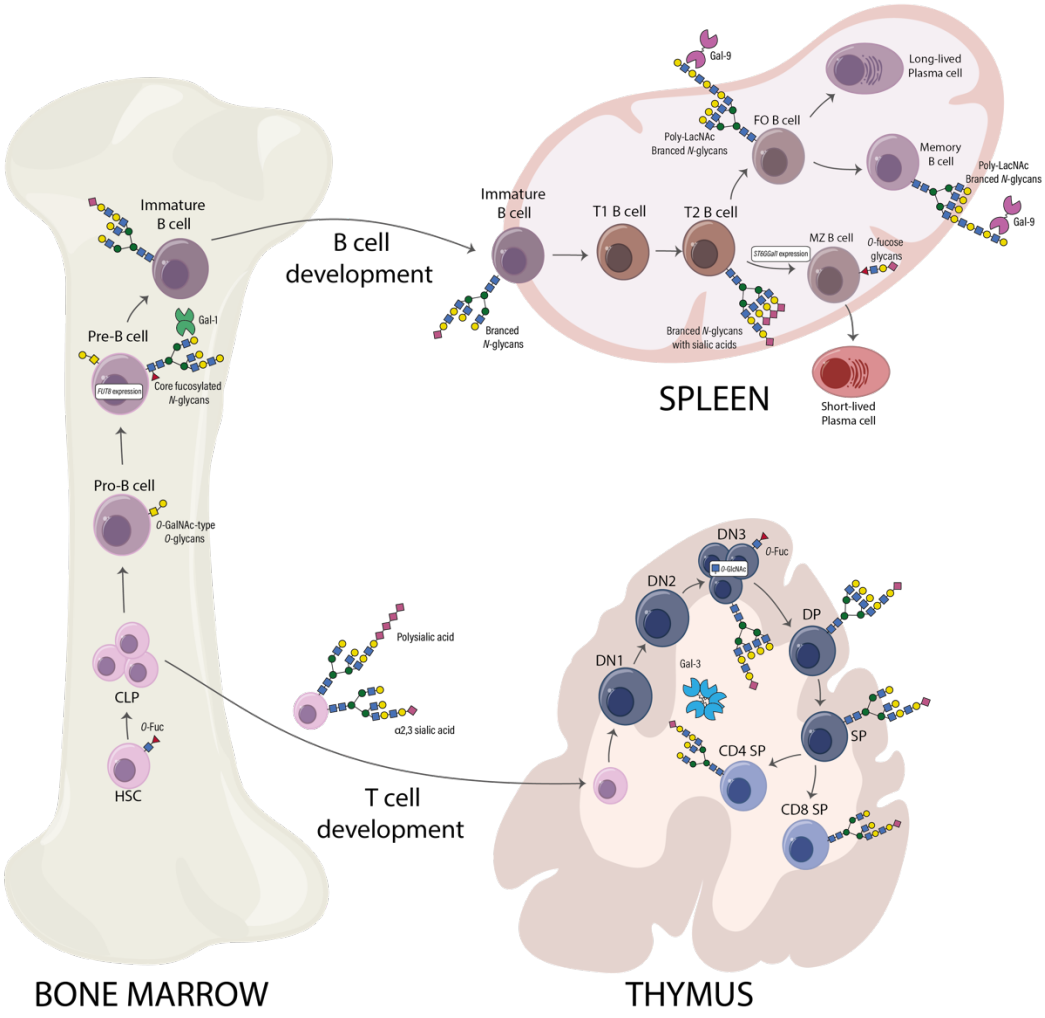
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Abstract

Glycans cover the surfaces of all mammalian cells through a process called glycosylation. Nearly all proteins and receptors that integrate the intricate series of co-stimulatory and co-inhibitory pathways of the immune system are glycosylated. There is growing evidence that the development of the immune system at the origins of T and B cell development is tightly regulated by glycosylation. In this *Opinion* we hypothesize the novel notion that the glycome of developing T and B cells is developmentally regulated, and will discuss how these molecules play fundamental roles in lymphocyte development. Moreover, we will address how glycans early define lymphocyte functionality in the multiple aspects of adaptive immunity.



Cracking the sugar code of T and B cells.

Glycans, or carbohydrates, are present in virtually all cellular surfaces, being important regulators of the immune system [1]. Not surprisingly, T and B cells contain a dense coat of surface glycans (glycocalyx) that tightly regulate their activity and function [2-5]. Glycosylation is the enzymatic pathway responsible for the attachment of glycans to proteins/lipids by a portfolio of glycosyltransferases and glycosidases enzymes [6]. The common classes of glycans are defined according to the nature of the linkage to the protein or lipid. A glycoprotein is a glycoconjugate in which a protein carries one or more glycans covalently attached to a polypeptide backbone, usually via *N*- (giving rise to *N*-glycans) or *O*-linkages (giving rise to *O*-glycans). These structures are usually built in a step-wise manner, including glycan extension/elongation and termination by the addition of sialic acid, fucose, galactose or *N*-acetylglucosamine (GlcNAc) monosaccharides. The composition of glycans structures within a certain cell or tissue is the so-called glycome that contains a huge amount of important biological information.

Glycans have been shown to be essential regulators of T and B cell activity and function [3, 7]. However, the fundamental role of glycans in immunity is broader and vast, spanning from its impact in early stages of T and B cell development, in differentiation, selection and in defining the pro- or anti-inflammatory properties of T cells and also the B cell production of antibodies.

In fact, protein glycosylation has the potential to take part in the majority of cellular processes involved in both humoral and adaptive immunity, by regulating immune cell-fate decisions, activity, function, but also in the early developmental processes in the thymus and bone marrow. In fact, there is evidence suggesting that the glycome composition of T and B cells is dynamic across developmental stages. Here, we hypothesize that the glycome alterations across development of lymphocytes developmentally regulated.

Based on the findings revealed so far, this regulation of glycan signatures leads to normal developmental transitions that, in the case of T and B cells, ensure proficient cellular populations to drive adaptive and humoral immunity. In the next sections, we will gather relevant pieces of work that support the view that the developmental regulation of glycans ensure the generation of proficient mature T and B cells in homeostasis, further discussing the consequences of a glycan switch in T and B cell development and function and its implications in diseases. We will present a guided tour through each developmental step of T and B cell development, highlighting the prominent role of glycans at the origins of humoral and adaptive immunity. Taken together, the time is now to crack the glycome code of T and B cells in immunity, which certainly will give rise to a tremendous amount of novel biological and mechanistic information. This will further lead to understanding on when, why and how glycosylation impacts basic mechanism of immunity with consequences on the development of major immunological diseases, including infection, inflammation, autoimmunity and cancer.

The step-wise regulatory functions of glycans along T cell development

T cells are one of the key players of the adaptive immune response, controlling the multiple insults simultaneously throughout the body and maintaining immune homeostasis. 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 [9].

Glycans in thymus seeding

The majority of hematopoietic cell lineages develop in the bone marrow, derived from several differentiation events, sitting on top of the "differentiation tree" the hematopoietic stem cell pool. T cells have a unique environment for development that is set in the thymus. In order to begin T cell development, an hematopoietic progenitor must reach the thymus, egressing from the bone marrow into the blood stream. The cellular subset that represents the supply of progenitors to the thymus is composed by the thymic seeding progenitor (TSP) cells [10]. This cellular pool has been proposed to be composed by multipotent progenitors (MPPs) and common lymphocyte progenitors (CLPs), with variable differentiation kinetics [11]. In order to enter the interaction of P-selectin, a glycan-recognizing receptor expressed by the thymic endothelium, and its ligand, the P-selectin glycoprotein ligand-1 (PSGL-1) [12]. Not surprisingly, the glycosylation profile of PSGL-1 was shown to be essential for its binding to P-selectin and thus the entry into the thymus [13], namely the expression of core 2 *O*-glycan structures [14], α1,3 fucosylated glycans [15] and α2,3 sialylation [16]. In fact, mice lacking FUT4 (fucosyltransferase 4), FUT7 (fucosyltransferase 7), or the ST3GAL4 (ST3 ß-galactoside alpha-2,3-sialyltransferase 4), showed impaired TSPs homing to the thymus, but no alteration in bone marrow engraftment [14, 15]. Moreover, in the alpha-2,8-sialyltransferase sialyltransferase *St8Sia4*$^{-/-}$ mouse model, it was also reported reduced thymic cellularity and deficient thymic immigration by hematopoietic progenitors [17]. This evidence supports the prominent role of glycans in the earliest events of T cell development, such as the thymus seeding.

Glycans in T cell lineage commitment

Once TSPs enter the thymus, they develop into early thymocyte progenitors (ETPs), a subset of the $CD4^{-}CD8^{-}$ double negative (DN) population, identified as $cKit^{+}CD44^{+}CD25^{-}$, which can give rise to multiple hematopoietic lineages [9], including myeloid ones [18]. The Notch signaling pathway is critical for T cell lineage commitment of these immature thymocytes [19]. Notch proteins (NOTCH1-4 in mammals) are evolutionary conserved heterodimeric glycoprotein receptors, which can bind to two families of Notch ligands: Delta family (in mammals: Delta-like [DLL]-1, DLL-3 and DLL4) and the Serrate family (in mammals: Jagged [Jag]-1 and Jag-2). In fact, previous studies on the genetic depletion of the *Notch1* receptor showed that it is required for T cell lineage specification [20]. Notch receptors are covered with a multitude of glycan structures, ranging from *O*-linked Fuc (*O*-fucose), *O*-Glc (*O*-glucose), extracellular *O*-GlcNac (*N*-acetylglucosamine), as well as *N*-glycans [21]. Within these several glycan structures, *O*-Fuc ones in epidermal growth factor-

like (EGF-like) repeats stand out in their regulatory role in membrane trafficking of Notch receptors [22] and in establishing differential affinities for Notch ligands [23]. Interestingly, mice that lack *Pofut1* (a conditional knockout induced by Mx^{Cre}), the fucosyltransferase responsible for the *O*-Fuc addition in the EGF-like repeats of Notch, show increased numbers of B cells in the thymus, and impaired T cell development [24]. These initial Fuc monosaccharides are used by the lunatic, manic, and radical Fringe glycosyltransferases, which transfer *N*-acetylglucosamine (GlcNAc) to *O*-linked fucose glycans, in a ß1,3 linkage [23]. The role of lunatic fringe, coded by the *Lfng* gene, in T cell development, namely in the T cell lineage commitment, has been the focus of several studies. An early report identified high *Lfng* expression in the thymic medulla, not in the cortex, by RNA *in situ* hybridization where the DN populations reside [25]. In a follow-up study, a transgenic murine model where *Lfng* was ectopically expressed in DN thymocytes (under a proximal *Lck* promotor) was generated, and an increase of B cells in the thymus of transgenic mice was observed [20]. Curiously, no major effects were observed in *Lfng*-overexpressing thymocytes. However, it was seen that the increased B cell population derived from TSPs, with no *Lfng* overexpression, as TSPs are not comprised by the proximal *Lck* promotor [26]. This suggested that *Lfng* overexpressing cells inhibited, non-autonomously, the NOTCH1 activation in TSPs, thus inhibiting T cell lineage commitment and promoting ectopic B cell development in the thymus, as the action of lunatic fringe increases the NOTCH1-DLL ligands interaction [27]. Furthermore, it was demonstrated that *Lfng*-overexpressing DP thymocytes blocked the T cell-oriented TSP development, through competition to functionally limiting DLL ligands [28]. Furthermore, a mouse model of triple fringe deficiency was shown to have reduced binding of Notch to DLL ligands, namely DLL-4, altering the frequencies of several T cell subsets in the thymus [29]. This body of work highlight the role of glycans, namely in the NOTCH1 receptor, as master regulators of T cell lineage commitment in the thymic microenvironment.

Glycans in ß-selection

Once DN2 thymocytes commit to the T cell lineage, the progression to the DN3 stage occurs [9], where a decisive developmental checkpoint is placed and glycans have also an important role in this step. Through the action of the RAG (recombination-activation genes) complex, chromosomal rearrangements of the *Tcrb* gene are attempted and, once there is a productive one, it results in the expression of a functional pre-TCR (TCRß chain and pre-Tα) complex [30]. Together with Notch glycoprotein and IL-7, pre-TCR signaling cascades initiate the ß-selection, by inducing the transition to the DN4 stage, with multiple rounds of

cell division, leading to the rise of the most abundant thymocyte population, the $CD4^+CD8^+$ DP thymocytes. We have previously described that a deficiency in the expression of complex branched *N*-glycans, in a mouse model of *Mgat1*-deficiency in DN2 thymocytes and onwards, using a $Rag1^{Cre}$ line, resulted in an abnormal expression of high-mannose glycans that was associated with defects in ß-selection [8]. The deletion of *Mgat1* gene, that encodes the first GlcNAc transferase in the *N*-glycan branches formation, blocks the pathway, only allowing the formation of high mannose glycans. We observed that highly mannosylated DN3 cells had lower levels of CD127 (IL-7 receptor **α** chain) and CD25 (IL-2 receptor), which contributed to defects in the generation of ß-selected DN3 thymocytes. Interestingly, when a second GlcNAc-branch in *N*-glycans is expressed, giving rise to hybrid-type *N*-glycans (modeled in $Mgat2^{fl/fl}Rag1^{Cre}$ deficient mouse) the defects in ß-selection were repaired [8]. Besides branching *N*-glycans, also core-fucosylated *N*-glycans have been described to have a role in ß-selection. The analysis of $Fut8^{-/-}$ mice showed increased DN2 population with a moderate decrease of DN4 thymocyte absolute numbers [31].

The glycome signature can be decoded and read by complementary glycan-binding proteins (GBPs) that translate glycan recognition into function. Nowhere is this more evident than in the immune system. Galectins, C-type lectins (including selectins) and Siglecs are the classical examples of GBPs through which glycans control immunity. Galectins have been shown to play an important role in assuring a proper T cell development. Specifically, in galectin-3 (Gal-3) deficient mice, DN3 cell numbers were found to be reduced, as it was their proliferation rates, indicating a deficiency in ß-selection [32]. The role of glycans in ß-selection, also comes from its relevance in assuring a proper (glyco)metabolism of DN3 thymocytes [33]. It was reported that the import of glucose and glutamine was increased in ß-selected thymocytes, with a parallel increase in protein *O*-GlcNAcylation [34]. This is an unique post-translation modification, where GlcNAc is added to Serines or Threonines of intracellular proteins. Being reversible (OGT, *O*-linked *N*-acetylglucosamine transferase, adds the GlcNAc monosaccharide, and OGA, *O*-GlcNAcase, removes it) and being shown to compete with phosphorylation modifications, it is a major regulator of cellular biology [35]. In fact, in the same study, it was shown that the conditional knockout of *Ogt* in DN4 led to a failure in the clonal expansion of those cells, leading to decreased numbers of DP thymocytes. This suggests that the increase in *O*-GlcNAc levels in ß-selected cells, driven by the increased uptake of glucose and glutamine, promotes c-Myc stabilization, which in turn regulates the nutrient uptake, in a positive feedback loop [34]. The conditional deletion of *Oga* in hematopoietic cells also led to a dramatic decrease in thymocyte numbers [36]. The UDP-GlcNAc metabolite is a common substrate for *O*-GlcNAcylation and the other

glycosylation pathways, as the *N*-glycan branching, of surface and extracellular proteins. Being a product of the hexosamine biosynthetic pathway, its *de novo* synthesis is regulated by a rate-limiting reaction catalyzed by the GFAT1 (glutamine-fructose-6-phosphate transaminase 1) enzyme. Deletion of the *Gfat1* gene in the DN2 stage was recently shown to result in increased presence of mannosylated *N*-glycans and decreased *O*-GlcNAcylation, associated with an increase in DN3 and decreased DP cell numbers, further indicating defects in ß-selected cells´ expansion [37], which is in line with our observations on the "pathogenic" role of mannosylated glycans along T cell development [8].

Glycans in DP selection

The ß-selected DN4 thymocytes undergo several rounds of cellular division, upregulating surface expression of CD8 (found in the transitional population of immature CD8 single positive cells) and finally CD4, reaching the DP stage, where mature TCRs (formed by the TCRß and TCR**α** chains) are generated [38]. The newly formed TCRs are then probed for their recognition potential in two selection steps, by thymic epithelial cells. These cells, as a whole population, can present every peptide in the genome in MHC-I or MHC-II [39]. This feature enables the screening of TCRs by their specificity and binding strength. The first selection step of DP thymocytes is termed "positive selection", where cells that express an **α**ßTCR capable of recognizing peptide/MHC ligands (immunocompetent TCRs) receive survival signals [38]. There, DPs also acquire MHC specificity, thus committing to the CD4 or CD8 SP lineage [40]. In the model of *Gfat1*-deficiency (with increased presence of mannosylated *N*-glycans), in early stages of T cell development, it was observed an overall decrease in TCRß surface expression in thymocytes, which was accompanied by lower levels of surface CD5 (proxy of TCR signaling). Moreover, CD4 and CD8 SP absolute numbers were decreased, showcasing the effect of the UDP-GlcNAc deficiency in DP selection steps [37], which is line with the previous observation by us and others on the role of *N*-glycan branching in DP selection [8, 41]. In addition, the levels of *O*-GlcNAcylation were seen to increase in positively selected cells, and the deletion of *Ogt* in DP thymocytes led to failure in positive selection, with a marked deficiency in mature SP populations generation [34].

The positively selected cells then undergo "negative selection", the second step, where thymocytes that display high levels of TCR signaling are eliminated, due to their autoreactive potential [38]. As the positive and negative selection processes require efficient TCR signaling, specific glycans are involved in this developmental checkpoint, given their role in the regulation of TCR-mediated cellular activation. Core fucosylation of *N*-glycans

were described to be involved in thymocyte maturation. Wildtype DP cells were shown to have high levels of *N*-glycan core fucosylation, which were maintained in SP cells. In the absence of FUT8 (fucosyltransferase 8, responsible for core fucosylation of *N*-glycans), positive selection was impaired and the frequency of mature SP thymocytes was decreased. In fact, TCR signaling was shown to be attenuated in *Fut8*$^{-/-}$ cells, which suggested a role of core-fucosylated *N*-glycans in mediating positive selection [31]. Complex branched *N*-glycans have been also known to regulate TCR clustering, mediating cellular thresholds for activation, as demonstrated by others and us [42, 43]. In fact, it was also shown that branched *N*-glycans regulate the range of TCR signaling strengths in positive selection, through the promotion of CD4 or CD8 surface expression. Moreover, the role of *N*-glycans in the tuning of TCR signaling, by mediating TCR clustering, was also described in the context of negative selection [41]. Both studies were done by using murine models of conditional deletion of *Mgat1* and *Mgat2*. In a recent study, we also observed higher rates of negative selection in *Mgat1*-deficent thymocytes (expressing mannosylated *N*-glycans), as well as defects in TCR repertoire variability in CD4 and CD8 SP cells.

Naturally-occurring Tregs (nTregs) are generated in the thymus, derived from a pool of CD4 SPs with high levels of activation, close to the threshold of negative selection [44]. In agreement with the observation that negative selection is promoted in the absence of branching *N*-glycans [8, 41], we further observed that in the absence of branched *N*-glycans (*Mgat1* deficiency) the generation of T regulatory cells (Tregs) was severely impaired [8]. The role of branched *N*-glycans in the regulation of TCR clustering is also highlighted by the binding TCR glycans to GAL-3 and the formation of the molecular lattices that control the threshold for T cell activation [45]. *Gal-3*$^{-/-}$ mice showed increased levels of apoptosis in DP thymocytes, which supports the importance of galectins in T cell development [42].

O-glycans have also deserved some attention in the field of T cell development. The thymocyte-specific deletion of *Cosmc,* the gene that encodes the C1GALT1C1 chaperone responsible for the stabilization of the C1GALT1 transferase that initiates *O*-glycan extension, only allows the formation of the Tn or STn glycan structures in these cells. When evaluated, these mice showed non-altered T cell developmental dynamics, but reductions in the peripheral compartments [46].

Glycans in thymic egress

Upon survival, selected SP thymocytes egress from the thymus to the periphery, upregulating CD62L (L-selection) and the sphingosine-1-phosphate receptor-1 (S1P1), required for the cells to reach the bloodstream, where the concentration of S1P1-ligand is

higher [47]. Recently, we observed that the perturbation of the branching *N*-glycosylation result in a significant decrease on the frequencies of CD62L-expressing SP thymocytes, in the absence of *Mgat1* [8]. In addition, *Cosmc*-deficient thymocytes, only able to generate truncated *O*-glycans, are able to fulfill thymic developmental processes but fail to populate the periphery, which highlights a potential role of these *O*-glycans in thymic egress [46].

The regulatory functions of glycans along B cell development

B lymphocytes are fundamental players of the adaptive immune system, responsible for the production of the entire repertoire of antibodies with antigen specificity. B cell development is a highly regulated process that takes place in the bone marrow and the spleen [48, 49]. Similarly to 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.

Glycans in Pro-B to pre-B transition

The transition from progenitor B cell (pro-B cell) to precursor B cell (pre-B cell) requires the rearrangement of immunoglobulin (Ig) µ-heavy chain (µHC) resulting in the formation of the pre-B cell receptor (BCR), still with no antigen specificity, which triggers a signaling cascade driving the rapid clonal expansion of pre-B cells. In this very early step of B cell development, glycans have been shown to play an important role. *N*-linked glycosylation on µHC was shown to be essential for pre-BCR assembly [50]. Specifically, core fucosylation, catalyzed by *FUT8* enzyme, plays a fundament role in pre-B cell generation. *Fut8*$^{-/-}$ mice showed abnormalities in pre-B cell generation, suggesting that core fucosylation of µHC mediates the assembly of pre-BCR, affecting the pre-BCR intracellular signaling and proliferation [51]. Additionally, the loss of core fucosylation was shown to impair the interaction between the very late antigen 4 (VLA-4 integrin) on pre-B cells and the vascular cell adhesion molecule 1 (VCAM-1) on stromal cells, leading to a defect on the colony expansion of pre-B cells [52]. The deletion of *Cosmc* in pro-B cells was also shown to reduce the absolute numbers of this population and also of pre-B cells [53]. Importantly, GBPs such as galectins have been demonstrated to regulate B cell development. Galectin-1 was found to be produced by bone marrow stromal cells, interacting with glycosylated forms of pre-B cell integrins, promoting pre-BCR clustering and the further efficient B cell development of normal pre-B cells [54-56]. Moreover, galectin-3 deletion resulted in abnormal levels of several B cell subsets [57]. Galectin-3 was described to act as an

inhibitor of stromal-derived IL-7 and Notch/ Jagged-1 signaling, suggesting that its expression may act on bone marrow stromal cells controlling B cell development [57].

Glycans on immature B cell generation and selection

After clonal expansion of pre-B cells, signaling activation of the pre-BCR leads to the rearrangement of Ig μ-light chain (μLC) to form a mature BCR that progresses into immature B cells [58]. Immature B cells enter into a selection process in the bone marrow to assess their autoreactive potential. If BCR binds a self-antigen, immature B cells die by negative selection, whereas cells that display a tolerant BCR are positively selected, which lead to central B cell tolerance [59]. It was demonstrated that *N*-glycan branching is required for B cell selection. Using a model of B cell-specific deletion of the *Mgat1* gene, that renders branched *N*-glycan deficiency in those cells, a decreased surface expression of the BCR co-receptor CD19 was observed, inhibiting positive selection. Moreover, the nerve growth factor IB (Nur77, related with B cell activation) was shown to be upregulated in immature B cells in the absence of branched *N*-glycans, indicating that complex N-glycans play a role in B cell threshold establishment, similarly to T cells [60]. In a B cell specific *Cosmc* deficient model, where *O*-glycans are truncated, pro- and pre-B cells were decreased and an increase in immature B cells was observed in the bone marrow, possibly related to the defects in B cell peripheral homing observed in these mice [53].

Glycans in bone marrow egress and B cell peripheral maturation

After leaving the bone marrow, immature B cells will reach the spleen, where they mature in the so-called transitional stages (T1 and T2) until reaching the mature B cell stage [61]. Again, the role of branched *N*-glycan in these fundamental steps of B cell development was found to be essential for the survival of T2 transitional peripheral B cells, but not mature B cells. Branched *N*-glycans were shown to be important in the maintenance of an homeostatic pre-BCR/BCR signaling threshold boundaries, to allow positive selection by inhibiting both death by neglect and negative selection of the B cells [60].

Sialic acids have also a major impact on B lymphocytes function. In fact, blocking the synthesis of these terminal carbohydrate structures specifically on B cells through the knockout of the nuclear enzyme CMP-sialic acid synthase (CMAS), resulted in a remarkable reduction of immature B cells and almost the complete absence of mature B cells triggered by an apoptosis-mediated mechanism due to absence of sialic acid [62]. Additionally, the sialyltransferase ST6Gal1, which generates α2,6-linked sialic acids in glycoconjugates, was

shown to be important as a pro-survival factor guiding B cell development [63]. It was observed that B cell non-autonomous ST6Gal1 contributes to the development of early transitional B cells. In fact, B cells depend on two central survival signals, the BCR and the survival cytokine BAFF [64, 65]. Sialylation, mediated by ST6Gal1, was shown to increase transitional B cells, upregulate BAFF and BCR-mediated pro-survival signaling, and consequently the survival of B cells after negative selection [63].

In the spleen, mature or naïve B cells commit into marginal zone (MZ) or follicular (FO) B cells before they differentiate into memory B cells or plasma cells upon antigen activation [66]. B cell intrinsic ST6Gal1 is required for MZ B cell development [63]. Furthermore, sialic-binding immunoglobulin-type lectin (siglec), CD22, a B cell inhibitory co-receptor that binds to sialic acids, either in *cis* or *trans* linkage, has shown to influence the amount of MZ B cell population in the spleen, through the regulation of B-cell Ca_2^+ signaling [67]. *O*-fucose glycans are also critical for the efficient binding of Notch receptors with their canonical Notch ligands. Deletion of *Pofut1* gives rise to aberrant MZ B cell formation, myeloid hyperplasia, and thymic hypoplasia [24].

Glycans in mature B cell activation and Germinal Center formation

Once on the lymphoid organs, mature naïve B cells will recognize an antigen and will become activated. This antigen-induced B cell activation and differentiation is mediated by dynamic changes in gene expression that give rise to the germinal center (GC) reaction. The GC is characterized by clonal expansion, class switch recombination (CSR), somatic hypermutation (SHM), and selection for increased affinity of a BCR for its unique antigenic epitope. During B cell activation and differentiation, glycosylation has a major role. Naïve and memory B cells were reported to express branched *N*-glycans at the cell surface, elongated with poly-*N*-acetyllactosamine (poly-LacNAc) structures, that serves as high affinity binding sites to galectins such as galectin-9 [68]. Galectin-9 binding was described to regulate BCR signaling, antagonizing naïve B cell activation [68]. In homeostasis, the B cell differentiation into memory B cells or plasma cells is also accompanied by changes in *O*-glycosylation, characterized by a significant loss of *O*-glycan structures, including loss of extended core 1 and core 2 *O*-glycans, regulated by ST3Gal1 and GCNT1 enzymes [69]. Taken together, the role of glycans along B cell development is far from being completely elucidated having the potential to unlock novel mechanisms underlying humoral immune response, that can be leveraged towards further understanding of the functional outcomes in terms of disease development.

Alterations in the glycosylation pathway along T and B cell development: impact in disease susceptibility

Defects in the development of T and B cells have been related to several human diseases. As both processes entail chromosomal rearrangements and/or the induction of genomic mutations, in combination with failed developmental checkpoints, the risk of malignancy-driving events is quite high. In fact, leukemogenesis can be promoted upon genetic mutations on *NOTCH1* gene, for instance, which can trigger differential receptor-ligand interactions or constitutive signaling [9]. Changes in the glycosylation profile of lymphocytic pre-malignant cells may promote cellular transformation, as it occurs in other cancer types [70]. Interestingly, chronic lymphocytic leukemia patients were shown to present overexpression of *GALNT11* enzyme (polypeptide *N*-acetylgalactosaminyltransferase 11, one of the responsible enzymes for the addition of the first GalNAc in *O*-glycosylation), giving rise to increased expression of the Tn glyco-antigen [71]. Moreover, in T cell acute lymphoblastic leukemia (T-ALL) patients, it was observed an increase in *POGLUT1* (O-glycosyltransferase 1, responsible for *O*-glucose of NOTCH1), when compared to healthy controls [72]. In a murine model of T-ALL, with a *Pten* (gene encoding an important negative regulator of cell proliferation [73]) deletion in a *Ogt*-deficient background in DN2 cells, the essential role for *O*-GlcNAcylation was observed, as in *Ogt*-deficient thymocytes malignant transformation was decreased [34]. In B-cell precursor ALL (B-ALL) patients, abnormal glycosylation profile was also observed, showing an increased *O*-glycome complexity as well as, an overall increase in sialylated *N*-glycans [74]. B-ALL patients have an increased activity of GCNT1 and GALNT7 enzymes [74]. Moreover, ALL cells showed increased binding to GBP expressed in immune cells such as the C-type lectins DC-SIGN and L-SIGN, which was associated with the poor survival of B-ALL patients [75]. Furthermore, in B-cell chronic lymphocytic leukemia (B-CLL), it was also reported that lower levels of IgM expression resulted from defects in glycosylation of μHC and CD79a leading to their retention in the ER [76]. Together, this evidence supports the relevance of glycans in assuring immune homeostasis, highlighting that the perturbation in the glycosylation pathway is connected with malignancy. However, the precise role of glycans in leukemic transformation is far from being understood. As the knowledge regarding the regulatory role of these structures in developmental processes is increased, the opportunities for the discovery of novel malignancy promotors/inhibitors will present themselves.

Congenital disorders of glycosylation (CDGs) is a group of genetic diseases with mutation in glycogenes that often present hypoglycosylation states (or defects in one or more glycosylation pathways), associated with development of some immune phenotypes, such as primary immune deficiencies or autoimmunity [77]. Patients displaying PGM3-CDG, a

disorder characterized by autosomal recessive mutations of the *PGM3* gene (which encodes the phosphoglucomutase 3, enzyme that catalyzes the conversion of GlcNAc-6-phosphate into GlcNAc-1-phosphate, a precursor of UDP-GlcNAc), show CD4 and CD8 T cell lymphopenia [78], in which disease correction with HSC transplantation has been reported [79, 80]. Further studies of CDG patients would be worth envisioning the investigation of the impact of genetic glycosylation deficiencies in human lymphocyte development. Interestingly, *Mgat5*$^{-/-}$ mice (with absence of β1,6 GlcNAc-branched *N*-glycans) were shown to display a spontaneous development of glomerulonephritis phenotypes [42], as also observed in *Man2a1*$^{-/-}$ mice (with a deficiency in alpha mannosidase II enzyme, responsible for the removal of mannose monosaccharides in order to enable the formation of complex *N*-glycans) [81]. In *Mgat5*$^{-/-}$ mice, the hyperactivation of T cells, mediated by decreased thresholds for TCR activation imposed by the absence of complex *N*-glycans, was shown to be the major driver of immunopathogenesis of chronic inflammation [82]. In fact, inflammatory bowel disease patients showed decreased levels of ß1,6-GlcNAc *N*-glycan branching in T cells, that was associated with mucosal T cell hyperactivation and disease severity [43, 82, 83]. Moreover, genetic variants of *MGAT5* glycogene were described in ulcerative colitis patients, and found to be associated with low transcription levels of *MGAT5* gene, and with agalactosylation of circulating IgGs, often associated with pro-inflammatory phenotypes [84]. In infection, and specifically in COVID-19 patients (non-vaccinated), a differential expression levels of branched *N*-glycans in peripheral T cells was described, when comparing infected *versus* non-infected individuals, as well as in asymptomatic ones [85]. Together, the precise relationship between T and B cell developmental events, influenced by glycans, and disease susceptibility is an exciting novel research subject that is worth explored.

The compelling body of evidence on the role of circulating IgG glycans in disease has been gaining some momentum in the last years. In fact, immunoglobulin G Fc domain contains a single, highly conserved, *N*-glycosylation site, that carries complex *N*-glycans which regulate its binding to Fc receptors and therefore its pro- or anti-inflammatory function [86]. In COVID-19, we and others have shown that IgG glycans, namely the levels of fucosylation, galactosylation and sialylation, are differentially present in patients with severe disease, when compared to mild ones [87-89]. In Rheumatoid Arthritis (RA), a chronic autoimmune disease, the presence of variable domain glycans (VDGs) has been described in B cell-producing anti-citrullinated protein antibodies (ACPAs), acting as major drivers of autoimmunity [90], due to somatic hypermutation events that results in the selective introduction of N-linked glycosylation sites [91]. Remarkably, ACPA IgG V-domain contains biantennary *N*-glycans with one or two terminal sialic acid residues and one core fucose

that has been proposed to be involved in the positive selection of autoreactive B-cell clones [92]. Presumably, this event occurs during the germinal center formation where B cells might escape from checkpoints. However, there is still a lack of knowledge of the functional mechanism behind this selection [91]. ACPA B cell response matures prior to the onset of the disease [93], however, the precise contribution of defects in development of B cells and ACPA IgGs and how glycans act as a master switch of B cell autoreactivity in RA deserves further studies.

Concluding remarks and Future directions

Although underappreciated for many years, exciting findings in the last couple of years have been underscoring the essential contributions of protein glycosylation in the fundamental cellular and molecular mechanisms underlying immune tolerance and homeostasis and have been changing the landscape of thinking on the pathogenesis of immune-related diseases. Glycans cover the surfaces of all mammalian cells giving rise to the glycome and from the very early stages of immune system development, glycans play an instrumental role. In the cellular immune system, the regulatory power of glycans is highlighted by the fact that when perturbing the normal glycosylation pathway along T and B cell development, significant abnormalities occur in cellular commitment, differentiation and selection that ultimately may result in major primary immunodeficient diseases or susceptibility to infection, inflammation and cancer. Technological advances in the past decade, brought us to the level where reliable and extensive glycan analysis can be routinely performed on a large number of samples and even single cell glycomics is within reach [94]. The bioorthogonal chemistry (click-chemistry) recently distinguished by the Nobel prize in chemistry 2022 has proven to be an essential tool for understanding the structures, localization, and biological functions of glycans at cellular surfaces allowing a non-invasive imaging of glycans in a living system. Statistical and bioinformatics tools for integration of glycomics with other omics data such as transcriptomics, metabolomics and immune-profile will allow a deeper glyco-immune-proteome mapping revealing the complete glyco-picture and unlocking the sugar code of immunity in a structure-function relationship. In this *Opinion*, we gave a perspective on the newest advances and paradigm shifts of glycans along T and B cell development discussing how this information can be truly leveraged towards further understanding of basic principles of immunity with many clinical implications in disease diagnosis, prognosis and treatment.

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CHAPTER 1.3

AIMS AND THESIS OUTLINE

General aims

The current advances on the understanding of the role of glycans in T cell biology has gained momentum in the last years. However, the degree of complexity of this subject indicates the requirement for further work. In fact, the mechanisms underlying the influence of glycans in T cell development and its impact on homeostasis and disease is worth exploring. Therefore, in the present doctoral thesis, the general aim is to disclose the contribution of glycans in the formation of mature T cells and its functional impact.

The doctoral work here presented started in September of 2018. When the world was faced with a pandemic, murine models were being generated and initial characterizations were being performed. In March of 2020, a strict lockdown was implemented in Portugal and murine colonies were forced to be reduced, to minimize required animal house personnel. At the time (and still ongoing) there was a worldwide call for the understanding of the new SARS-CoV-2 virus and the human immune responses to its infection. In this scope, in this doctoral thesis, and taking advantage of the know-how in Glycobiology, we raised a new general aim: to understand the role of glycans in adaptive immune responses to SARS-CoV-2 infection.

Specific aims

1. **To survey the current knowledge on the role of glycans in T cell development**

The growing body of work on the contribution of glycans in T cell development has not deserved extensive review. In fact, episodic revisions of this subject usually focus on specific glycans and/or specific developmental checkpoints.

In order to fill this gap in the literature, the evidences of glycan involvement in T (and B) cell development, their mechanistic impact and implications in disease were revised and discussed. In **Chapter 1.2** we surveyed the findings regarding glycans in the key thymic developmental checkpoints, and, being an Opinion piece, raised the hypothesis that glycans are in fact developmentally regulated. We also outlined the future research directions

required to increase the understanding of an additional layer of T cell development regulation, based on glycans and the glycome.

2. **To determine the role of *N*-glycans in T cell development**

Driven by the lack of information of human and murine thymocyte glycomes, in steatdy-state conditions, in Chapter 2 we have investigated the potential role of *N*-glycans along thymocyte development, and its impact in disease susceptibility. In **Chapter 2**, we described glycome alterations in human and murine T cell development and, using novel mouse models of thymocyte-specific conditional knockout of *Mgat1* and *Mgat2*, demonstrated a striking influence of high-mannose *N*-glycans within the major developmental checkpoints.

3. **To uncover the glycan dynamics of circulating T cell subsets in COVID-19**

Due to the temporal context of the doctoral work presented in this thesis, we aimed to study glycosylation dynamics in circulating T cells of COVID-19 patients, at diagnosis. At the time, beginning of the pandemic, with no vaccination strategies available, there was an urgent need to stratify patients according to disease severity, to facilitate the management of clinical resources. In **Chapter 3**, we addressed whether SARS-CoV-2 infection would drive glycome alterations in circulating T cells, and if such changes were related to clinical presentation of the disease.

4. **To disclose the circulating IgG *N*-glycome in COVID-19**

In the same line as Specific Aim 4, in the beginning of the COVID-19 pandemic there was an urgent need for disease severity biomarkers. Given the potential of IgG *N*-glycans in other inflammatory diseases, we aimed to perform the analysis of these molecules in COVID-19 patients, at diagnosis. In **Chapter 4**, we investigated the disease course predictive potential of IgG *N*-glycome alterations upon SARS-CoV-2 infection, as well as functional aspects of the identified glycans in immune cell activation.

CHAPTER 2

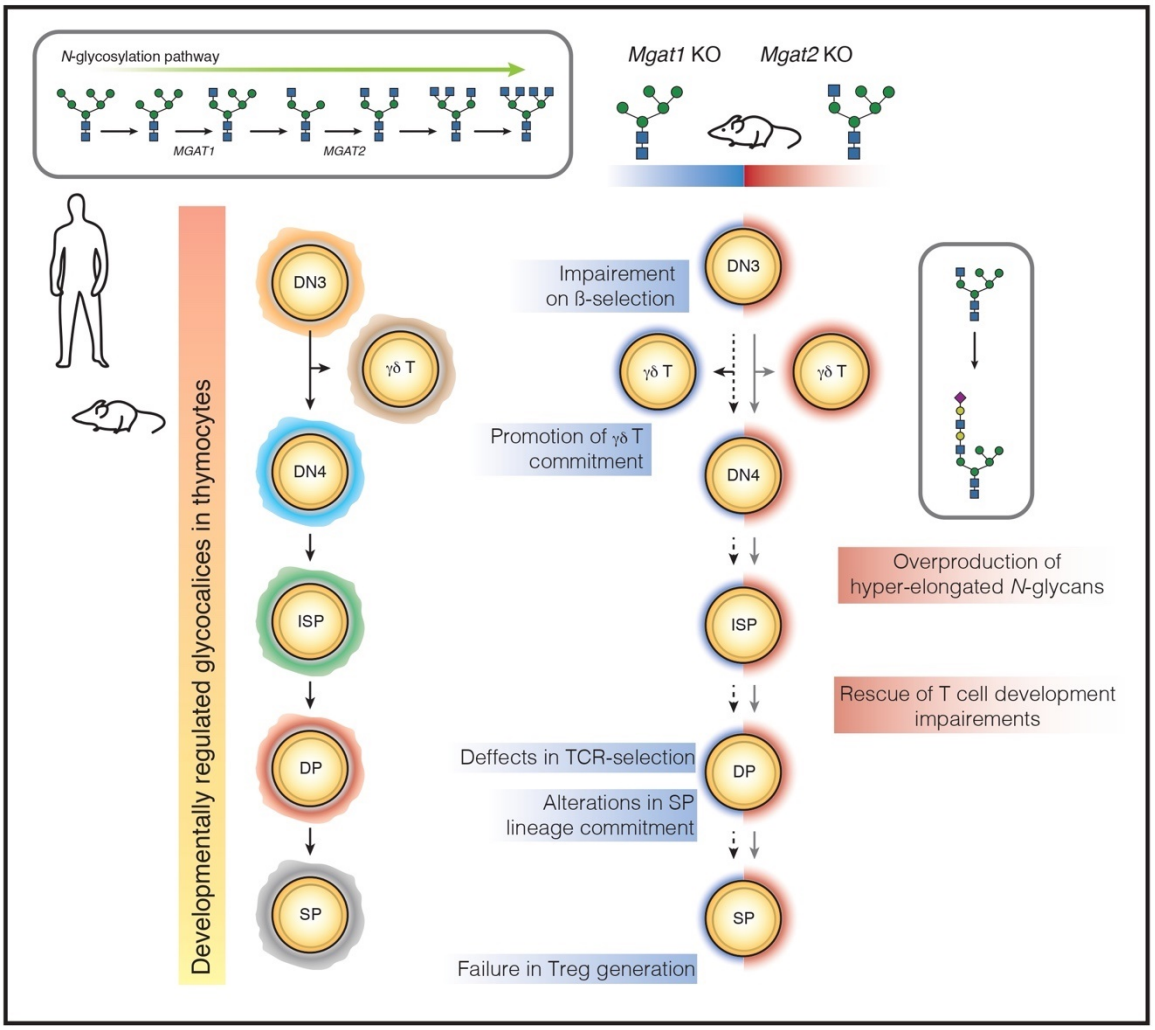
[RESEARCH PAPER]

MANNOSYLATED GLYCANS IMPAIR NORMAL T-CELL DEVELOPMENT BY REPROGRAMMING COMMITMENT AND REPERTOIRE

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Graphical Abstract



Mannosylated glycans impair normal T-cell development by reprogramming commitment and repertoire

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Running title: Mannosylated glycans impair normal T-cell development

Keywords: *N*-glycosylation, T-cell development, thymocytes, glycocalyx, Inflammation

Summary: Glycocalyx complexity identifies thymocyte populations in human and mice. High-mannose *N*-glycans on thymocytes impair normal T-cell development and promote peripheral inflammatory phenotypes.

Abstract

T-cell development ensures the formation of diverse repertoires of T-cell receptors, that recognize a variety of antigens. Glycosylation is a major post-translational modification present in virtually all cells, including T-lymphocytes, regulating activity/functions. Although these structures are known to be involved in TCR-selection in DP thymocytes, it is unclear how glycans regulate other thymic development processes, and how it influences susceptibility to disease. Here, we discovered stage-specific glycome compositions along T-cell development in human and murine thymocytes, as well as dynamic alterations. After restricting the *N*-glycosylation profile of thymocytes to high-mannose structures, using specific glycoengineered mice (*Rag1*^{Cre}*Mgat1*$^{fl/fl}$), we showed remarkable defects in key developmental checkpoints, including ß-selection, regulatory T-cell generation and γδT-cell development, associated with increased susceptibility to colon and kidney inflammation, and infection. We further demonstrated that a single *N*-glycan antenna (modelled in *Rag1*^{Cre}*Mgat2*$^{fl/fl}$) is the *sine-qua-non* condition to ensure normal development. In conclusion, we revealed that mannosylated thymocytes render a dysregulation in T-cell development, associated with inflammation susceptibility.

Introduction

Thymic T-cell development is a tightly regulated process that ensures the formation of a T-cell pool with a diverse repertoire of T-cell receptors (TCRs), essential in adaptive immunity (1,2). Thymocytes enter through a series of developmental stages, mainly defined by variations in coreceptors expression. In the $CD4^-CD8^-$ double-negative-3 (DN3) stage, cells commit to the αß or γδ lineages. Then massive proliferation of DN4 cells leads to the generation of $CD4^+CD8^+$ double-positive (DP) thymocytes, where a successful rearrangement of the *Tcra* locus generates a mature- and cell-unique TCR. An intermediate population is found on the DN4-to-DP transition, the $CD8^+CD3^-$ (mouse) or $CD4^+CD3^-$ (human) immature-single-positive (ISP) cells. Development of DP into mature $CD4^+$ or $CD8^+$ single-positive (SP) thymocytes is highly regulated by two steps: "positive-" and "negative-selection", where non- or self-reactive T-cells are eliminated. Moreover, cells with TCR affinities close to the threshold for negative selection, develop into natural-regulatory T-cells (nTregs) (3).

Glycans are present in essentially all cellular surfaces, being important regulators of the immune system. In fact, T-cells contain a dense coat of glycans (glycocalyx) that tightly regulate activity and function (4-6). Glycosylation is the enzymatic pathway responsible for the attachment of glycans to proteins/lipids by a portfolio of glycosyltransferase and glycosidase enzymes (7). Protein glycosylation has been shown to be essential in T-cell activation and differentiation (8-11). However, its precise impact in T-cell development and disease susceptibility remains poorly understood. Previous evidences demonstrated essential contributions of α2,3-

linked sialic-acid to T-cell development (12). Furthermore, seminal work showed *N*-glycan branching to be a master regulator of DP-selection, tunning the thresholds of TCR signaling (13). However, the role of *N*-glycans in other thymic developmental processes, such as ß-selection, γδT-cell development, natural Treg generation, and the peripheral consequences of a central deficiency of these structures (disease susceptibility) remains undefined. In fact, complex-branched *N*-glycans have been described to be chief regulators of T-cell activity and function (4). Specifically, we and others have shown that the deficiency of β1,6-GlcNAc-branched complex *N*-glycans promote TCR clustering and signaling, resulting in lower activation thresholds, associated with increased susceptibility to multiple sclerosis (14) and inflammatory bowel disease (10,11,15).

Overall, these evidences pinpoint *N*-glycans as key regulators of peripheral T-cell activity and function, but the knowledge of their role in central tolerance and T-cell development associated with disease is still unclear. Particularly, thymocyte glycocalyces composition and their influence in disease susceptibility remains largely unexplored. Here, we showed that human and murine thymocyte subsets display unique and distinct glycosylation signatures that define developmental stages. We further demonstrated that high-mannose restricted *N*-glycomes in thymocytes globally impair their normal development, predominantly in ß-selection, Treg cells generation and γδT-cell lineage choice and selection, that was associated with spontaneous autoimmune phenotypes. In addition, we showed that a single *N*-acetylglucosamine (GlcNAc) antenna is the *sine-qua-non* condition

needed to rescue homeostasis and T-cell developmental dynamics and peripheral phenotypes, due to a glycosylation pathway compensatory effect.

Materials and Methods

Animals

C57BL/6 wild-type (WT) mice were acquired from the Jackson laboratory. Mice containing floxed *Mgat1* (JAX:006891) and *Mgat2* (JAX:006892) were kindly provided by Dr. Michael Demetriou (UC Irvine, USA). *Rag1*^{Cre} transgenic mice, with a *Cre* recombinase gene introduced under the promoter of the *Rag1* gene (32), were kindly provided by Dr. Marc Veldhoen (IMM, Portugal). Mice (males) with two floxed alleles for *Mgat1* or *Mgat2* were crossed with *Rag1*^{Cre} ones (females), with only one allele with the *Cre*, to generate *Rag1*^{Cre} with heterozygous floxed (both for *Mgat1* or *Mgat2*) progeny. These mice (females) were then crossed with homozygous floxed *Mgat1* or *Mgat2* ones (males) to generate *Rag1*^{Cre}*Mgat1*$^{f/f}$ (*Mgat1*$^{\Delta/\Delta}$) and *Rag1*^{Cre}*Mgat2*$^{f/f}$ (*Mgat2*$^{\Delta/\Delta}$) progeny. The littermates containing only the two floxed alleles (*Mgat1*$^{f/f}$ and *Mgat2*$^{f/f}$) were used as controls. Mice were housed at the animal facility of the Institute for Research and Innovation in Health of the University of Porto. All mouse procedures were approved by the i3S ethics committee for animal experimentation under Portuguese regulation.

Human thymocyte collection

Thymic specimens (173 to 578 days old) were obtained during routine thymectomy performed during pediatric corrective cardiac surgery at Hospital de Santa Cruz

(HSC), Carnaxide, Portugal, after parent's written informed consent, using thymic tissue that would be otherwise discarded. The study was approved by the Ethical Boards of Faculty of Medicine of the University of Lisbon and of HSC.

Total thymocytes were recovered through tissue dispersion and separation on a Ficoll-Paque PLUS (GE Healthcare) density gradient. Cells were then frozen in drop by drop added freezing medium (86% FCS, 14% DMSO), placed for 48h at -80º in isopropanol container and stored in liquid nitrogen until use, and cells were thawed immediately before use.

Mice organ isolation and processing

After CO_2-mediated euthanasia, thymi and spleens were collected from age and sex matched mice and rinsed in PBS 2%FBS. Organs were macerated in a 70 µm nylon mesh, in order to generate single cell suspensions. For erythrocyte lysis, cells were incubated for 3 minutes at room temperature with 1xACK (150 mM NH_4Cl; 10 mM KHCO3; 0.1 nM Na_2EDTA), and washed in PBS 2%FBS. Cells were counted and stored in PBS 2%FBS on ice until further immediate use. Serum was collected after centrifugation of whole blood. Colons and kidneys were flushed with PBS, opened longitudinally, cut into small pieces and digested with 0.9 mg/mL of collagenase IV (Sigma) in RPMI (RPMI-1640 GlutaMAX; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/mL Penicillin/Streptomycin (Pen/Strep; Gibco), 1 mM CaCl2 and 1 mM MgCl2, during 45 min with agitation at 37 ºC. Cell suspensions were then filtered in 70 µm cell strainers and washed with RPMI. Afterwards, cells were submitted to a Lymphoprep (Alere Technologies) gradient density centrifugation at 900g, without acceleration or brake, for 30min at RT. Immune cells were collected in the interphase. After washing once with PBS

and centrifuging at 300g for 10 min in 4ºC, cells were resuspended in PBS, displayed in a 96-well plate, and proceeded for flow cytometry staining. For cytokine-producing cell analysis, cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C for 5h under stimulation with 10 ng/ml phorbol myristate acetate (PMA; Sigma) and 1µg/ml ionomycin (Merk), in the presence of 10 µg/ml Brefeldin A (Sigma), A portion of colon and kidney tissue was placed in 300-500 µL of RPMI for 18 hours in 48-well plates. The weight of tissue pieces was determined, and supernatants were stored at -80°C for later analysis by ELISA.

Flow cytometry

Cell suspensions were stained with lectins and monoclonal antibodies from eBioscience and Biolegend, as described (4). Briefly, for viability detection, cells were ressuspended in PBS and incubated with Fixable Viability Dye APC-Cy7 for 30mins on ice in the dark. For lectin staining, performed in murine and human thymocytes, $1x10^6$ cells were isolated and incubated with conjugated lectins for 30mins on ice, prior to antibody staining. Antibodies were titrated and optimal concentrations were set, and cells were incubated for 30mins on ice in the dark. For intracellular staining, cells were fixed and permeabilized with the FoxP3 Fix/Perm buffer set (eBioscience). For TCRvß screen, cells were stained for specified markers, washed, and incubated for 30 minutes on ice in the dark with supplied antibodies suspensions. Cells were analyzed in a FACSCanto II (BD Bioscience), and data was analyzed with the FlowJo v10 software.

ELISA and fecal calprotectin concentration evaluation

Serum and supernatants from tissue explants were collected and the concentrations of IFNg and IL17A (mouse ELISA Ready-SET-Go! Kits, eBioscience) were measured by ELISA, according to the manufacturer's protocol and using as substrate 3,3',5,5'-tetramethylbenzidine (TMB, eBioscience) and stop solution 2 N H2SO4. The absorbance was detected using a microplate reader (Biotek Instruments) at 450 nm and 570 nm, the concentration was determined using a standard curve, and the results were posteriorly normalized to the tumor explant dry weight (g). For fecal calprotectin concentration determination, feces were collected, weighed and the protein was extracted using an extraction buffer (0.1M Tris, 0.15M NaCl, 1.0M urea, 10mM CaCl2, 0.1M citric acid monohydrate, 5g/l BSA - pH 8.0). After that, the levels of calprotectin (mouse S100A8/A9 Heterodimer DuoSet ELISA kit, R&D systems) were measured by ELISA, according to the manufacturer’s protocol.

Tissue histology

Colon and kidney samples were immediately fixed in 10% neutral buffered formalin FFPE sample blocks and were sectioned at 3 µm using a microtome (microtome Microm HM335E) and placed in slides. These slides were used for hematoxylin and eosin (H&E) staining (colon) and periodic acid-Schiff (PAS) staining (kidney). The kidney PAS-stained-sections were evaluated by a pathologist (SS) using the following protocol: a semiquantitative analysis was performed translated into a 0 to 3 score. The percentage of interstitial inflammation and mesangial inflammation was scored in the following way <5% → grade 0; <25% → grade 1, 25%–50% → grade 2, or >50% → grade 3. Finally, the total score is given by the sum of each criteria score/grade.

188

189 ***Neospora caninum* infection**

190 *N. caninum* tachyzoites (NcT) (Nc-1, ATCC® 50843) were propagated by serial
191 passages in VERO cell cultures, maintained in Minimal Essential Medium
192 containing Earle's salts (Sigma, St. Louis, MO, USA), supplemented with 10% fetal
193 calf serum (BioWest, Nuaillé, France), L-Glutamine (2mM), Penicillin (100 IU/ml)
194 and Streptomycin (100 µg/ml) (all from Sigma), in a humidified atmosphere with
195 5% CO2 at 37°C. Free parasites were obtained as previously described (26). *N.*
196 *caninum* challenge infections of 8-week old mice were performed by i.p. inoculation
197 of 1×10^7 freshly isolated NcT in 500 µL of PBS. Mouse weights and overall
198 conditions were monitored daily until day 7 post infection. At euthanasia, spleens,
199 lungs, and brains were collected for DNA isolation and parasite load quantification
200 by qPCR.

201

202 **Quantification and Statistical analysis**

203 Statistical analyzes were performed using the GraphPad Prism 9 software. Further
204 details of the statistics can be found in the Fig legends. Multiple comparisons were
205 done using the Kruskal-Wallis test. Significance values were computed using
206 Mann-Whitney t-test. No statistical method was used to predetermine the sample
207 size. All experiments were performed at least 2 times, with mice from independent
208 progenies.

209

210 **Results**

The unique glycocalyx landscape in human and murine T-cell development

The glycocalyx composition and function of human and murine thymocytes is far from being completely deciphered. To gain insights into the composition and function of glycan structures at each T-cell developmental stage, an extensive lectin-based characterization of human and murine thymocytes glycome was performed (Fig 1A). A combination of different lectins recognizing specific glycan structures were used to assess the levels of relevant glycans along the *N*-glycosylation pathway (Fig 1B). Thawed human thymocytes were used to perform the glycoprofiling of the main developmental populations (SupFig 1A). We found that thymocyte subsets display unique and stage-specific glycocalyces (Fig 1C-F). Specifically, complex-branched *N*-glycans detected by L-PHA staining (Fig 1C) and terminally α2,6 sialylated glycans by the SNA lectin (Fig 1D), structures known to be involved in T-cell biology (4), displayed a clear and distinctive signature along T-cell development (SupFig 1B). We observed differential profiles of complex-branched *N*-glycans, containing ß1,6-GlcNAc antennae (L-PHA binding), across subsets, showing high representation of these *N*-glycans in DN cells, which decrease in ISP4, and finally increase in mature populations (Fig 1C). Notably, between the DP pre-positive selection and both DP post-positive selection stage and mature SP cells, we found a significant increase in L-PHA binding (Fig 1C), which reveals differential expression dynamics of complex-branched *N*-glycans on thymocytes, when compared to peripheral cells (8). By analyzing SNA reactivity, we found an α2,6-linked sialic acid enrichment in mature SP populations, when compared to ISP4 and DP subsets (Fig 1D). Moreover, the levels of less complex, high-mannose *N*-glycans structures (GNA binding) was highly represented in

mature SP populations, differing from ISP4 and DP populations (Fig 1E). The presence of poly-LacNAc elongated glycans (LEL binding) was higher in DN and ISP4 populations, reaching a peak in the latter (Fig 1F). These results show that human thymocytes display a unique and dynamic glycan signatures, across development.

Then, and to evaluate whether the glycan profile of murine thymocytes recapitulates the one displayed in humans, we glycophenotyped biologically equivalent murine thymocyte populations (SupFig 1C). In the mouse, the presence of complex-branched *N*-glycans (L-PHA binding) was enriched in DN, ISP8 (equivalent to human ISP4) and DP cells. As in peripheral T-cells, upon TCR signaling (positive selection), DP-post selection show lower levels of ß1,6-GlcNAc branching, when compared to DP-pre selection cells (Fig 1G). On the other hand, terminal α2,6-sialylation was found to be scarce in DN and ISP8 populations, reaching high levels in mature SP populations (Fig 1H). Moreover, high-mannose *N*-glycans remained at (low) constant levels across all T-cell developmental stages, however with differences between populations (Fig 1I). Elongated glycans were found to have a developmental peak in ISP8 cells, being reduced from DP onwards (Fig 1J). Interestingly, both human and mouse thymocytes shared major glycocalyx alterations displaying high levels of mature complex-branched and elongated *N*-glycans in the ISP population that precedes the DP stage, and increased terminal α2,6-sialylation in mature subsets. Interestingly, the relative presence of complex-branched *N*-glycans in mature SP (increase/maintenance in

human, decrease in mouse) may underlie differences between organisms regarding thymic egress and peripheral naïve T-cell properties.

Taken together, this analysis of human and murine thymocytes suggests the existence of a developmentally-regulated glycome, which supports the existence of functional effects of glycans in the regulation of T-cell development.

High-mannose restricted *N*-glycome significantly impairs T-cell development, which can be rescued by compensatory mono-antennary *N*-glycan structures

The degree of complexity in the alterations of thymocyte *N*-glycomes during T-cell development, both in human and mice, prompted us to investigate their functional relevance in the context of T-cell development. Previous work revealed the role of complex N-glycans in TCR-selection, in DP thymocytes, showcasing the ability of these structures to fine tune the thresholds of TCR signaling, thus controlling thymic DP selection (13). This work, however, left questions to be answered, namely the role of *N*-glycans in ß-selection, γδT-cell development, natural Treg generation, and peripheral consequences of a central deficiency of these structures (disease susceptibility). Therefore, we hypothesized that high-mannose restricted *N*-glycomes might have a pathogenic role during T-cell development beyond DP selection. To test this, we have genetically modified the *N*-glycosylation pathway in murine models, at early DN stages, using a *Rag1*^{Cre} transgene strain crossed with *Mgat1*$^{fl/fl}$ or *Mgat2*$^{fl/fl}$ ones, thus eliminating the glycogenes as early as in the DN2 stage. The model with the *Rag1*^{Cre}-mediated knockout of *Mgat1* (*Mgat1*$^{\Delta/\Delta}$) has a complete truncation of complex-branched *N*-glycans, only

presenting high-mannose *N*-glycans, whereas the conditional knockout of *Mgat2* (*Mgat2*$^{\Delta/\Delta}$) exhibits a partial pathway truncation, generating hybrid-type *N*-glycans with only one GlcNAc antenna, possible to be further extended. We set out to evaluate alterations of the most prevalent thymocyte populations in both glycoengineered models (Fig 2A; SupFig 2A). Notably, *Mgat1*$^{\Delta/\Delta}$ mice display a significant increase in DN and DP thymocyte populations, which is accompanied by a significant decrease in CD8 SP and CD4 SP populations (Fig 2B). Within the DN compartment, complete truncation of complex-branched *N*-glycans resulted in a significant drop of DN2 and DN3 subsets and increased frequency of DN4 thymocytes (Fig 2C), suggesting a significant role of these glycans in ß-selection. Moreover, no differences were detected in the ISP8 population, but a significant decrease in CD8 mature SP cells, CD4 immature (CD3^{lo}CD24^{hi}, post-selection and pre-egress) and mature (CD3^{hi}CD24^{lo}) SP cells was observed (Fig 2D), which may reflect increased DP negative selection, compatible to previous models of *Mgat1* conditional deletion in DP thymocytes (13). Regarding the numbers of thymocyte populations, we detected a significant increase in ISP8 cells and significantly decreased CD4 and CD8 mature SP populations (Fig 2I). Since the absence of complex-branched *N*-glycans may influence endocytosis of cell surface glyco-receptors, we evaluated the expression levels for the CD4 and CD8 co-receptors, relevant for DP-selection/lineage commitment, and found a significant decrease of both CD4 and CD8 SP co-receptors in the respective SP populations (SupFig 2I and 2J), as in previous studies (13).

We then evaluated the impact of *Mgat1* deletion on glycocalyx composition of thymocytes. As expected by the genetically-mediated interruption of the *N*-glycosylation pathway, we observed a sticking decrease in complex-branched *N*-

glycans (L-PHA binding) in all developmental stages beyond DN2 (Fig 2E). Accordingly, we detected a remarkable increase in high-mannose *N*-glycans (GNA binding) in all developmental stages beyond DN2 (Fig 2F). Elongated glycans (LEL binding) were unaltered in DNs, suggesting poly-LacNAc in *O*-glycans in those cells, but were severely decreased in ISP8 thymocytes (Fig 2G). The blockade in the *N*-glycosylation pathway was also accompanied by ablation of terminal α2,6-sialic acid, as determined by SNA binding, in DP, CD4 SP and CD8 SP thymocyte's glycocalyces (Fig 2H).

These results led us to investigate whether the presence of a single GlcNAc-antenna in *N*-glycans would influence normal developmental processes. Interestingly, the frequencies of thymocyte populations in *Mgat2*$^{\Delta/\Delta}$ mice were not altered when compared to their wild-type counterparts (SupFig 2A-D). Thus, the presence of hybrid *N*-glycans, containing the first GlcNAc antenna, transferred onto the oligomannose core (creating the β-linked GlcNAc-β-1,2-Man-α-1,3-Man-β-R product) generated by GnT-I (the glycosyltransferase encoded by *Mgat1*), was sufficient and a *sine-qua-non* condition to guarantee normal thymic T-cell development. Notably, the absolute thymocyte numbers of *Mgat2*$^{\Delta/\Delta}$ mice was unaltered for all detected populations, with the exception of ISP8 cells (SupFig 2K). Moreover, we observed a decreased expression of the CD4 and CD8 co-receptor surface expression in their respective SP subsets upon *Mgat2* deficiency (SupFig 2L and 2M). We then set out to define the *N*-glycome alterations upon the loss of *Mgat2*. As expected, we observed a significant decrease in complex-branched *N*-glycans (L-PHA binding) and increased levels of mannose residues (GNA binding) in all developmental stages beyond DN2 (SupFig 2E and 2F). Interestingly, we found a remarkable increase of elongated poly-LacNAc structures (LEL binding)

from DN3 onwards (SupFig 2G), with no significant alterations in terminal α2,6-sialylation (SupFig 2H). Thus, the unique GlcNAc-antenna is found to be hyper-elongated, similar to what was described in *Mgat2*-deficient mature T-cells (16), generating a compensatory *N*-glycan structure, that is sufficient to guarantee normal T-cell development.

Collectively these data suggest that a high-mannose restricted *N*-glycome, achieved in the *Mgat1*$^{\Delta/\Delta}$ model, has a drastic influence on T-cell development, precluding the selection of peripheral T-cells. This effect was not observed in the *Mgat2*$^{\Delta/\Delta}$ model, suggesting that one GlcNAc antenna *N*-glycan is sufficient to guarantee developmental dynamics, highlighting a pathogenic role of high-mannose *N*-glycans.

Mannosylated thymocytes exhibit a deficient ß-selection

Given the alterations in the frequency of DN cells imposed by *Mgat1* deficiency, particularly the decrease of DN3 and the increase in DN4 subpopulations, we sought out to investigate the impact of a high-mannose restricted *N*-glycome in ß-selection. This key checkpoint translates the functionality of the newly generated ß-chain TCRs, a product of *Tcrb* gene rearrangements (1,2). ß-selected DN3 cells begin to express pre-TCR complexes, detected by the expression of intracellular (ic)TCRß chain (1,17). To evaluate the role of high-mannose *N*-glycans in ß-selection, we first determined the frequency of DN3 cells bearing icTCRß. We observed in the *Mgat1*$^{\Delta/\Delta}$ model, a significant decrease of icTCRß+ DN3 cells but not DN4 cells (Fig 3A-B), suggesting that clonal expansion of ß-selected thymocytes was not compromised, but rather their generation. Moreover, in the absence of *Mgat1*, decreased CD25 surface expression was detected in DN3 cells

(Fig 3C), suggesting ß-selection defects (18). Pre-TCR signaling, responsible for induction of DN3 proliferation and DN4 transition, can be detected by proxy CD5 expression (19). In the *Mgat1*$^{\Delta/\Delta}$ model, we found a decrease of CD5 in DN3, but not in DN4 cells (Fig 3D). Thus, high-mannose restricted *N*-glycomes interrupt the generation of ß-selected thymocytes, but do not alter their clonal expansion. Moreover, as the disruption of ß1,6-GlcNAc branching *N*-glycans promotes mature TCR clustering and T-cell hyperactivation (10,14), the clustering of pre-TCR complexes could be playing a role, which deserves further investigation. As IL-7 signaling is one of the key regulators of ß-selection (1,2) we evaluated the IL-7Rα (CD127) expression in DN3 cells and found a decrease in *Mgat1*$^{\Delta/\Delta}$ thymocytes (Fig 3E), which agrees with a perturbation of ß-selection. In accordance with previous results, in the presence of the mono-antennary *N*-glycans on *Mgat2*$^{\Delta/\Delta}$ mice, we detected no differences in the parameters regarding ß-selection (SupFig 3A-E). Thus, these results pinpoint the hampering of ß-selection and pre-TCR signaling by high-mannose *N*-glycans, thus revealing the role of *N*-glycan complexity in this developmental checkpoint.

High-mannose *N*-glycans bias T cell development favoring the positive selection of γδT-cell development

T-cell commitment towards the γδT-cell lineage occurs early at the DN2 and DN3 stages, where the *Tcrg* and *Tcrd* may also be targeted for genetic rearrangements (3). The precise mechanisms underlying γδ lineage commitment are not yet fully clarified (20). Since the absence of *Mgat1* leads to a deficient generation of DN3 ß-selected cells (Fig 3A-E), we analyzed the presence of DN2/3 cells bearing

intracellular (ic)TCRγδ, but no differences were detected in either *Mgat1*- (Fig 3F and 3G) and *Mgat2*-deficient thymocytes (SupFig 3F and 3G). However, an increased proportion of thymic mature γδT-cells was observed in both glycoengineered models (Fig 3H; SupFig 3H), although no changes in the absolute numbers of these cells (Fig 2I). These cells were shown to display absence of L-PHA binding (Fig 3I; SupFig 3I) and increased mannose expression (GNA binding), in both models (Fig 3J; SupFig 3J). When we evaluated the TCR-mediated activation status of these mannosylated cells we observed increased CD5 in γδT-cells of *Mgat1*$^{\Delta/\Delta}$ (Fig 3K), but not in *Mgat2*-deficient mice (SupFig 3K), suggesting higher TCR signaling in *Mgat1*-deficient γδT-cells, which has been associated with the promotion of this T-cell subset development (20). The regulation of γδTCR signaling intensities by *N*-glycans poses itself as a potential mediator in the development of those cells.

Our results suggest that high-mannose *N*-glycomes in DN2/3 cells favor γδT lineage commitment, and should play a major role in the regulation of their activation, as in peripheral αβTCR T-cells (4).

High-mannose *N*-glycans disturb TCR repertoire diversity

Previous evidences showed that *N*-glycans regulate positive and negative DP-selection, in a *Lck*$^{Cre/+}$*Mgat1*$^{f/f}$ background, where ~70% DPs were defective in complex-branched *N*-glycans (13). In our *Mgat1*$^{\Delta/\Delta}$ model, in which >90% DP cells are deficient in complex-branched *N*-glycans, the DP-selection effect was validated. We analyzed the differential expression of the TCRß chain and CD69 (SupFig 4A and 4D), to identify the thymocyte populations undergoing selection.

As expected, *Mgat1* deficiency caused a significant increase in the frequency of TCRß$^{-}$CD69^{-} cells (SupFig 4B), with higher apoptosis levels (SupFig 4C), indicating increased death by neglect. Accordingly, a decrease in positively selected cells, TCRß^{hi}CD69^{hi}, was observed (SupFig 4B). Finally, post-selection thymocytes, TCRß^{hi}CD69^{-} were reduced in *Mgat1*$^{\Delta/\Delta}$ mice (SupFig 4B), suggesting a more pronounced negative selection. Again, these differences were not observed in the *Mgat2*$^{\Delta/\Delta}$ mice, as the populations of DP-selection as well as apoptosis levels were similar to their control counterparts (SupFig 4E-F).

Once positively selected, DP thymocytes undergo CD8/CD4 lineage choice, a process regulated by TCR signaling dynamics (21). We then analyzed the CD8/CD4 lineage commitment, by analyzing the relative distributions of the three populations described above, according to CD8/CD4 expression (Fig 4A). Interestingly, positively selected cells were differentially distributed according to their CD8 and CD4 surface expression in both models (Fig 4A), being the CD8 SP quadrant underrepresented in post-negative selection cells in the *Mgat1*-deficiency model (Fig 4A), with no major differences in *Mgat2*$^{\Delta/\Delta}$ mice (SupFig 4G) These results indicate a role for *N*-glycans on CD4/CD8 lineage commitment.

One of the most important events occurring during TCR-selection is the generation of a diverse TCR-repertoire. Given the impact of *N*-glycans in TCR-selection, we evaluated whether selection defects observed in *Mgat1*$^{\Delta/\Delta}$ mice could generate impaired TCR diversity. For this, we screened for TCRß chains in the mature SP subsets. Notably, in *Mgat1*$^{\Delta/\Delta}$ mice we found a significant impact on the diversity of the TCRß-variants-expressing cells in mature CD4 SP (Fig 4B) and CD8 SP cells (Fig 4C). However, no major differences on TCR-repertoire were observed for *Mgat2*$^{\Delta/\Delta}$ thymocytes (SupFig 4H and 4I). We cannot exclude that the defects

on ß-selection of *Mgat1*$^{\Delta/\Delta}$ mice may contribute to this phenotype and further studies on TCR-repertoire diversity analysis using next-generation-sequencing would provide further insights. These results further reveal the central role of *N*-glycan structures in DP selection, namely in lineage commitment and TCR-repertoire diversity.

High-mannose *N*-glycans hamper regulatory T-cell development

The ability of complex-branched *N*-glycans to control the thresholds of TCR positive and negative selection prompted us to raise the question as whether these structures could influence thymic development of T regulatory cells (Tregs). Naturally-occurring Tregs (nTregs) are generated in the thymus, comprising a set of CD4 SP cells with high levels of activation, close to the threshold of negative selection (22). In fact, the upregulation of complex *N*-glycans of thymic Tregs, when compared to conventional CD4 SP thymocytes, in wild-type mice (Fig 4D), showcases a dynamic glycoprofile alteration. In this work, and previous ones (13), it is shown that negative selection is promoted in *Mgat1*-deficient DPs (SupFig 4A-C), so we argued that Treg generation would be impaired. The initial precursors of Tregs are DP cells which show considerable expression of the FoxP3 transcription factor (22). Interestingly, this population was decreased in an *Mgat1*-deficient scenario (Fig 4E), whereas no differences were found for *Mgat2* deficiency (SupFig 4J). As mature Tregs may arise from $CD4^+CD25^+FOXP3^+$ and $CD4^+CD25^-FOXP3^{lo}$ progenitors, we evaluated the presence of these two subsets in the thymic compartment. We detected lower frequency of both populations in *Mgat1*$^{\Delta/\Delta}$ mice (Fig 4F), and no differences were found in *Mgat2*$^{\Delta/\Delta}$ ones (SupFig 4K). Moreover, the absolute numbers of thymic Tregs were severely diminished in

Mgat1$^{\Delta/\Delta}$ mice, when compared to their wild-type controls (Fig 1I). Thus, our results highlight an impact of high-mannose *N*-glycans on thymic Treg generation and this effect is not observed in the presence of hyper-elongated hybrid *N*-glycans.

High-mannose *N*-glycans impair thymic egress of mature thymocytes

The final step of T-cell development is thymic egress, where thymocytes that surmounted negative selection upregulate CD62L expression and leave the thymus (23). The presence of mature and selected CD4 and CD8 SP thymocytes in both murine models was assessed and the relative expression of CD3 and CD69 was used to detect post-selection cells. A remarkable decrease in the frequency of CD62L^{+} cells was observed in post-selection CD4 SP in the *Mgat1*$^{\Delta/\Delta}$ mice, that was fully compensated in *Mgat2*$^{\Delta/\Delta}$ ones, whereas no differences in the CD8 SP subset (Fig 4G, SupFig 2L) were detected, although a trend toward a decrease in CD8 SP subset could be observed in the absence of branched *N*-glycans. Thus, as the previous developmental checkpoints, thymic egress is markedly compromised when complex *N*-glycans are absent.

Mannosylated thymocytes promote peripheral γδT-cell activity and immune response

Given the number of defects observed in T-cell development upon *Mgat1* deficiency, we further explored the biological consequences of this abnormal expression of mannosylated glycans within the peripheral compartment in both models. We have observed a drastic reduction in splenic CD4+ and CD8+ T-cell numbers in *Mgat1*$^{\Delta/\Delta}$ mice (Fig 5A), with no differences observed in *Mgat2*$^{\Delta/\Delta}$ ones

(SupFig 5A), as also reported by others (13). We then evaluated the impact on TCRvß repertoire in splenic CD4+ and CD8+ T-cells and found critical differences in *Mgat1*$^{\Delta/\Delta}$ mice (Fig. 5B), suggesting a significant reduction in diversity of peripheral TCRvß repertoires in comparison with control mice, with no alterations in *Mgat2*$^{\Delta/\Delta}$ (SupFig 5B). Interestingly, in *Mgat1*$^{\Delta/\Delta}$ glycoengineered mice, we observed no alterations in the total numbers of splenic γδT-cells (Fig 5A), showcasing a resistance of this subset to a pathogenic high-mannose restricted *N*-glycome. In fact, in agreement with the increased activation status of mature thymic γδT-cells (Fig 3L), *Mgat1*-deficient splenic γδT-cells showed increased levels of CD25 expression, which is related with cellular activation (Fig 5C). Both these differences were not observed in *Mgat2*$^{\Delta/\Delta}$ mice (SupFig 5A and 5C). To our knowledge, this is the first report demonstrating the impact of mannosylated *N*-glycans pathway in promoting peripheral γδT-cell activity.

Given this effect on the activity of γδT-cell at the periphery, we next explored the pathophysiologic consequences, at baseline, of these glycoengineered murine models. The histological analysis of colon sections of *Mgat1*-pro and -deficient models showed increased immune infiltrates in the latter group, related with inflammation (Fig 5D), which was not observed in the *Mgat2*$^{\Delta/\Delta}$ model (SupFig 5D). Moreover, immunohistochemistry analysis of colon sections showed higher $CD3^+$ immune infiltrates (SupFig 5N). In addition, flow cytometry analysis of $CD45^+$ cells from the colonic tissue revealed increased γδT-cell frequencies (Fig 5E), for *Mgat1*$^{\Delta/\Delta}$ mice, that was accompanied with increased IFNγ-producing CD4+ and γδT-cells (Fig 5F). No differences in the frequency of IL-17-producing CD4+ and γδT-cells, nor in IL-17 expression (Fig 5G) were observed. Moreover, and in

agreement with the flow cytometry data, colon explants from *Mgat1*$^{\Delta/\Delta}$ mice significantly released more IFNγ (Fig 5H). No differences in the serum levels of these two cytokines were observed in *Mgat1*$^{\Delta/\Delta}$ (SupFig 5Q). In *Mgat2*$^{\Delta/\Delta}$, although the analysis of colon sections revealed some degree of inflammation (SupFig 5D), no significant differences were detected in the analysis of the immune infiltrates, IL-17/IFNγ-producing cells or explant cytokine release (SupFig 5E-H). The analysis of fecal calprotectin levels, commonly used in the clinics as a marker of intestinal inflammation (24), revealed a slight tendency of increase in in *Mgat1*$^{\Delta/\Delta}$ in comparison with controls, and absent in *Mgat2*$^{\Delta/\Delta}$ (SupFig 5O and Sup 5P).

Having established colonic features of spontaneous inflammation in *Mgat1*$^{\Delta/\Delta}$, that were compensated in *Mgat2*$^{\Delta/\Delta}$, we decided to analyze other putative target organs, such as the kidney, which is commonly affected in the context of chronic inflammation and autoimmunity, in a process that can be mediated by abnormal glycosylation (25). The analysis of both the interstitial and mesangial compartments of renal parenchyma showed increased inflammatory signs in *Mgat1*$^{\Delta/\Delta}$ mice (Fig 5I). Similar to the colon scenario, we observed increased γδT-cell kidney infiltration (Fig 5J), and although no differences were seen in the frequencies of IFNγ-producing γδT-cells (Fig 5K), an increased release of IFNγ in kidney explants from *Mgat1*$^{\Delta/\Delta}$ mice was seen (Fig 5M). Regarding IL-17-producing cells, no differences were found (Fig 5L). No significant differences were observed in the *Mgat2*$^{\Delta/\Delta}$ glycoengineered mice (SupFig 5I-M). Together, these results showed phenotypic consequences of the restriction of high-mannose *N*-glycomes

in thymocytes, highlighting the pathogenic role of these structures in T-cells, revealing γδT-cells as potential drivers of spontaneous inflammation.

In order to further understand the immunological responses of the glycoengineered models, we tested an infection model. Both *Mgat1*$^{\Delta/\Delta}$ and *Mgat2*$^{\Delta/\Delta}$ mice were challenged with the obligate intracellular protozoan *N. caninum*, as resistance to this parasite was previously shown to be highly T-cell-dependent (26). Our results showed that *Mgat1*$^{\Delta/\Delta}$ mice exhibited an increased parasite colonization in key organs analyzed, suggesting a tendency for an increased susceptibility to infection, when compared to their wild-type counterparts (SupFig 5R), that was not observed in *Mgat2*$^{\Delta/\Delta}$ mice (SupFig 5T). Since *N. caninum*-related immune responses are highly dependent of an intact Th1 response (26), we further analyzed the immunophenotype of the splenocytes of infected mice, and observed an expansion in both CD4$^+$ and CD8$^+$ T-cell frequencies and numbers in *Mgat1*$^{\Delta/\Delta}$ mice, when compared to the steady state ones (SupFig 5S), that presented a higher frequency CD69$^+$ cells in CD4$^+$ T-cells, upon infection, which agrees with the role of complex *N*-glycans in TCR clustering. Moreover, we detected negligible levels of naïve T-cells (CD62L$^+$CD44$^-$) in the spleens of these mice (SupFig 5S). These results support an increased pro-inflammatory response in *Mgat1*$^{\Delta/\Delta}$ mice that, together with lowered thresholds for T-cell activation, may explain the increased susceptibility to infection.

Altogether, our results show that an early thymocyte high-mannose restricted *N*-glycome, creates a pathogenic glycophenotype characterized by abnormal expression of mannosylated *N*-glycans on T cells which has profound central and peripheral immune consequences, by conferring increased susceptibility to

infection, chronic inflammation and autoimmunity. Hyper-elongation of *N*-glycans efficiently rescues this T-cell phenotype and prevents higher susceptibility to disease development.

Discussion

T-cell development is a key biological process that ensures a functional and diverse TCR repertoire of T-cells, enabling robust mechanisms of immune protection and tolerance. The discovery of cellular developmental mediators of thymocyte maturation has deserved attention since the 1960´s, shaping modern immunology (27). However, in spite of considerable progress, the function of cell-specific *N*-glycans in early T-cell lineage commitment and selection, and their relevance in T-cell-mediated immunopathology, has been largely unexplored. Here we present a comprehensive characterization of the glycocalyx composition of both human and murine thymocytes, demonstrating the existence of unique glycosylation signatures that distinguish each developmental stage. In fact, the analysis of the thymocyte populations' glycome highlighted high *N*-glycan complexity and diversity across different thymocyte stages, namely high levels of poly-LacNAc structures in immature subsets, elevation of terminal α2,6-sialylation in mature SP populations, both accompanied by a considerable presence of complex ß1,6-GlcNAc branching. Moreover, the overall low levels of high-mannose structures, suggests *N*-glycan complexity as a *sine-qua-non* feature for functional developmental processes, associated with homeostasis. In fact, we have previously reported altered T-cell glycoprofiles in several disease scenarios,

573 related with activation, which highlight the regulatory power of glycans in
574 determining T-cell state identities (4,28).
575 In order to model the expression of *N*-glycans in thymocytes, we generated unique
576 glycoengineered murine models with conditional ablation of branched *N*-glycans
577 structures in T-cells, through the *Rag1*^{Cre}-mediated disruption of *Mgat1* and *Mgat2*
578 in DN2 thymocytes. A deficiency in the synthesis of complex-branched *N*-glycans,
579 and the consequent restriction of high-mannose *N*-glycan presence, modelled in
580 *Mgat1*$^{\Delta/\Delta}$ mice, resulted in increased percentages of DN cells relative to total
581 thymocytes that were associated with an impairment of ß-selection. In total DN3
582 cells, we showed a decreased pre-TCR signaling in the absence of *Mgat1*, that
583 was restored in DN4, which can be due to pre-TCR clustering, as described for
584 mature TCR's, upon the deficiency of complex-branched *N*-glycans (4,13).
585 Moreover, decreased expression of the IL-7Rα may also contribute to impaired ß-
586 selection, and DN3 survival. Interestingly, IL-7Rα has 3 *N*-glycosylation conserved
587 sites in mice and 6 in humans. The high-mannose glycans in *Mgat1*$^{\Delta/\Delta}$ mice in this
588 receptor may may influence its dynamics, such as IL-7 affinity (29) or endocytosis,
589 which deserves further studies. As *N*-glycans control αßTCR thresholds, the same
590 regulatory effect might be extrapolated to γδTCRs, promoting sustained signaling
591 to γδT-cells (in the *Mgat1*-deficient model), and favoring development of this subset
592 (20). To our knowledge, this is the first report on the role of mannosylated *N*-
593 glycans in promoting γδT-cell development, suggesting the critical role of the *N*-
594 glycans structures in the regulation of this T-cell subset activity and function.
595 DP thymocytes are bi-potential progenitors for the $CD4^+$ and $CD8^+$ T-cell lineages,
596 during positive selection. We found that the presence of only mannosylated

structures in cells that have been positively selected was associated with an increased percentage of cells in the DN and DP quadrants, suggesting enhanced co-receptor internalization/endocytosis, as previously observed in other settings (13), which altered the fate of DP cells to the CD4 SP and CD8 SP lineages. Importantly, our results further identify an additional and novel role of *N*-glycans in TCR selection, demonstrating that high-mannose *N*-glycomes significantly alter the diversity of TCRvß variants in mature SP cells. Additionally, we found that thymic Treg generation is also compromised in the absence of complex-branched *N*-glycan structures. Impairment in the generation of nTregs could be explained by the reduction of the TCR affinity threshold for negative selection imposed by the absence of *N*-glycan branching (13). Finally, CD62L-expressing mature CD4 SP thymocytes in *Mgat1*$^{\Delta/\Delta}$ were diminished, suggesting an impairment of egression, which we cannot exclude to be influenced by the previous selection processes. Altogether, our results demonstrated that thymocyte glycocalyces are developmentally and dynamically regulated and that high-mannose *N*-glycomes do not ensure proper transitions between developmental stages, nor do regulate central selection and commitment processes. Elegant studies from Demetriou´s group on the contribution of *N*-glycans in TCR selection showed its impact in the regulation of the thresholds for positive and negative selection (13). Our work, targeting *Mgat1* at earlier stages of T-cell development (*Rag1*^{Cre}), has illuminated the contribution of *N*-glycans, specifically highly mannosylated *N*-glycan structures in several critical developmental processes, including lineage commitment, TCR repertoire determination, thymic Treg generation, γδT-cell development and thymic egress, as well and the phenotypic consequences in term of disease susceptibility.

621 In this study we showed that GlcNAc-branching of *N*-glycans is a *sine-qua-non*
622 condition for appropriate T-cell development as highlighted by the fact that
623 *Mgat2*$^{\Delta/\Delta}$ mice, proficient in synthesizing in mono-antennary *N*-glycan structures in
624 T-cells, do not display major developmental defects, exhibiting a normal ß-
625 selection, production of diverse TCR repertoires, normal generation of Tregs and
626 thymic egress, as well as γδT-cell development. This suggests that hyper-
627 elongated mono-antennary *N*-glycans are key glycan determinants essential in T-
628 cell development and able to rescue the majority of developmental processes
629 markedly impaired in *Mgat1*-deficient mice. The synthesis of a mono-antennary *N*-
630 glycan structure appears to create a compensatory mechanism that allows glycan
631 elongation and terminal sialylation, essential to feed a proper T-cell selection
632 program. These glycosylation compensatory pathways have been described in
633 other physiopathological settings (16,30).
634 The phenotypic impact of such a drastic impairment on T-cell development was
635 demonstrated in *Mgat1*$^{\Delta/\Delta}$ mice, that exhibited an increased susceptibility, at
636 baseline, to inflammation and infection. A central deficiency of complex-branched
637 *N*-glycans on T-cells results in remarkable reduced splenic αβT cells. The TCRvß
638 reduced variability in thymic SP cells was conserved in splenic T-cells, in the
639 *Mgat1*-deficient model. Moreover, we detected a phenotype of colonic and kidney
640 inflammation, mainly involving increased IFNγ presence/production, in *Mgat1*$^{\Delta/\Delta}$
641 mice. The absence of complex *N*-glycans in T-cells has been shown to decrease
642 their activation thresholds (4), which agrees with the inflammatory cues we have
643 observed. Moreover, the tendency for increased susceptibility to *N. caninum*
644 infection, with increased T-effector polarization, agree with the overall phenotype

of increased inflammation. Finally, we disclosed a previously unknown feature of mannosylated *N*-glycans, that influence the activity status of γδT-cells associated with inflammation.

The role of the immune system in Congenital Disorders of Glycosylation (CDG) is receiving some attention from the clinical and scientific community (31). One of the aspects that work highlights is the pathogenic role of mannosylated N-glycans (also aberrantly present in several forms of CDGs) in T-cell development, with peripheral consequences, namely in inflammatory cues. Altogether, the evidences on the role of glycans in immune development highlight the need of further studies of CDG patients.

Taken together, this study unveils the regulatory power of branched *N*-glycans in T-cell development, highlighting their role as key determinants for immunity and as essential players in the control of ß-selection, Treg generation, TCR repertoire diversity and γδT-cell development. Our findings constitute novel features of thymocyte "identity" and function, by revealing branched *N*-glycans as essential structures of T-cells. We also reveal the implications of its deficiency giving rise to "pathogenic" mannose structures on T cells that drive increased susceptibility to the development of major diseases, such as infection and inflammation.

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669 ÂF, AMD and AC in animal experimentation; EP and AC in infection experiments.
670 SS performed the histopathological score of murine tissues. SSP, AC, GAR, MV
671 and AES contributed with reagents/analytical tools. AC, ARMA, GAR and MV
672 provided intellectual input to research and expertise in data analysis. MMV and
673 SSP wrote the manuscript with contributions from all authors.

674

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Figure legends

Fig 1: Human and murine T-cell developmental stages entail differential glycans profiles. (A) Scheme of the isolation of thymocytes both from human (N=4) and murine (N=8, 8 weeks old) thymi and subsequent workflow. **(B)**Golgi-located major steps of the *N*-glycosylation pathway, of GlcNAc antennae formation, elongation and termination. Boxes indicate the specific lectin binding glycans. **(C to F)**Histograms of lectin staining for major human thymocyte populations (top), Median Fluorescence Intensity (MFI) quantifications normalized for the mean DN levels (middle) and heatmap of mean rank differences showing the Kruskal-Wallis test multiple comparisons between populations, done as "population in line" *vs*. "population in column" (bottom), for L-PHA **(C)**, SNA **(D)**, GNA **(E)** and LEL **(F)**. As an example for the heatmap interpretation: comparison between L-PHA levels of CD8 SP and ISP4 shows a blue color, indicating a negative mean rank difference, *i. e.*, ISP4 levels L-PHA are lower than CD8 SP. **(G to J)**Histograms of lectin staining for major murine thymocyte populations (top),

834 Median Fluorescence Intensity (MFI) quantifications normalized for the mean DN
835 levels (middle) and heatmap of mean rank differences showing the Kruskal-Wallis
836 test multiple comparisons between populations, done as "population in line" *vs*.
837 "population in column" (bottom), for L-PHA **(G)**, SNA **(H)**, GNA **(I)** and LEL **(J)**.
838 Kruskal-Wallis test, *q*-value *< 0.05, **< 0.005, ***< 0.001.
839
840 **Fig 2: Impairment of T-cell development in high-mannose restricted**
841 **thymocytes. (A)**Thymocyte populations discrimination in *Mgat1*$^{f/f}$ (N=8, 6-8 weeks
842 old) and *Mgat1*$^{\Delta/\Delta}$ (N=8, 6-8 weeks old). **(B)**Frequencies of DN, DP, CD8 SP and
843 CD4 SP subsets, within live cells. **(C)**Frequencies of DN1, DN2, DN3 and DN4
844 subsets, within the total DN population. **(D)**CD8 SP and CD4 SP subset
845 frequencies: ISP8, CD8 MSP, CD4 ISP and CD4 MSP, within live cells. **(E to**
846 **H)**Levels of lectin binding (MFI levels) normalized to indicated population (left) and
847 representative histograms of lectin binding profiles in indicated thymocyte subsets,
848 for L-PHA **(E)**, GNA **(F)**, LEL **(G)** and SNA **(H)**. **(I)**Absolute numbers of cells within
849 the indicated populations. Each dot represents one mouse in all graphs. Mann-
850 Whitney t-test, *p*-value *< 0.05, **< 0.005 and ***< 0.001.
851
852 **Fig 3: Lack of complex-branched N-glycans impairs ß-selection without**
853 **afecting determination of γδ T-cells. (A)**Gating strategy for the identification of
854 ß-selected (icTCRß+) DN3 cells and **(B)**frequencies of those cells in DN3 and DN4
855 subsets, in *Mgat1*$^{f/f}$ (N=8) and *Mgat1*$^{\Delta/\Delta}$ (N=8). **(C)**Quantification of CD25 surface
856 levels (MFI) in the DN3 subset, normalized to the mean of *Mgat1*$^{f/f}$ DN3 levels.
857 **(D)**Quantification of CD5 and **(E)**CD127 surface levels (MFI) in DN3 and DN4

thymocytes, normalized to the mean of *Mgat1*$^{f/f}$ DN3 levels, and CD127 DP levels, respectively. **(F)**Gating strategy for the identification of icTCRγδ+ cells within the DN2/3 subset and **(G)**frequencies, in *Mgat1*$^{f/f}$ (N=8) and *Mgat1*$^{\Delta/\Delta}$ (N=8). (**H**)Frequency of total thymic mature TCRγδ+ cells within live cells. **(I)**Quantification of CD27+ cells within the mature TCRγδ+ population. **(J)**Levels of L-PHA and **(K)**GNA binding levels, in thymic TCRγδ+ cells, normalized to the mean of *Mgat1*$^{f/f}$ levels. **(L)**CD5 surface levels (MFI) of TCRγδ+ cells, normalized to the mean of *Mgat1*$^{f/f}$ levels. Each dot represents one mouse. Mann-Whitney t-test, p-value *< 0.05, **< 0.005 and ***< 0.001.

Fig 4: TCR-selection is impaired in high-mannose restricted *N*-glycome, with severe effects in TCR repertoire diversity and thymic Treg generation. **(A)**Distribution of each selection subset, pre-selection, post-positive selection and post-negative selection thymocytes, according to its CD4 and CD8 expression levels, and quantification in *Mgat1*$^{f/f}$ (N=8) and *Mgat1*$^{\Delta/\Delta}$ (N=8). **(B)**Screen of TCRvß+ expressing cells, within thymic mature CD4 SP and **(C)**CD8 SP cells, in *Mgat1*$^{f/f}$ (N=4) and *Mgat1*$^{\Delta/\Delta}$ (N=4). **(D)**Levels of L-PHA binding (MFI) in wildtype thymic CD4 SP CD25-FOXP3- and Tregs populations. **(E)**Quantification of the frequency of FoxP3+ cells in the DP population. **(F)**Frequencies of subsets within CD4 SP thymocytes. **(G)**Identification of CD62L^{+} cells in mature CD4 SP and CD8 SP, in *Mgat1*WT (N = 4) and *Mgat1* cKO (N = 4), and *Mgat2*WT (N = 4) and *Mgat2* cKO (N = 4) mice, respectively. Each dot represents one mouse. Mann-Whitney t-test, p-value *< 0.05, **< 0.005 and ***< 0.001.

Fig 5: Impairments on T-cell development by the absence of complex N-glycans lead to peripheral alteration in homeostasis. (A) Splenic T-cell numbers cell numbers in *Mgat1$^{f/f}$* (N=8) and *Mgat1$^{\Delta/\Delta}$* (N=8). **(B)**Screen of TCRvß+ expressing cells, in splenic mature CD4+ (right) and CD8+ T-cells (right), in *Mgat1$^{f/f}$* (N=4) and *Mgat1$^{\Delta/\Delta}$* (N=4). **(C)**Levels of CD25 surface expression (MFI) in splenic γδ T-cells, normalized to the mean of *Mgat1$^{f/f}$* levels. **(D)**H&E staining of colon sections, at 20x (top) and 40x magnification (bottom), and specimen distribution according to overall detection of inflammation cues (right). Scale bars indicate 100 um. **(E)**T-cell population frequencies within CD45+ cells, isolated from colon tissues. **(F)**Frequencies of IFNγ-producing cells within CD4+ and γδ T-cells and IFNγ expression (MFI) in the same subsets. **(G)** Frequencies of IL-17-producing cells within CD4+ and γδ T-cells and IL-17 expression (MFI) in the same subsets. **(H)**IL-17 and IFNγ concentration in colon explant culture supernatants, normalized to tissue weight. **(I)**PAS staining of colon sections, at 20x (top) and 40x magnification (bottom), and scores of intersticial and mesangial inflammation (right). Scale bars indicate 100 um. **(J)**T-cell population frequencies within CD45+ cells, isolated from kidney tissues. **(K)**Frequencies of IFNγ-producing cells within CD4+ and γδ T-cells and IFNγ expression (MFI) in the same subsets. **(L)**Frequencies of IL-17-producing cells within CD4+ and γδ T-cells and IL-17 expression (MFI) in the same subsets. **(M)**IL-17 and IFNγ concentration in colon explant culture supernatants, normalized to tissue weight. Each dot represents one mouse. Mann-Whitney t-test, p-value *< 0.05, **< 0.005 and ***< 0.001.

Figure 1

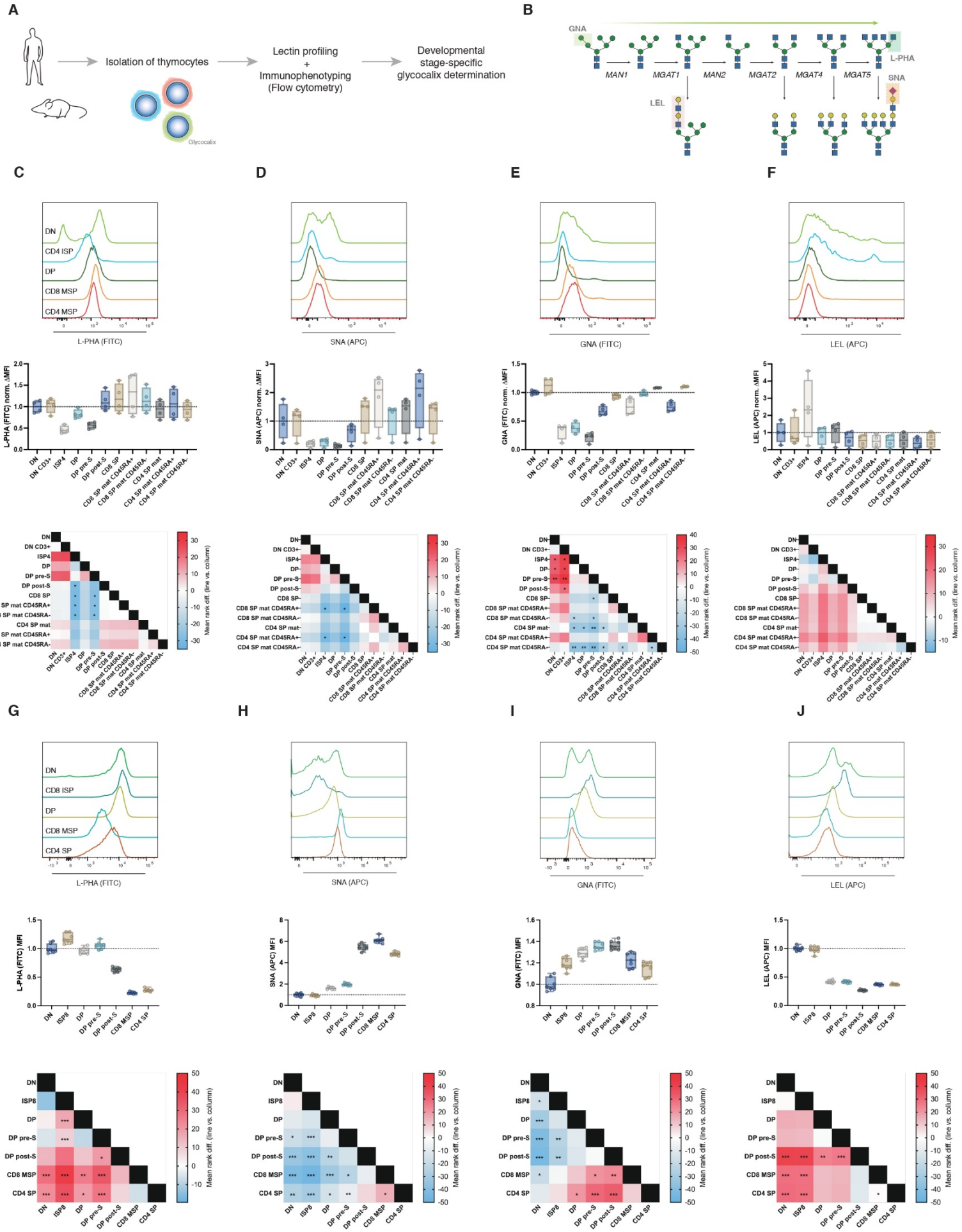

A
Isolation of thymocytes
Lectin profiling + Immunophenotyping (Flow cytometry)
Developmental stage-specific glycocalix determination
Glycocalix
B
GNA
MAN1
MGAT1
MAN2
MGAT2
MGAT4
MGAT5
L-PHA
SNA
LEL
C
D
E
F
DN
CD4 ISP
DP
CD8 MSP
CD4 MSP
L-PHA (FITC)
SNA (APC)
GNA (FITC)
LEL (APC)
L-PHA (FITC) norm. ΔMFI
SNA (APC) norm. ΔMFI
GNA (FITC) norm. ΔMFI
LEL (APC) norm. ΔMFI
DN
DN CD3+
ISP4
DP
DP pre-S
DP post-S
CD8 SP
CD8 SP mat CD45RA+
CD8 SP mat CD45RA-
CD4 SP mat
CD4 SP mat CD45RA+
CD4 SP mat CD45RA-
Mean rank diff. (line vs. collumn)
G
H
I
J
DN
CD8 ISP
DP
CD8 MSP
CD4 SP
L-PHA (FITC) MFI
SNA (APC) MFI
GNA (FITC) MFI
LEL (APC) MFI
DN
ISP8
DP
DP pre-S
DP post-S
CD8 MSP
CD4 SP
Mean rank diff. (line vs. collumn)

Figure 2

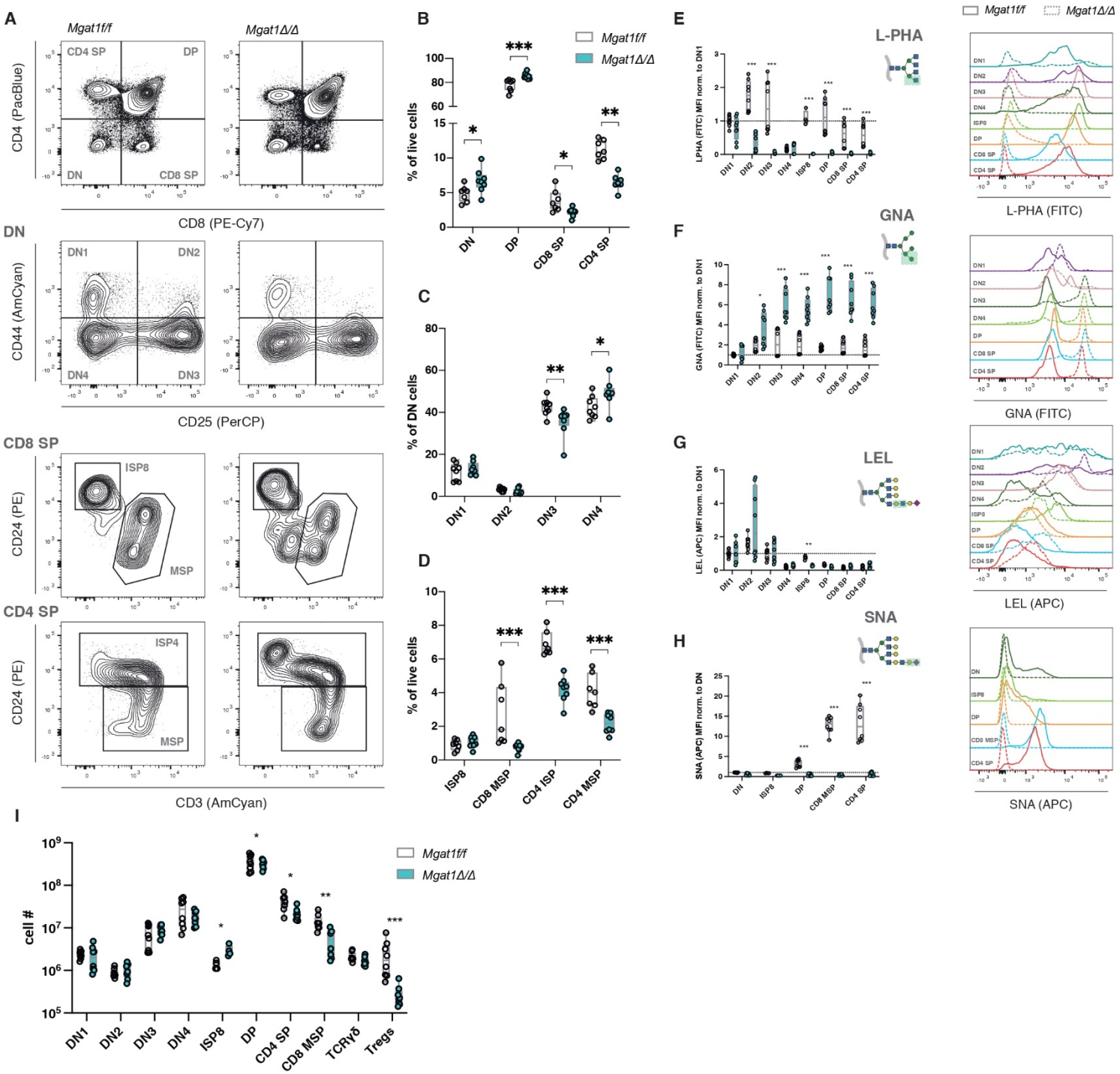

A
Mgat1f/f
Mgat1Δ/Δ
CD4 SP
DP
DN
CD8 SP
CD4 (PacBlue)
CD8 (PE-Cy7)
DN
DN1
DN2
DN4
DN3
CD44 (AmCyan)
CD25 (PerCP)
CD8 SP
ISP8
MSP
CD24 (PE)
CD4 SP
ISP4
MSP
CD24 (PE)
CD3 (AmCyan)
B
Mgat1f/f
Mgat1Δ/Δ
% of live cells
DN
DP
CD8 SP
CD4 SP
C
% of DN cells
DN1
DN2
DN3
DN4
D
% of live cells
ISP8
CD8 MSP
CD4 ISP
CD4 MSP
E
L-PHA
LPHA (FITC) MFI norm. to DN1
F
GNA
GNA (FITC) MFI norm. to DN1
G
LEL
LEL (APC) MFI norm. to DN1
H
SNA
SNA (APC) MFI norm. to DN
Mgat1f/f
Mgat1Δ/Δ
L-PHA (FITC)
GNA (FITC)
LEL (APC)
SNA (APC)
I
Mgat1f/f
Mgat1Δ/Δ
cell #
DN1
DN2
DN3
DN4
ISP8
DP
CD4 SP
CD8 MSP
TCRγδ
Tregs

Figure 3

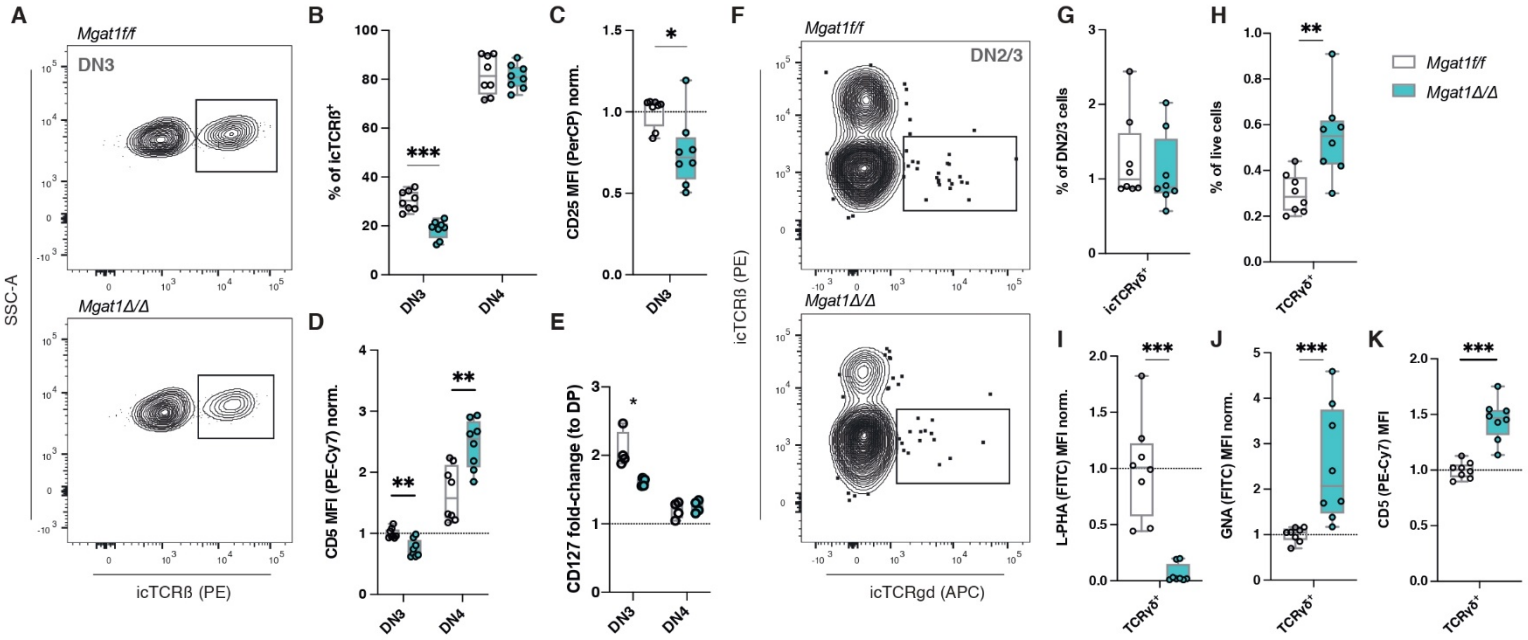

A
Mgat1f/f
DN3
Mgat1Δ/Δ
SSC-A
icTCRß (PE)
B
% of icTCRß+
***
DN3
DN4
C
CD25 MFI (PerCP) norm.
*
DN3
D
CD5 MFI (PE-Cy7) norm.
**
**
DN3
DN4
E
CD127 fold-change (to DP)
*
DN3
DN4
F
Mgat1f/f
DN2/3
Mgat1Δ/Δ
icTCRß (PE)
icTCRgd (APC)
G
% of DN2/3 cells
icTCRγδ+
H
**
% of live cells
TCRγδ+
Mgat1f/f
Mgat1Δ/Δ
I
***
L-PHA (FITC) MFI norm.
TCRγδ+
J
***
GNA (FITC) MFI norm.
TCRγδ+
K
***
CD5 (PE-Cy7) MFI
TCRγδ+

Figure 4

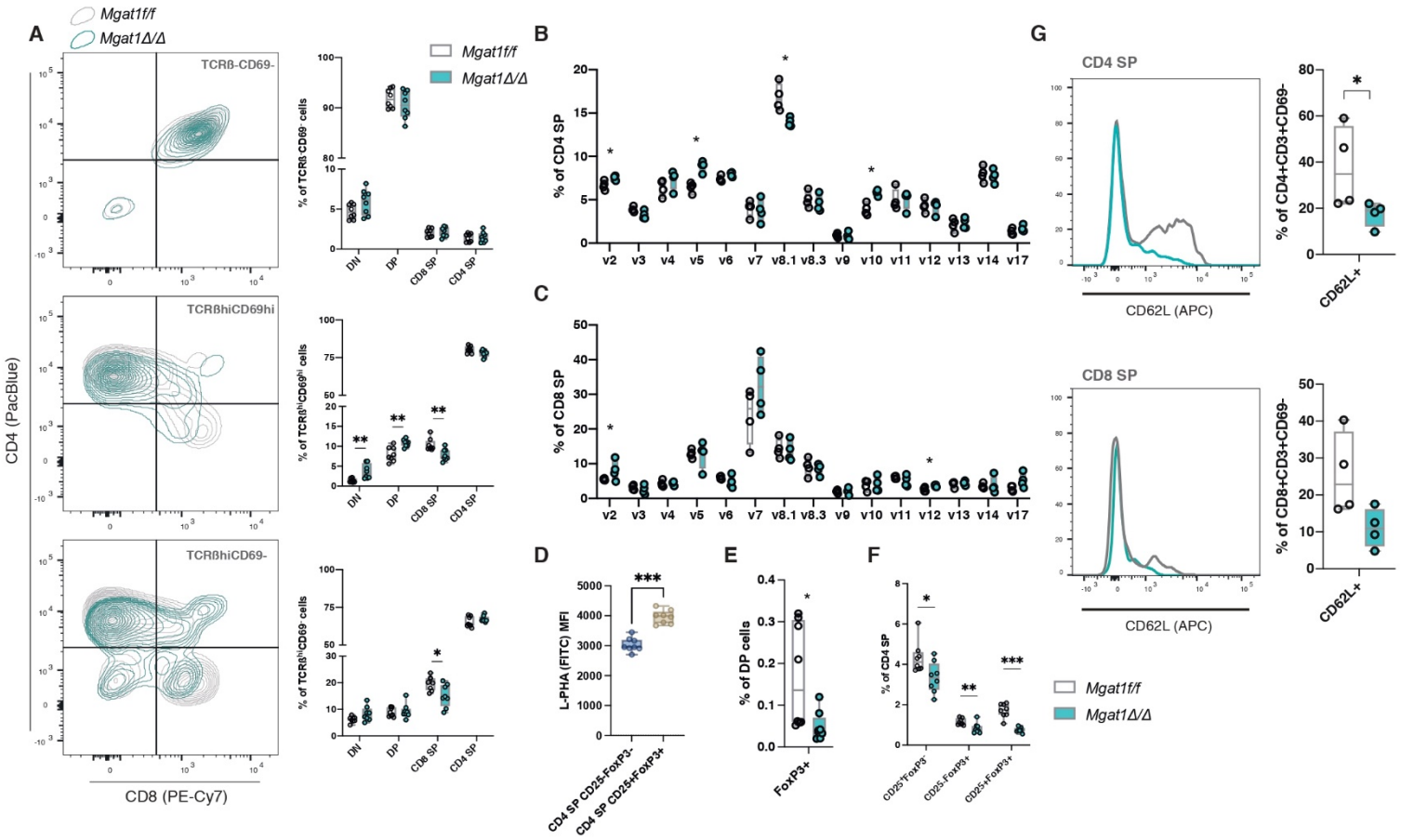
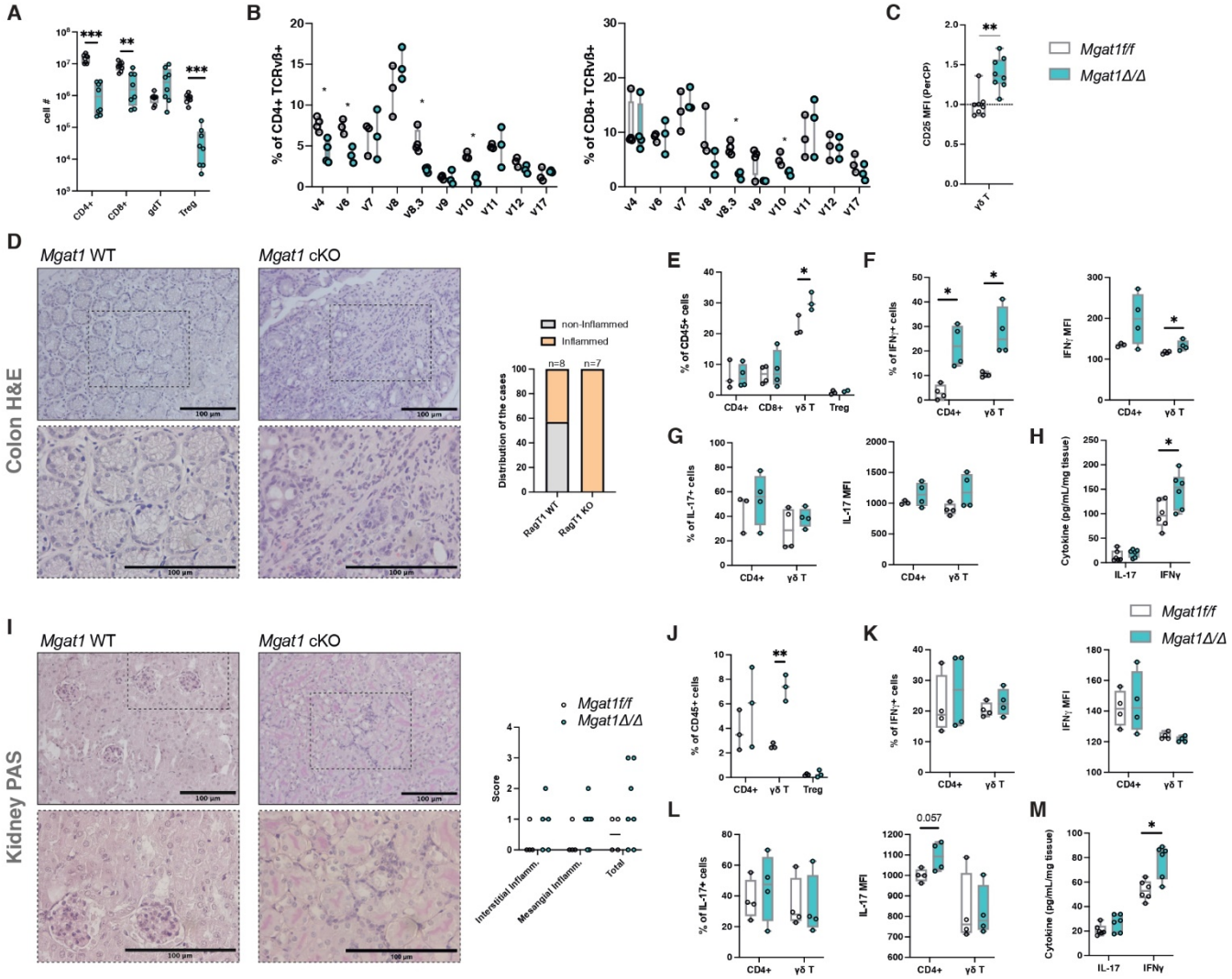

A
Mgat1f/f
Mgat1Δ/Δ
TCRß-CD69-
TCRßhiCD69hi
TCRßhiCD69-
CD4 (PacBlue)
CD8 (PE-Cy7)
% of TCRß CD69 cells
% of TCRßhiCD69hi cells
DN
DP
CD8 SP
CD4 SP
B
% of CD4 SP
v2 v3 v4 v5 v6 v7 v8.1 v8.3 v9 v10 v11 v12 v13 v14 v17
C
% of CD8 SP
D
L-PHA (FITC) MFI
CD4 SP CD25-FoxP3-
CD4 SP CD25+FoxP3+
E
% of DP cells
FoxP3+
F
% of CD4 SP
CD25-FoxP3-
CD25-FoxP3+
CD25+FoxP3+
G
CD4 SP
CD8 SP
CD62L (APC)
% of CD4+CD3+CD69-
% of CD8+CD3+CD69-
CD62L+
Mgat1f/f
Mgat1Δ/Δ

Figure 5


A
B
C
D
E
F
G
H
I
J
K
L
M
Mgat1 WT
Mgat1 cKO
Colon H&E
Kidney PAS
Mgat1f/f
Mgat1Δ/Δ
non-Inflammed
Inflammed
Distribution of the cases
Rag1 WT
Rag1 KO
Score
Interstitial Inflamm.
Mesangial Inflamm.
Total
CD4+
CD8+
γδ T
Treg
IL-17
IFNγ
0.057

Supplementary information

Supplementary Figures legends

Sup. Fig. 1: (A) Gating strategy for human thymocyte subsets identification. On top of dot plots the parent gate is detailed. **(B)** Unsupervised flow cytometry analysis of human thymocytes using tSNE, combining the data from 4 human thymi, with the main thymocyte populations annotated manually by the gating strategy defined in (A). Colormap for L-PHA and SNA (right) binding levels in the same tSNE representation. Colorbar indicates the levels of binding. **(C)** Gating strategy for murine thymocyte subsets identification. On top of dot plots the parent gate is detailed.

Sup. Fig. 2: (A) Thymocyte populations discrimination in *Mgat2*$^{f/f}$ (N = 8, 6-8 weeks old) and *Mgat2*$^{\Delta/\Delta}$ (N = 8, 6-8 weeks old). **(B)** Frequencies of DN (CD4-CD8-), DP (CD4+CD8+), CD8 SP (CD4-CD8+) and CD4 SP (CD4+CD8-) thymocyte subsets, within live cells. **(C)** Frequencies of DN1 (CD44+CD25-), DN2 (CD44+CD25+), DN3 (CD44-CD25+) and DN4 (CD44-CD25-) subsets, within the total DN population. **(D)** CD8 SP and CD4 SP subset frequencies: ISP8 (CD8+CD24+CD3-), CD8 MSP (CD8+CD24int/-CD3+), CD4 ISP (CD4+CD24hiCD3lo/hi) and CD4 MSP (CD4+CD24loCD3hi), within live cells. Levels of lectin binding (MFI levels) normalized to indicated population (left) and representative histograms of lectin binding profiles in indicated thymocyte subsets, for L-PHA **(E)**, GNA **(F)**, LEL **(G)** and SNA **(H)**. **(I)** Surface expression (MFI) of the CD4 and **(J)** CD8 co-receptors in indicated populations, normalized to the value of DP cells. **(K)** Absolute numbers of thymocytes in the indicated subsets. **(L)** Surface expression (MFI) of the CD4 and **(M)** CD8 co-receptors in indicated populations, normalized to the value of

DP cells.Each dot represents one mouse in all graphs. Mann-Whitney t-test, *p*-value *< 0.05, **< 0.005 and ***< 0.001.

Sup. Fig. 3: (A) Gating strategy for the identification of ß-selected (icTCRß+) DN3 cells and **(B)** frequencies of those cells in DN3 and DN4 subsets, in *Mgat2*$^{f/f}$ (N=8) and *Mgat2*$^{\Delta/\Delta}$ (N=8). **(C)** Quantification of CD25 surface levels (MFI) in the DN3 subset, normalized to the mean of *Mgat2*$^{f/f}$ DN3 levels. **(D)** Quantification of CD5 and **(E)** CD127 surface levels (MFI) in DN3 and DN4 thymocytes, normalized to the mean of *Mgat2*$^{f/f}$ DN3 levels, and CD127 DP levels, respectively. **(F)** Gating strategy for the identification of icTCRγδ+ cells within the DN2/3 subset and **(G)** frequencies, in *Mgat2*$^{f/f}$ (N=8) and *Mgat2*$^{\Delta/\Delta}$ (N=8). Frequency of total thymic mature TCRγδ+ cells within live cells. **(I)** Quantification of CD27+ cells within the mature TCRγδ+ population. **(J)** Levels of L-PHA and **(K)** GNA binding levels, in thymic TCRγδ+ cells, normalized to the mean of *Mgat2*$^{f/f}$ levels. **(L)** CD5 surface levels (MFI) of TCRγδ+ cells, normalized to the mean of *Mgat2*$^{f/f}$ levels. Each dot represents one mouse. Mann-Whitney t-test, p-value *< 0.05, **< 0.005 and ***< 0.001.

Sup. Fig. 4: (A) and **(D)** Identification of pre-selection (TCRß$^{-/lo}$CD69^{-}), post-positive selection (TCRß$^{int/hi}$CD69$^{int/hi}$) and post-negative selection (TCRß^{hi}CD69^{-}) thymocytes in *Mgat1*$^{f/f}$ (N = 8) and *Mgat1*$^{\Delta/\Delta}$ (N = 8), and *Mgat2*$^{f/f}$ (N = 8) and *Mgat2*$^{\Delta/\Delta}$ (N = 8) mice, respectively. **(B)** and **(E)** Quantification of the frequencies of the populations identified in **(A)** and **(D)**. **(C)** and **(F)** Quantification of Annexin V+ cells within the thymocyte populations in *Mgat1*$^{f/f}$ (N = 4) and *Mgat1*$^{\Delta/\Delta}$ (N = 4), and *Mgat2*$^{f/f}$ (N = 4) and *Mgat2*$^{\Delta/\Delta}$ (N = 4) mice, respectively. **(G)** Distribution of each selection subset, pre-selection, post-

positive selection and post-negative selection thymocytes, according to its CD4 and CD8 expression levels, and quantification in *Mgat2*$^{f/f}$ (N = 8) and *Mgat2*$^{\Delta/\Delta}$ (N = 8). **(H)** Screen of TCRvß+ expressing cells, within thymic mature CD4 SP and **(I)** CD8 SP cells, in *Mgat2*$^{f/f}$ (N = 4) and *Mgat2*$^{\Delta/\Delta}$ (N = 4). **(J)** Quantification of the frequency of FoxP3+ cells in the DP population. **(K)** Frequencies of CD25+FOXP3-, CD25-FOXP3+ and CD25+FOXP3+ subsets with CD4 SP thymocytes. **(L)** Identification of CD62L^{+} cells in mature CD4 SP and CD8 SP, in *Mgat2*$^{f/f}$ (N = 4) and *Mgat2*$^{\Delta/\Delta}$ (N = 4) mice. Each dot represents one mouse. Mann-Whitney t-test, p-value *< 0.05, **< 0.005 and ***< 0.001.

Sup. Fig. 5: (A) Splenic T-cell numbers cell numbers in *Mgat2*$^{f/f}$ (N = 8) and *Mgat2*$^{\Delta/\Delta}$ (N = 8). **(B)** Screen of TCRvß+ expressing cells, within splenic mature CD4+ (right) and CD8+ T-cells (right), in *Mgat2*$^{f/f}$ (N = 4) and *Mgat2*$^{\Delta/\Delta}$ (N = 4). **(C)** Levels of CD25 surface expression (MFI) in splenic γδ T-cells, normalized to the mean of *Mgat2* WT levels. **(D)** H&E staining of colon sections, at 20x (top) and 40x magnification (bottom), and specimen distribution according to overall detection of inflammation cues (right). Scale bars indicate 100 um. **(E)** T-cell population frequencies within CD45+ cells, isolated from colon tissues. **(F)** Frequencies of IFNγ-producing cells within CD4+ and γδ T-cells and IFNγ expression (MFI) in the same subsets. **(G)** Frequencies of IL-17-producing cells within CD4+ and γδ T-cells and IL-17 expression (MFI) in the same subsets. **(H)** IL-17 and IFNγ concentration in colon explant culture supernatants, normalized to tissue weight. **(I)** PAS staining of colon sections, at 20x (top) and 40x magnification (bottom), and scores of intersticial and mesangial inflammation (right). Scale bars indicate 100 um. **(J)** T-cell population frequencies within CD45+ cells, isolated from kidney tissues. **(K)** Frequencies

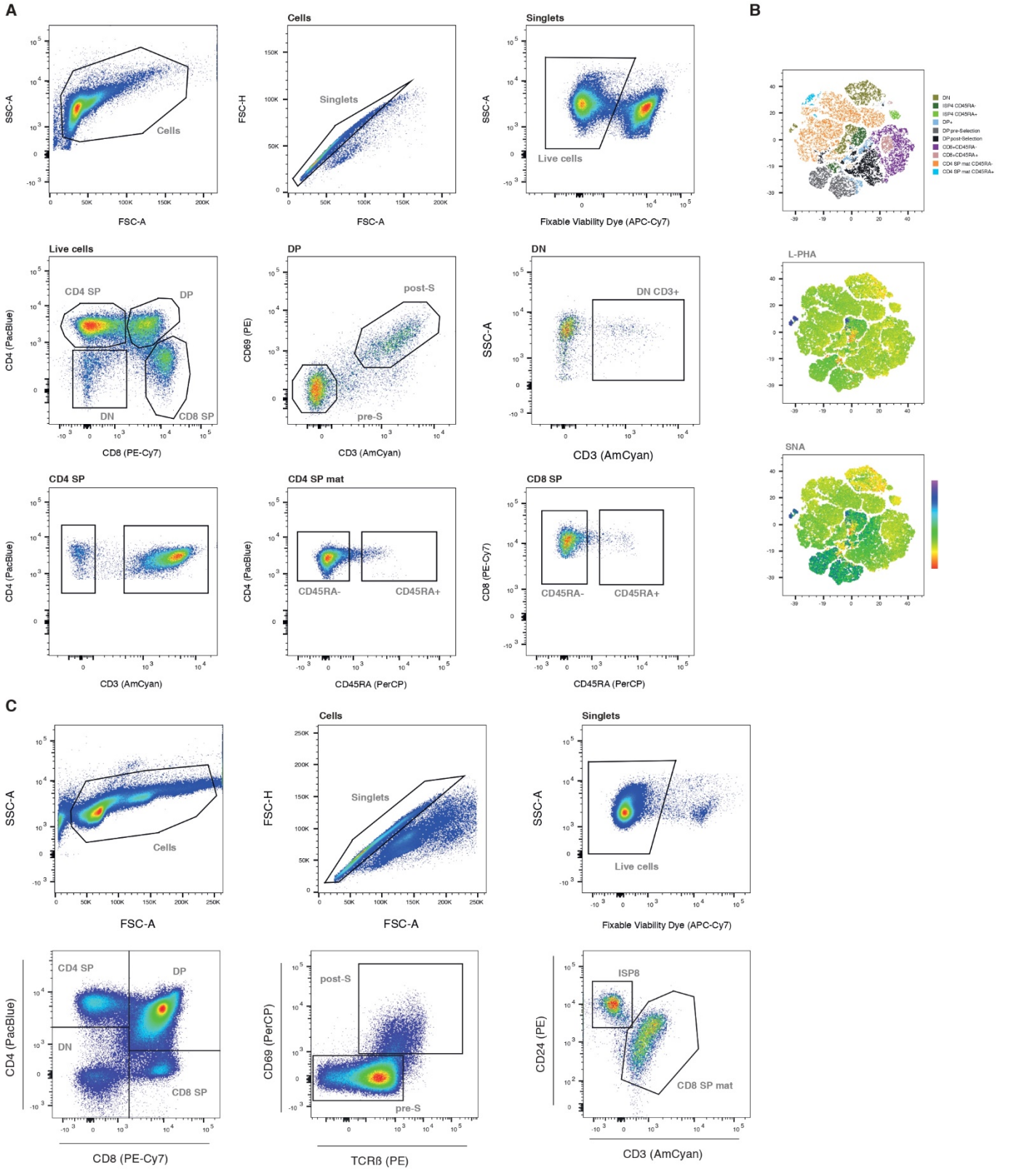
of IFNγ-producing cells within CD4+ and γδ T-cells and IFNγ expression (MFI) in the same subsets. **(L)** Frequencies of IL-17-producing cells within CD4+ and γδ T-cells and IL-17 expression (MFI) in the same subsets. **(M)** IL-17 and IFNγ concentration in colon explant culture supernatants, normalized to tissue weight. **(N)** Immunohistochemistry staining for CD3+ cells in colon sections. **(O)** and **(P)** Fecal calprotectin concentration analysis for *Mgat1*$^{f/f}$ (N = 5) and *Mgat1*$^{\Delta/\Delta}$ (N = 7), and *Mgat2*$^{f/f}$ (N = 5) and *Mgat2*$^{\Delta/\Delta}$ (N = 4). **(Q)** Serum concentrations of IFNγ and IL-17. **(R)** *N. caninum* organ colonization determination, through the quantification of total parasite DNA in 1 mg of total host DNA, in *Mgat1*$^{f/f}$ (N = 3 - 4) and *Mgat1*$^{\Delta/\Delta}$ (N = 4). **(S)** T-cell frequencies of the spleens of infected mice and frequencies of CD69+ cells within CD4+ and CD8+ splenic T-cells. **(T)** *N. caninum* organ colonization determination, through the quantification of total parasite DNA in 1 mg of total host DNA, in *Mgat2*$^{f/f}$ (N = 3) and *Mgat2*$^{\Delta/\Delta}$ (N = 3). Each dot represents one mouse. Mann-Whitney t-test, p-value *< 0.05, **< 0.005 and ***< 0.001.

Supplementary Table 1

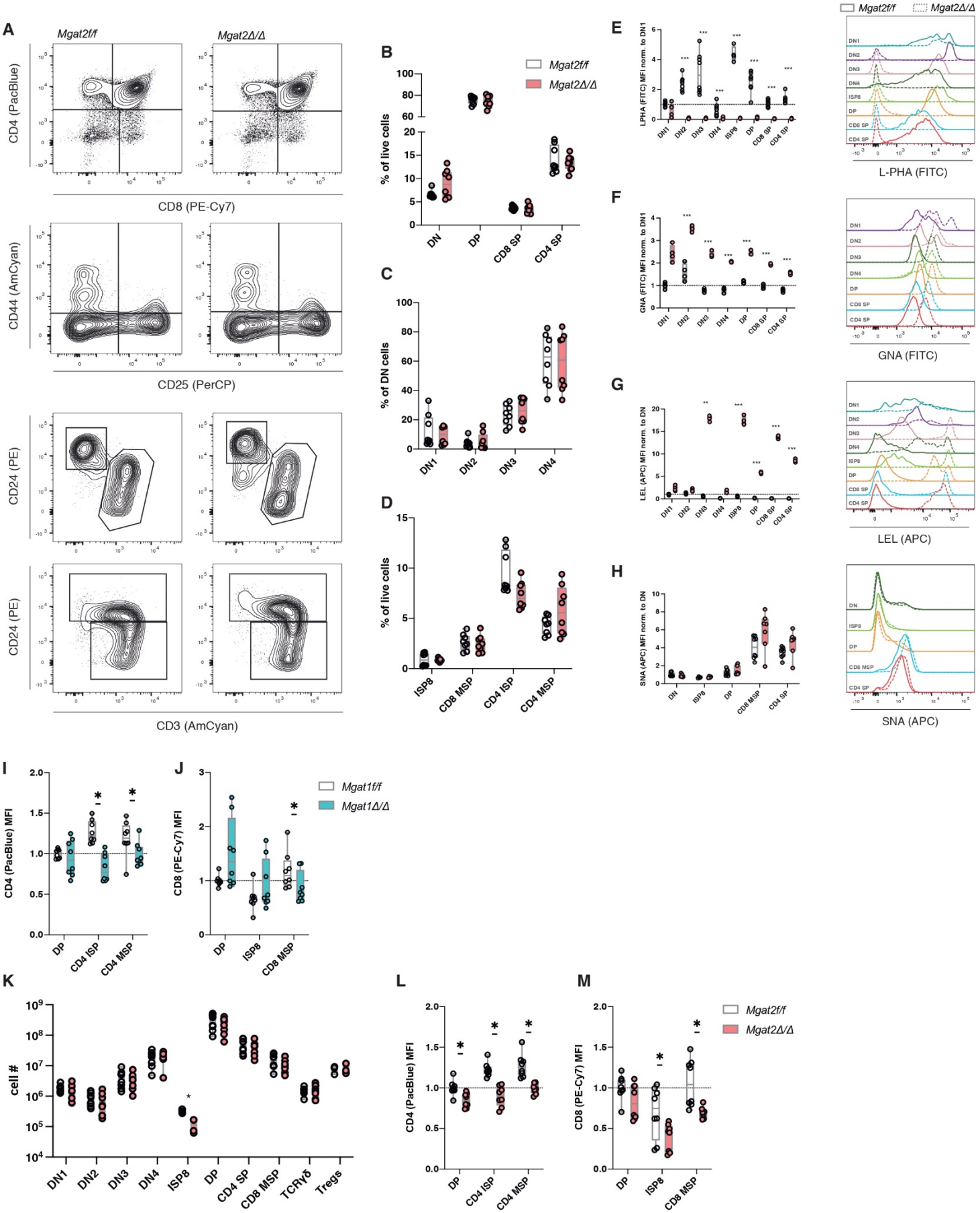
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
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human CD8 (RPA-T8) PE-Cy7 1:400	BD Pharmigen	557750
human CD69 (FN50) PE 1:100	Biolegend	310905
human CD3 (OKT3) Brilliant Violet 510 1:100	BD Pharmigen	566779
human CD45RA PerCP 1:100	eBioscience	45-0458-42
mouse CD4 (RM4-5) eFluor 450 1:300	eBioscience	48-0042-80
mouse CD8alpha (53-6.7) PE-Cy7 1:500	eBioscience	25-0081-81

mouse CD69 (H1.2F3) PerCP-Cy5.5 1:200	eBioscience	45-0691-80
mouse CD3 (17A2) Brilliant Violet 510 1:300	Biolegend	100233
mouse CD24 (M1/69) PE 1:800	eBioscience	12-0242-81
mouse TCRß (H57-597) PE 1:800	eBioscience	12-5961-81
mouse CD25 (PC61.5) PE-Cy5 1:400	eBioscience	35-0251-80
mouse CD44 (IM7) eFlour 506 1:100	eBioscience	69-0441-80
mouse CD5 (53-7.3) PE-Cy5 1:200	Biolegend	100609
mouse CD127 (A7R34	eBioscience	11-1271-81
mouse TCRgd (eBioGL3) APC 1:400	eBioscience	17-5711-81
mouse CD27 (LG.7F9) SuperBright 436 1:200	eBioscience	62-0271-80
mouse FoxP3 (FJK-16S) APC 1:200	eBioscience	17-5773-80
mouse CD62L (MEL-14) APC 1:200	eBioscience	A14720
mouse CD25 (PC61.5) PE 1:400	eBioscience	12-0251-81
mouse CD19 (eBio1D3) APC 1:500	eBioscience	17-0193-80
Phaseolus vulgaris Leucoagglutinin FITC 1:1000	Vector Laboratories	FL-1111-2
Galanthus nivalis lectin FITC	Vector Laboratories	FL-1241-2
Sambucus nigra lectin Cy5 1:1000	Vector Laboratories	CL-1305-1
Lycopersicon esculentum lectin DyLight594 1:1000	Vector Laboratories	DL-1177-1

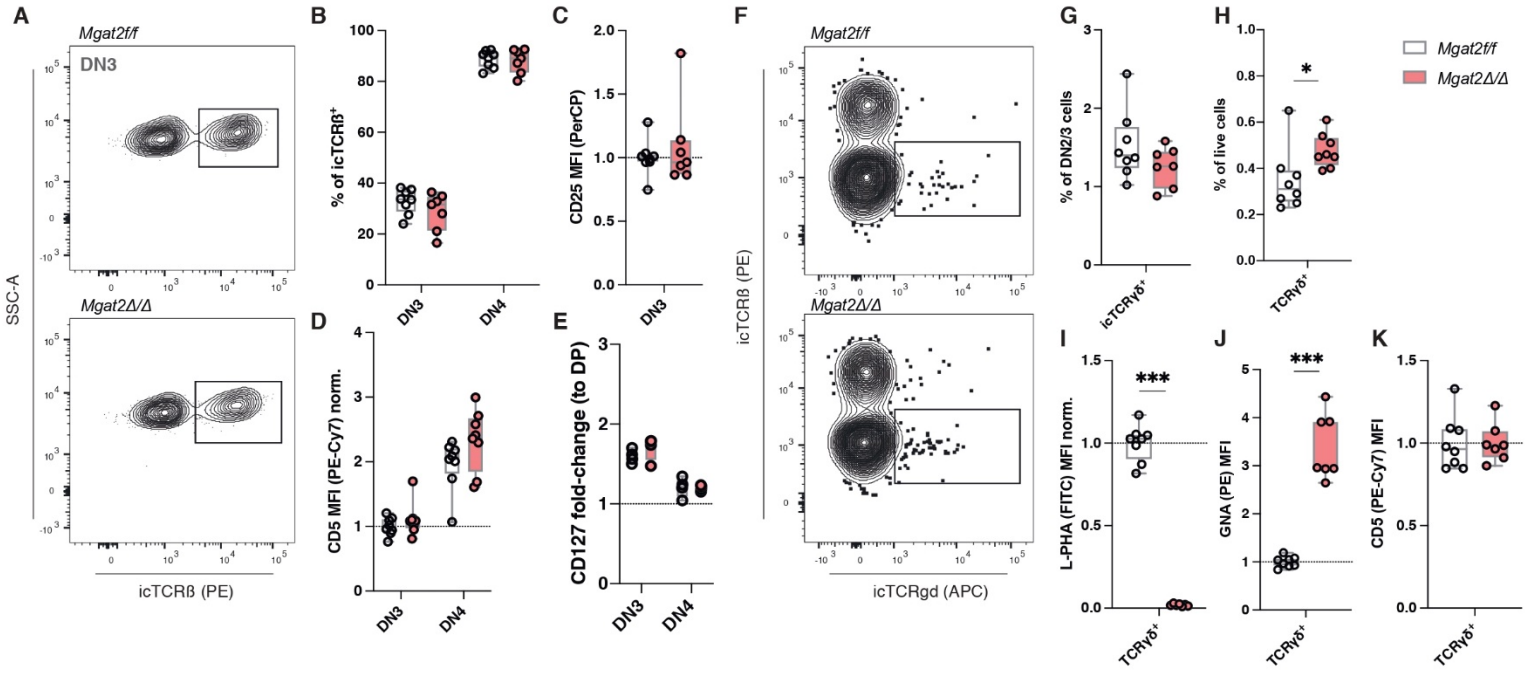
SuppFigure 1



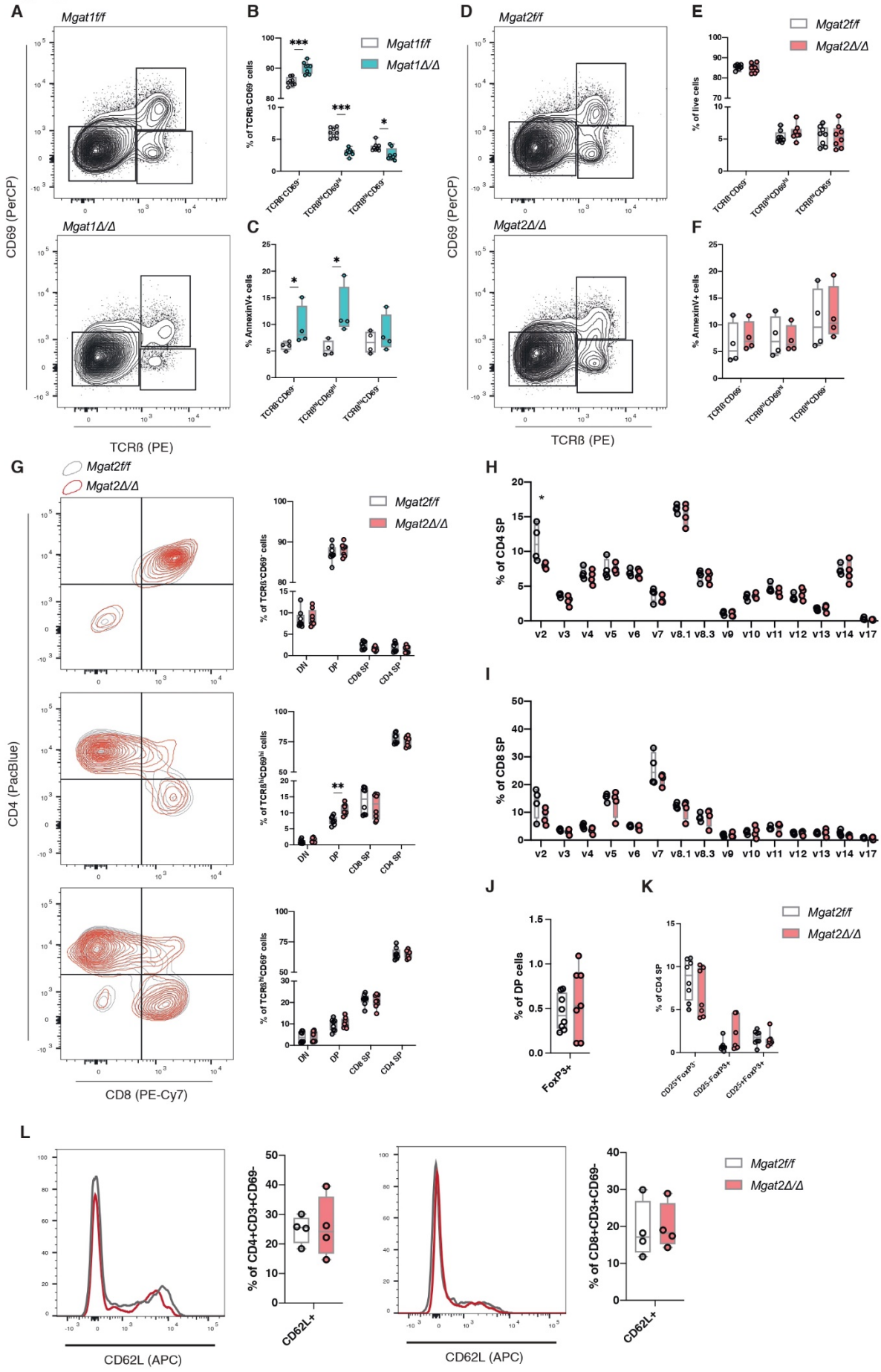
SuppFigure 2



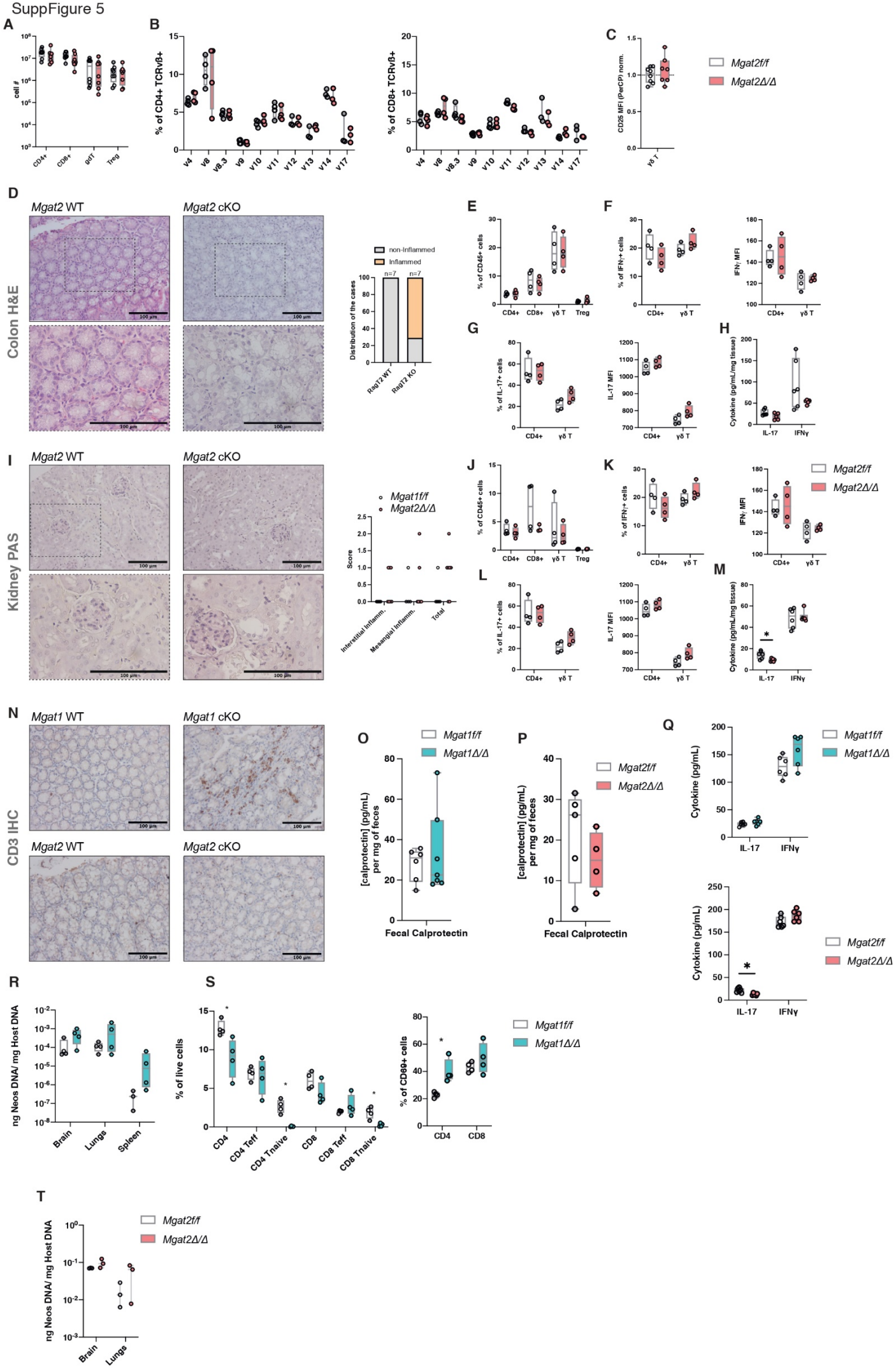
SuppFigure 3



SuppFigure 4



SuppFigure 5



CHAPTER 3

[RESEARCH PAPER]

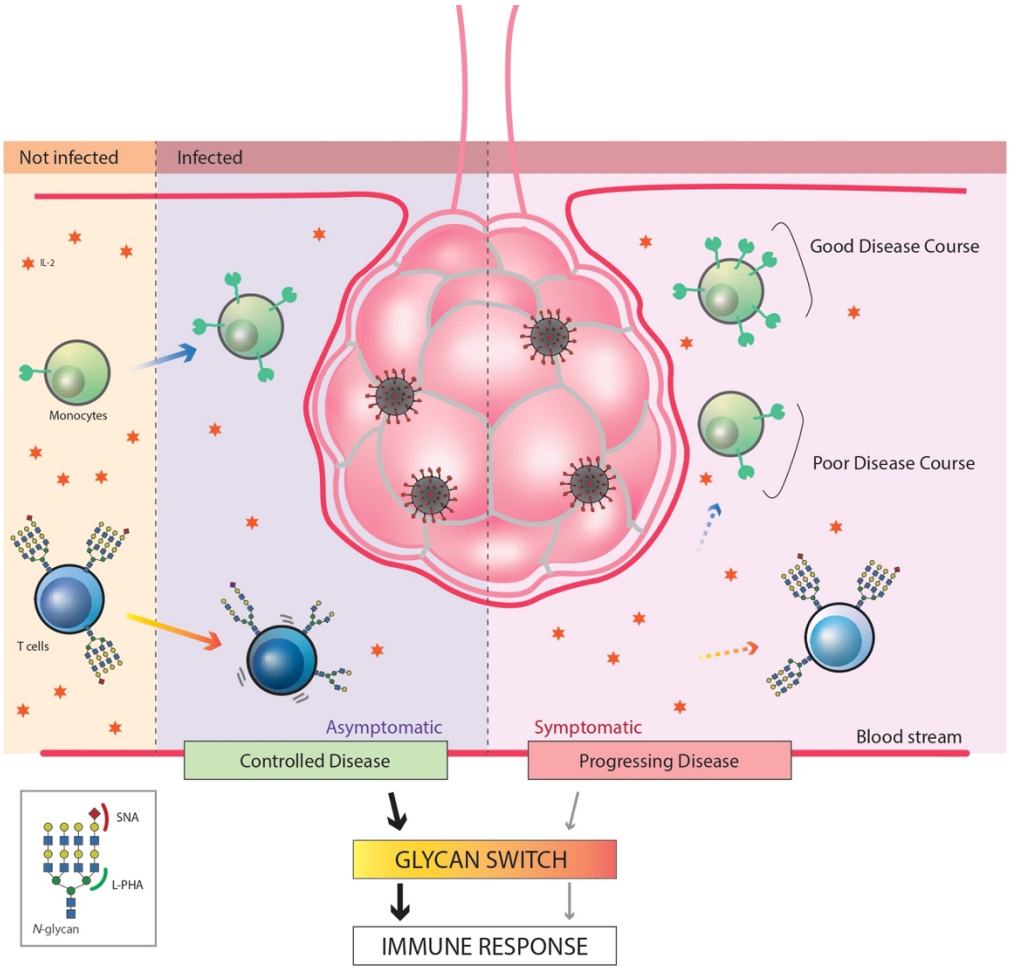
SARS-COV-2 INFECTION DRIVES A GLYCAN SWITCH OF PHERIPHERAL T CELLS AT DIAGNOSIS

Inês Alves*, **Manuel M. Vicente***, Joana Gaifem, Ângela Fernandes, Ana M. Dias, Cláudia S. Rodrigues, José C. Oliveira, Nair Seixas, Luis Malheiro, Miguel A. Abreu, Rui S. Castro, Salomé S. Pinho

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* These authors contributed equally

Graphical Abstract



SARS-CoV-2 Infection Drives a Glycan Switch of Peripheral T Cells at Diagnosis

Inês Alves,[*,†,‡,1] Manuel Machado Vicente,[*,†,§,1] Joana Gaifem,[*,†] Ângela Fernandes,[*,†] Ana Mendes Dias,[*,†] Cláudia Sousa Rodrigues,[*,†,§] José Carlos Oliveira,[¶] Nair Seixas,[‖] Luis Malheiro,[#] Miguel Araújo Abreu,[§,] Rui Sarmento e Castro,[§,**] and Salomé Soares Pinho[*,†,‡,§]**

COVID-19 is a highly selective disease in which SARS-CoV-2 infection can result in different clinical manifestations ranging from asymptomatic/mild to severe disease that requires hospitalization. In this study, we demonstrated that SARS-CoV-2 infection results in a glycosylation reprogramming of circulating lymphocytes at diagnosis. We identified a specific glycosignature of T cells, defined upon SARS-CoV-2 infection and apparently triggered by a serological factor. This specific glycan switch of T cells is detected at diagnosis being more pronounced in asymptomatic patients. We further demonstrated that asymptomatic patients display an increased expression of a viral-sensing receptor through the upregulation of DC-SIGN in monocytes. We showed that higher levels of DC-SIGN in monocytes at diagnosis correlates with better COVID-19 prognosis. This new evidence pave the way to the identification of a novel glycan-based response in T cells that may confer protection against SARS-CoV-2 infection in asymptomatic patients, highlighting a novel prognostic biomarker and potential therapeutic target. ***The Journal of Immunology*****, 2021, 207: 1591–1598.**

Coronavirus SARS-CoV-2 is the etiologic agent responsible for the global pandemic of coronavirus disease 2019 (COVID-19), that, as of February 9, has infected (IF) over 106 million people worldwide (1, 2). The overall mortality of COVID-19 is between 0.5 and 3.5% (1, 2). COVID-19 is a highly selective disease. In fact, only some IF individuals get sick, and although most of the critically ill are elderly, some patients that die are previously healthy and/or relatively young (3). There is an urgent need to improve the understanding of the pathophysiology of this disease, envisioning better management in terms of patient care, treatment options, vaccination strategies, and the allocation of health care resources. Vaccine development against SARS-CoV-2 poses a great promise for the resolution of the pandemic, and several vaccines are now being distributed among the world population, namely mRNA- and protein-based approaches (4, 5). However, its long-term protection, effectiveness to reinfection, efficacy to new variants, and availability for the general public is still on trial. This lack of knowledge highlights the importance of understanding patient-specific immune response to infection, envisioning the identification of novel mechanisms of disease. Moreover, comprehensive insights on COVID-19 molecular mechanism may be translated into diagnostic or prognostic biomarkers, therapeutic targets to improve patient's clinical management, and patients stratification for vaccination.

SARS-CoV-2 infection results in a broad range of symptoms (6). Several studies on immune profiling of IF patients consistently revealed an immunological dysregulation, observed in PBMCs. A decrease of T cells and dendritic cells frequencies and an increase of monocytes and neutrophils have been observed in hospitalized IF individuals, as well as a cytokine dysregulation, the so-called "cytokine storm" associated with critical illness (7–10). However, the mechanism underlying this immunological dysregulation remains largely unknown.

Despite being overlooked, the field of glycobiology can provide missing answers to immunological questions. Glycosylation is a major posttranslational mechanism characterized by the enzymatic addition of glycans (carbohydrates) to proteins or lipids of essentially all cells, including immune cells. In fact, glycans are master regulators of immune cell functions, defining the activation and differentiation of T cells (11, 12). In the cellular immune system, we and others have previously demonstrated the regulatory power of

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I.A., M.M.V., and S.S.P. designed research. I.A., M.M.V., J.G., Â.F., A.M.D., and C.S.R. performed research. S.S.P. contributed new reagents/analytical tools. J.C.O., N.S., L.M., M.A.A., and R.S.eC. provided and characterized clinical samples. I.A. and M.M.V. analyzed data. I.A., M.M.V., and S.S.P. wrote the manuscript with contributions from all authors.

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The online version of this article contains supplemental material.

Abbreviations used in this article: AAL, *Aleuria aurantia* lectin; CHUP, Centro Hospitalar e Universitário do Porto; CHVNG, Centro Hospitalar de Vila Nova de Gaia/Espinho; COVID-19, coronavirus disease 2019; DC-SIGN, dendritic cell–specific ICAM-3–grabbing nonintegrin; GNA, *Galanthus nivalis* lectin; IF, infected; L-PHA, *Phaseolus vulgaris* leucoagglutinin; SNA, *Sambucus nigra* lectin.

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glycans in adaptive immune response through the modulation of T cell activation thresholds associated with the immunopathogenesis of autoimmune diseases and cancer (13–19). Moreover, being present in several pathogens, glycans can also be sensed by immune cells through their recognition by specific glycan-recognizing receptors expressed in those cells (20, 21). Like the majority of viruses, SARS-CoV-2 viral capsids are also covered with glycans. More specifically, the spike protein of the SARS-CoV-2 envelope was shown to be highly glycosylated (harboring 22 *N*-glycosylation sites) with glycan structures such as oligomannose and some branched *N*-glycans (22, 23). These structures can be specifically recognized by glycan binding proteins of the host's immune cells, such as the C-type lectin dendritic cell–specific ICAM-3–grabbing nonintegrin (DC-SIGN), present in innate immune cells, promoting virus recognition and elimination (24, Amraei, R., W. Yin, M. A. Napoleon, E. L. Suder. J. Berrigan, Q. Zhao, J. Olejnik, K. B. Chandler, C. Xia, J. Feldman, et al., manuscript posted on medRxiv, DOI: 10.1101/2020.06.22.165803, and Hoffmann, D., S. Mereiter, Y. Jin Oh, V. Monteil, R. Zhu, D. Canena, L. Hain, E. Laurent. C. Grünwald-Gruber, M. Novatchkova, et al., manuscript posted on medRxiv, DOI: 10.1101/2021.04.01.438087).

Defining the glycosylation profiles of immune cells in SARS-CoV-2–IF individuals and how they impact their effector functions remains completely unknown, but it is of the utmost importance for the understanding of COVID-19 immunopathogenesis as well as for the improvement of the clinical management of the disease and for pandemic control measures, namely as a stratification biomarker.

In this study, we discovered that circulating T cells exhibit a glycan switch upon SARS-CoV-2 infection that is detected at COVID-19 diagnosis. This change in the glycosylation profile of T cells appears to be triggered by a serum inflammatory factor present in IF patients. This specific T cell glycan switch is more pronounced in asymptomatic patients rather than in those who exhibit symptoms. Importantly, from a clinical standpoint, we also unveiled that COVID-19 patients with "good prognosis" exhibit an upregulation of DC-SIGN expression in circulating monocytes.

Materials and Methods

Cohort description and patient's selection criteria

The present study integrates a total of 32 patients diagnosed with SARS-CoV-2 from two individual Portuguese cohorts (between May 2020 and July 2020), 20 patients from the Infectious Disease Department of Centro Hospitalar e Universitário do Porto (CHUP) (Porto, Portugal), and 12 patients from the Infectious Disease Department of Centro Hospitalar de Vila Nova de Gaia/Espinho (CHVNG) (Vila Nova de Gaia, Portugal). The total cohort includes patients of both genders and age between 22- and 89-y-old. All the samples were included in all the FACS analysis discussed.

Blood was collected, and plasma and PBMCs were isolated at the time of diagnosis, within the first 72 h of PCR^+ SARS-CoV-2 test, and 14 d ($n = 2$) after diagnosis. Similar analysis was conducted in a subset of patients ($n = 2$) that recovered.

The eligibility criteria for inclusion in this study were SARS-CoV-2–positive patients asymptomatic or symptomatic with different levels of severity (mild, moderate, and severe). Asymptomatic patients ($n = 5$) were defined as positive for the SARS-CoV-2 PCR test but no signs of diseases. The following criteria were used to stratify symptomatic SARS-CoV-2 patients in terms of the disease severity and accordingly with World Health Organization guidelines (25): 1, mild ($n = 18$): individuals with no evidence of pneumonia; 2, moderate ($n = 6$): individuals without need of invasive mechanical ventilation and without need of admission to hospital intensive care unit, but there is evidence of pneumonia and need of supplementary oxygen; and 3, severe ($n = 1$): individuals with need of invasive mechanical ventilation and with need of admission to hospital intensive care unit. Severity was determined both at diagnosis and 14 d postdiagnosis. Patient demographics and relevant clinical data are summarized in Supplemental Table I.

Healthy controls ($n = 5$) are represented by volunteer individuals with no history of infection disorders that went to CHUP for routine analysis. Additionally, to evaluate the predictive value of DC-SIGN expression, patients were grouped according to disease severity at day 14 postdiagnosis. Patients were then classified as having a good prognosis when they were asymptomatic at diagnosis and remained without symptoms throughout the follow-up and until day 14 postdiagnosis, as well as symptomatic patients at diagnosis that improved over the disease course, alleviating symptoms at day 14 postdiagnosis. Patients that either did not improve symptoms and disease severity or aggravated symptoms by displaying worsened disease at day 14 postdiagnosis were classified as "poor prognosis."

All participants gave informed consent about all clinical procedures, and research protocols were approved by the ethics committee of CHUP and CHVNG in Portugal.

Human PBMCs isolation

Human PBMCs from COVID-19 patients and healthy donors were isolated by gradient centrifugation using 1 vol of Lymphoprep (STEMCELL Technologies) for 2 vol of blood for 30 min at 900 × *g* with the brake off. The upper phase containing the serum was collected and stored at −80°C. PBMCs (interphase) were collected, washed twice with PBS, and incubated with FVD–allophycocyanin-eFluor780 (eBioscience) for 30 min. Cells were washed with PBS, fixed with 2% formaldehyde (PanReac AppliChem), and resuspended in FACS buffer (PBS 2% FBS). All procedures were performed under biosafety level 3 conditions.

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-fluorescein, FITC), biotinylated *Sambucus nigra* lectin (SNA), and biotinylated *Aleuria aurantia* lectin (AAL) for 15 min. Biotinylated lectins were incubated with streptavidin-PE-Cy7 (eBioscience) for 30 min. For surface marker staining, cells were stained for 30 min on ice while protected from light with the following Abs: allophycocyanin anti-human TCRγδ (clone B1), BV510 anti-human CD69 (clone FN50), PE anti-human CD25 (clone BC96), BV421 anti-human CD14 (clone 63D3), PE anti-human CD56 (clone HCD56), and PE-Cy7 anti-human CD206 (clone 15-2) from BioLegend; eFluor 450 anti-human CD4 (clone RPA-T4), PerCP-eFluor 710 anti-human PD-1 (clone J105), PE-Cy5 anti-human CD19 (clone HIB19), eFluor 450 anti-human IgM (clone SA-DA4), eFluor 660 anti-human galectin 3 (clone M3/38), allophycocyanin anti-human CD11c (clone BU15), and PE-Cy5 anti-human CD86 (clone IT2.2) from eBioscience; PE anti-human TCRα/β (clone BW242/412) from Miltenyi Biotec; and BV510 anti-human CD3 (clone OKT3) from BD Biosciences. For DC-SIGN staining, cells were incubated with DC-SIGN rabbit IgG (Bio-Rad Laboratories) followed by incubation with polyclonal swine anti-rabbit IgG (FITC; Dako) for 30 min on ice. Cells were resuspended in FACS buffer prior to analysis. Data were obtained on a BD FACS Canto II instrument (Becton Dickinson) and analyzed using FlowJo v10.0 (Tree Star).

IL-2 serum quantification

Plasma from four non-IF and eight COVID-19 patients (four asymptomatic and four moderate disease patients) were analyzed by flow cytometry using the BD Cytometric Bead Array Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences), following the manufacturer's instructions.

In vitro assessment of glycan modulation of T cells

PBMCs from healthy and independent from the COVID-19 cohort human donors were isolated from fresh collected blood, as described above. PBMCs were cultured in RPMI-1640 (Life Technologies) supplemented with 10% (v/v) heat-inactivated FBS, 1% penicillin/streptomycin, and 10% of plasma from four asymptomatic or moderate patients for each condition. Different cell culture conditions were tested, namely human plasma concentration (25 and 10%) as well as time of culture (4 d, 2 d, 24 h, and 12 h). The optimal culture condition (with lower cell death and stronger glycan modulation) was selected (10% human plasma and 24 h cell culture). After 24 h, cell glycoprofile was evaluated using the same Abs and lectin panel selected above. Serum IL-2 was neutralized by incubating PBMCs from healthy donors with 20 μg/ml of anti–IL-2 (5334, R&D Systems) and 10% serum from non-IF and COVID-19 patients (four asymptomatic and four moderate patients) for 24 h. After 24 h, the cellular glycoprofile was evaluated as above. Data were obtained on a BD FACS Canto II instrument (Becton Dickinson) and analyzed using the FlowJo 10.0 software (Tree Star).

Data visualization and statistical analysis

Data visualization and statistical analyses (nonparametric Mann–Whitney *t* test) were done using GraphPad Prism 9 software.

The prediction capacity of DC-SIGN levels to discriminate patients who develop a poor disease course from those that have a good disease course

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was determined by plotting the receiver operating characteristic curves and calculating the area under the curve. The cut-off that revealed the best balance between sensitivity and specificity was selected for the subsequent statistical analysis. Sensitivity, specificity, and positive and negative predictive values were calculated. Univariate binary logistic regression analysis was performed to test DC-SIGN levels and disease progression (good: asymptomatic maintained asymptomatic and/or severity decreased at least one category; poor: symptoms were not improved and/or escalate to a higher severity). In logistic regression, model goodness-of-fit was assessed by the Hosmer–Lemeshow statistical test. Results are presented as odds ratios for each category as compared with a predefined reference category and their respective 95% confidence intervals. Odds ratios above one and below one are indicative, respectively, of higher and lower odds of developing a poor disease course as compared with a reference category. Statistical analysis was performed using the statistical software SPSS version 25 (IBM, IBM SPSS Statistics for Windows, Version 25.0, Armonk, NY; released 2017) The threshold used for statistical significance was p value < 0.05.

Results

SARS-CoV-2–IF individuals display decreased β1,6-GlcNAc–branched and α2,6-sialic acid N*-glycans on peripheral T cells at diagnosis*

Taking into consideration the fact that the underlying mechanisms that explain a differential interindividual clinical presentation of COVID-19 at diagnosis are still unclear, we, in this study, characterized the glycosylation profile of T cells from patients' peripheral blood. A lectin-based flow cytometry of the T cell populations was performed to evaluate glycosylation profiles (gating strategy in Supplemental Fig. 1A). We started by analyzing the levels of the expression of β1,6-GlcNAc–branched *N*-glycan structures, known to have a major impact on the regulation of T cell activity and function (13, 16, 26), using the L-PHA lectin. Our results demonstrated that, overall, T cells from IF subjects have a decrease in branched *N*-glycan structures, particularly on $CD8^+$ and γδ T cells, when compared with non-IF ones (Fig. 1A, top and bottom left panel). Moreover, T cells from asymptomatic patients have lower levels of β1,6-GlcNAc–branched *N*-glycans that gradually tend to increase along disease severity (Fig. 1A, right). Notably, $CD8^+$ T cells display a significant decrease in the levels of the expression of complex branched *N*-glycans in asymptomatic and mild disease patients when compared with non-IF individuals (Fig. 1A, top).

Concerning α2,6-sialylation (recognized by the SNA lectin), already described to play a role in the regulation of T cell immune response (27), our results demonstrate that $CD8^+$ T cells from IF patients display a significantly decreased SNA binding, in comparison with non-IF individuals (Fig. 1B, top left). Consistently, asymptomatic patients present the lowest levels of SNA binding compared with non-IF (Fig. 1B, top right). Even though the levels of SNA binding were shown to be associated with age (Supplemental Fig. 1E), there are no significant differences between the mean age of asymptomatic and symptomatic patients (Supplemental Table I). Curiously, for both glycans, the two COVID-19 convalescent patients seem to recover (or surpass) the levels of expression of β1,6-GlcNAc–branched and α2,6-sialic acid glycan structures in all T cell subsets (Fig. 1A, 1B, right). These results showed that $CD8^+$ T cells from asymptomatic patients display a defect in branched and sialylated *N*-glycans when compared with non-IF.

The differences observed in the glycosylation profile of T cells across severity were not related to the abundance or activation of T cell populations because $CD3^+$, $CD4^+$, $CD8^+$, and γδ T cells showed similar frequencies as well as similar levels of PD1 and CD69 expression between the groups, with the exception of lowered $CD3^+$ population and increased $CD69^+$ within $CD8^+$ T cells in the mild disease group (Supplemental Fig. 1A–C).

In addition, because glycosylation is a dynamic process that can change along with disease status, we took advantage of a limited longitudinal follow-up analysis using a single patient, representative of each severity group (asymptomatic, mild, moderate, and severe), which revealed that levels of expression of β1,6-GlcNAc and α2,6-sialylation are restored in patients with improved disease severity at day 14 postdiagnosis (Supplemental Fig. 1D).

No major differences were observed for other glycan structures such as mannosylation (GNA binding) and fucosylation (AAL binding) in the different T cell subsets at diagnosis (Supplemental Fig. 1F, 1G).

These results showed that SARS-CoV-2–IF individuals led to an en bloc decreased expression of specific glycan structures such as β1,6-GlcNAc–branched *N*-glycans and α2,6-sialic acid in T cells, predominately in $CD8^+$ T cells and asymptomatic patients. The absence of differences on high mannose structures (GNA binding) or fucose residues on T cells (AAL binding) supports the specificity of the glycan switch. In fact, decreased levels of β1,6-branched *N*-glycans in T cells have been pointed out as a mechanism of TCR threshold regulation by hampering the TCR complex clustering and lowering the necessary amount of Ag recognition to signal a cellular response (16). Moreover, surface α2,6-sialylation also regulates the T cell immune response through galectins binding modulation (28, 29).

To further clarify the underlying mechanism that imposes the changes in the glycosylation profile of circulating T cells upon SARS-CoV-2 infection, we have performed an in vitro assay in which PBMCs isolated from healthy individuals were incubated with plasma from four patients from either each group: non-IF, asymptomatic, or moderate disease. Our results showed that T cells, upon incubation with plasma from asymptomatic patients, displayed a significant decreased expression of β1,6-GlcNAc–branched *N*-glycans (Fig. 2A) and α2,6-sialylation (Fig. 2B) when compared with T cells cultured with plasma from non-IF individuals and moderate disease patients. These results suggest the existence a specific serum factor that in asymptomatic patients drives a remarkable drop of complex branched and sialylated glycans structures on T cells. In fact, it is known that asymptomatic patients display a differential serum composition of cytokines and chemokines when compared with symptomatic (10, 30, and B. Pérez-Cabezas, R. Ribeiro, I. Costa, S. Esteves, A. R. Teixeira, T. Reis, R. Monteiro, A. Afonso, V. Pinheiro, M. I. Antunes, et al, manuscript posted on medRxiv). Moreover, T cell glycosylation is known to be modulated by specific ILs, namely IL-2 and IL-7, that regulate the transcription of specific Golgi glycosyltransferases (17, 31). In addition, IL-2 was described to be a specific biomarker of SARS-CoV-2 cellular response (B. Pérez-Cabezas, R. Ribeiro, I. Costa, S. Esteves, A. R. Teixeira, T. Reis, R. Monteiro, A. Afonso, V. Pinheiro, M. I. Antunes, et al, manuscript posted on medRxiv). Given that IL-2 has been pointed out to be involved in SARS-CoV-2 immune responses and that its signaling drives a glycan modulation in T cells, we evaluated the levels of this cytokine in the serum of the individuals in our cohort. Interestingly, we found that asymptomatic patients that exhibited decreased levels of branched *N*-glycans on T cells (Fig. 2A) had lower levels of IL-2 in the serum when compared with non-IF and moderate patients (Fig. 2C). Therefore, we hypothesized that levels of IL-2 would be involved in T cell glycan switching. To test this, we set up the same in vitro approach by incubating PBMCs with serum from either asymptomatic or moderate patients, in which IL-2 was inhibited using anti–IL-2–neutralizing Ab. Our results showed that the inhibition of IL-2 resulted in decreased levels of β1,6-GlcNAc branching in both $CD8^+$ and $CD4^+$ T cells (Fig. 2D, 2E). This evidence pinpoint IL-2 as a potential serum

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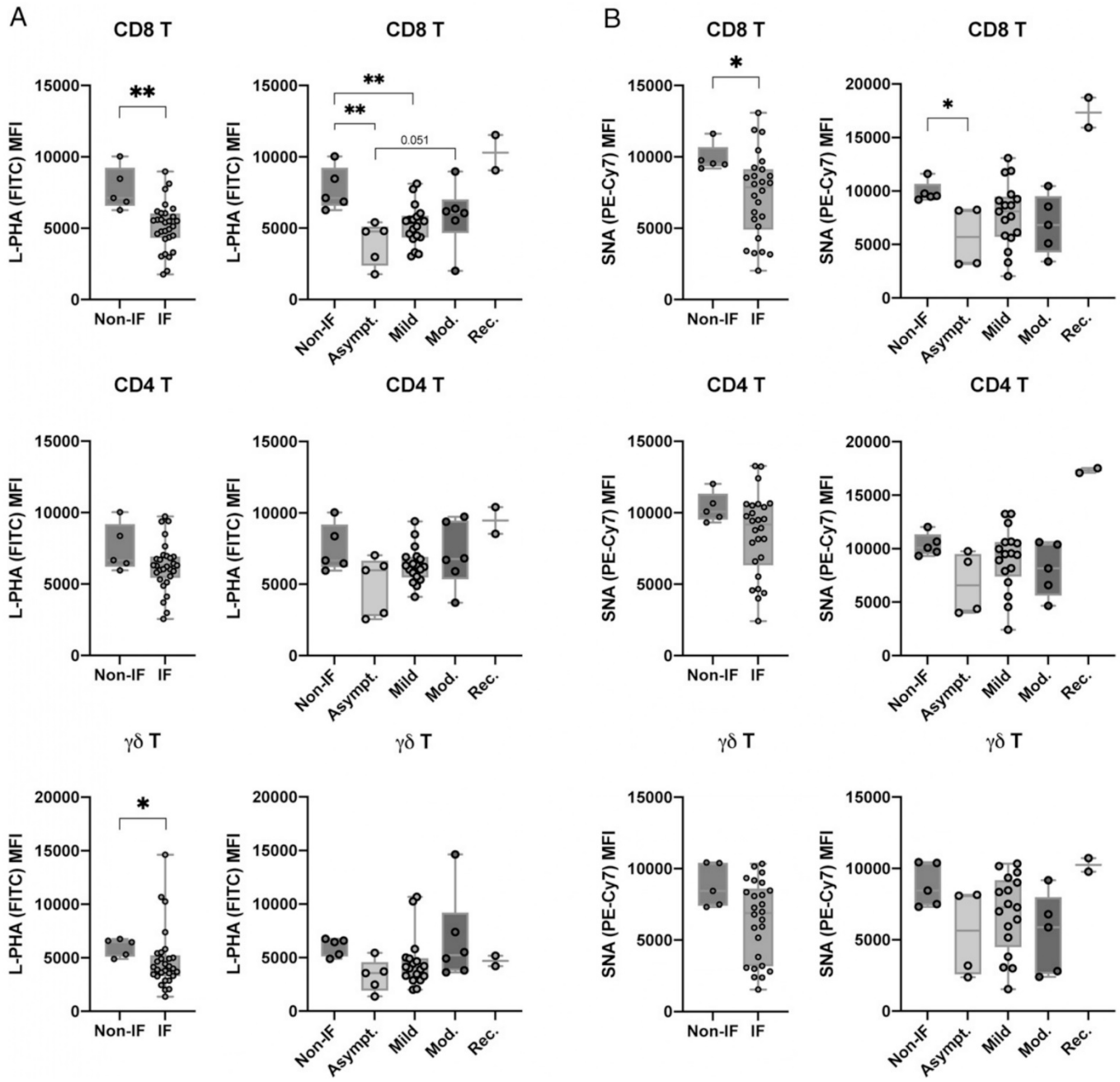


FIGURE 1. Peripheral T cell glycoprofile is altered upon SARS-CoV-2 infection at diagnosis. Levels (mean intensity fluorescence, MFI) of (**A**) L-PHA lectin binding detecting β1,6-GlcNAc–branched *N*-glycans and (**B**) SNA lectin binding detecting α2,6-sialylation in $CD8^+$, $CD4^+$, and γδ T cell subsets of non-IF ($n = 5$) and IF ($n = 30$) patients. Levels of (A) L-PHA and (B) SNA lectin binding for each disease severity group: asymptomatic (Asympt.; $n = 5$), mild ($n = 18$), moderate (Mod.; $n = 6$), and recovered (Rec; $n = 2$). Each dot represents one patient. Mann–Whitney *t* test was performed to evaluate statistically significant differences between each group pair. $*p < 0.05$, $**p < 0.005$.

factor involved in T cells glycan switching, highlighting a promising clinical target for hampering inefficient immune responses through glycosylation remodeling of T cells, controlling severe symptoms observed in COVID-19 patients.

Levels of DC-SIGN expression in circulating monocytes predict COVID-19 prognosis. To gain further insights in the viral–glycan recognition mechanism, we have characterized the expression of specific glycan binding proteins expressed by innate immune cells that are known to sense and recognize specific viral glycans, instructing an immune response (32). Regarding the abundance of innate cells, we have observed an increase in the frequency of dendritic cells as well as monocytes in asymptomatic patients when compared with non-IF individuals, and no differences in terms of NK and NKT cells subsets (Supplemental Fig. 1H, 1I). Regarding their activation state, analyzed by CD86 expression, only monocytes are shown to be activated in asymptomatic and mild disease patients (Supplemental Fig. 1C). This is in accordance with previous findings (33).

Specifically, DC-SIGN, a C-type lectin that recognizes mannose structures, is already known to play a role in the SARS-COV-2 recognition (24, Amraei, R., W. Yin, M. A. Napoleon, E. L. Suder. J. Berrigan, Q. Zhao, J. Olejnik, K. B. Chandler, C. Xia, J. Feldman, et al., manuscript posted on medRxiv, DOI: 10.1101/2020.06.22.165803, and Hoffmann, D., S. Mereiter, Y. Jin Oh, V. Monteil, R. Zhu, D. Canena, L. Hain, E. Laurent. C. Grünwald-Gruber, M. Novatchkova, et al., manuscript posted on medRxiv,

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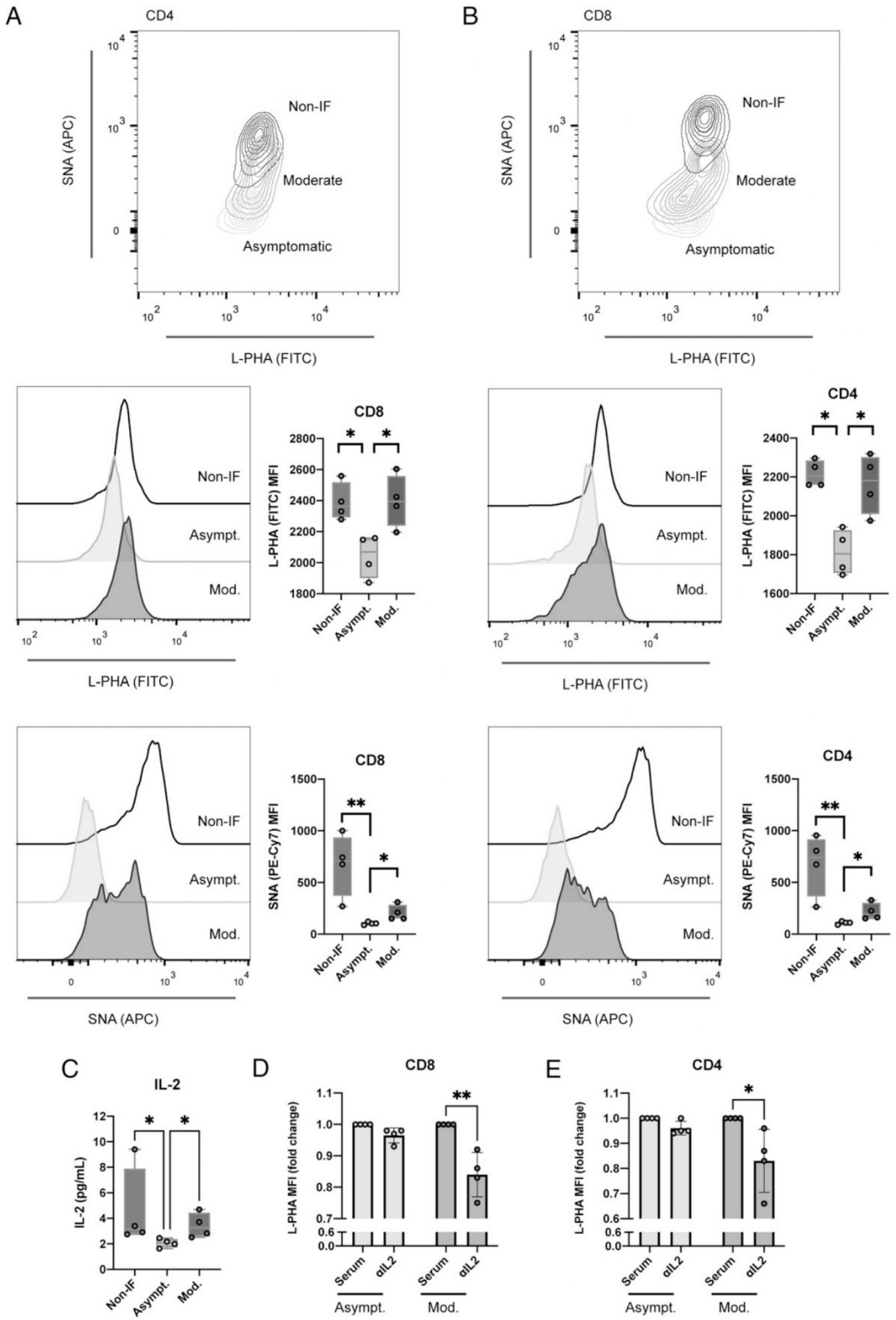


FIGURE 2. Patient-derived serum induces a differential T cell glycosylation comparing asymptomatic and symptomatic patients (**A**) L-PHA lectin and (**B**) SNA lectin binding (mean fluorescence intensity, MFI) of $CD8^+$ and $CD4^+$ T cells from a healthy donor, independent of our cohort, incubated for 24 h with 10% plasma from four patients from each group (non-IF, asymptomatic, or moderate). (**C**) Plasma levels of IL-2 from four patients from each group (non-IF, asymptomatic, or moderate). (**D**) Effect of neutralizing anti–IL-2 on the L-PHA levels of $CD4^+$ and (**E**) $CD8^+$ T cells in asymptomatic and moderate patients. Each dot represents a patient. Gating strategies for each population are included in Supplemental Fig. 1A. Mann–Whitney *t* test was performed to evaluate statistically significant differences between each group pair. $*p < 0.05$, $**p < 0.005$.

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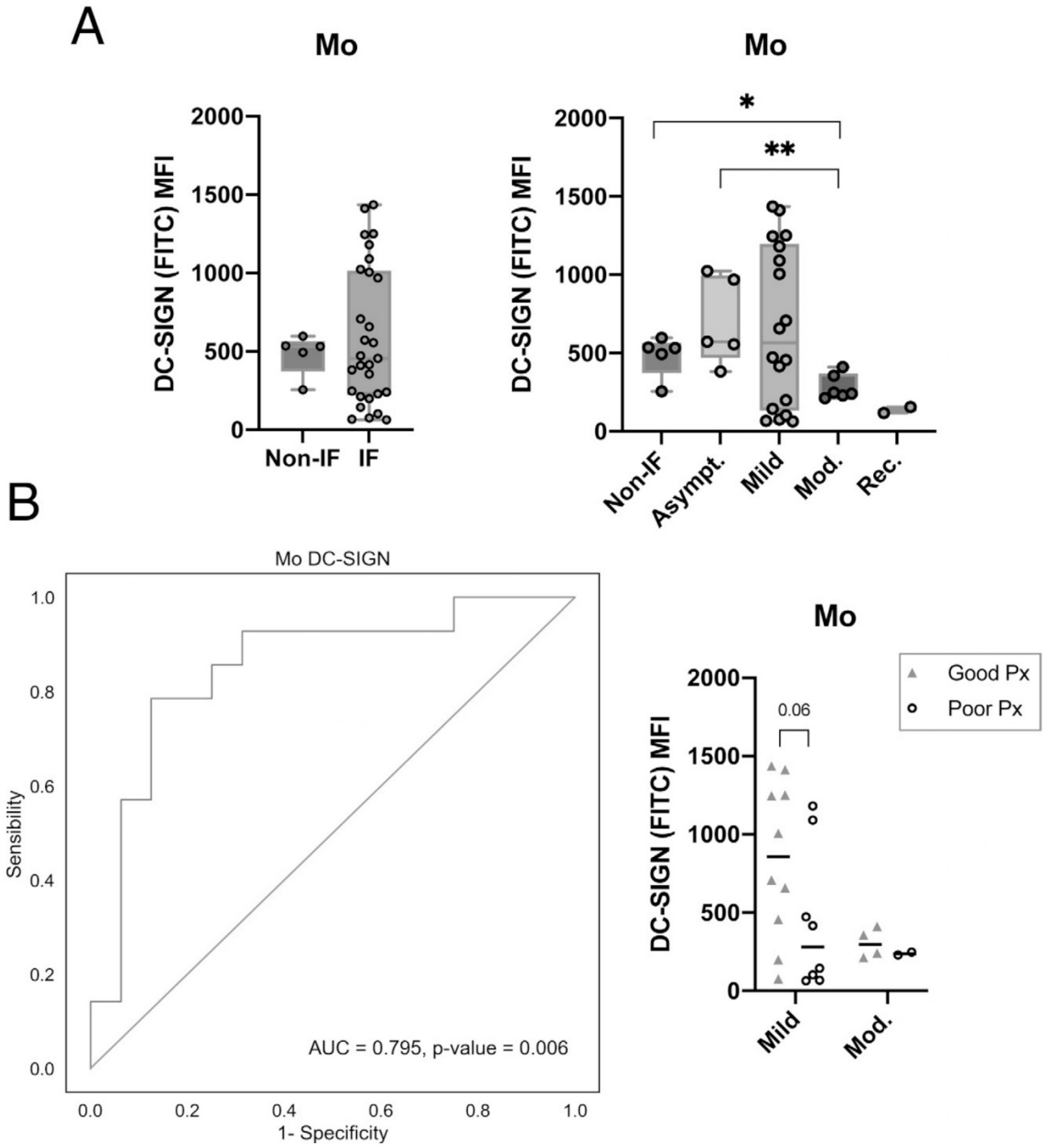


FIGURE 3. DC-SIGN expression in peripheral monocytes as a predictor of good versus poor prognosis in COVID-19 patients. (**A**) Levels of DC-SIGN surface expression (mean fluorescence intensity, MFI) in monocytes (Mo). (**B**) Receiver operating characteristic curve plotted for the DC-SIGN expression levels in monocytes from COVID-19 patients. The distribution of DC-SIGN levels are represented regarding the prognosis (Px) (day 14; good Px in green and poor Px in gray) as well as the severity at diagnosis. Each dot represents one patient; $n = 24$. Gating strategies for each population are included in Supplemental Fig. 1A. Mann–Whitney t test was performed to evaluate statistically significant differences. $^*p < 0.05$, $^{**}p < 0.005$, or represented.

DOI: 10.1101/2021.04.01.438087), being expressed by monocytes, immature dendritic cells, and macrophages. Our results showed that patients from the asymptomatic group displayed higher levels of DC-SIGN expression in monocytes compared with moderate disease (Fig. 3A), suggesting an increased capacity of asymptomatic patients to sense and recognize the virus.

Taking into consideration the biological relevance of DC-SIGN expression in monocytes as the first sensing mechanism upon SARS-CoV-2 infection and given the heterogenous expression of DC-SIGN among IF individuals (at diagnosis) (Fig. 3A), we next analyzed its prognostic value. A longitudinal cohort was used with clinical information in terms of disease progression (development of a poor versus good prognosis at day 14 postdiagnosis, characterized in Supplemental Table I). The symptoms and disease severity at day 14 postdiagnosis were considered as criteria to define the prognosis. Patients with good prognosis were those individuals that either remained asymptomatic or improved symptoms/disease severity between diagnosis and day 14 postdiagnosis. The poor prognosis

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group includes patients that did not improve across disease course or displayed a worsened disease and aggravated symptoms at day 14 postdiagnosis. Remarkably, the expression levels of DC-SIGN in monocytes were found to be able to stratify patients at diagnosis in terms of their likelihood of developing a good versus a poor disease prognosis with a specificity and sensitivity of 78.9% and 63.6%, respectively (Fig. 3B). The univariate analysis demonstrated that high levels of DC-SIGN expression in monocytes at diagnosis increase the odds ratio associated with the prediction of having a good prognosis (odds ratio = 6.548; $p = 0.011$). To assess the influence of cellular activation in DC-SIGN upregulation, we have evaluated the correlation between DC-SIGN expression levels and CD86 in monocytes, and no correlation was observed (Supplemental Fig. 1J, 1K). Moreover, taking into consideration that the presence of comorbidities has been associated with COVID-19 prognosis, as also observed in our cohort (Supplemental Fig. 1L), we evaluated whether the presence of comorbidities would influence DC-SIGN expression, and the results showed no significant association between DC-SIGN levels and the presence of comorbidities (Supplemental Fig. 1M), which excludes this variable as a potential contributor for the different DC-SIGN expression in monocytes among these patients. In fact, the recognition of the coronavirus glycans by innate immune cells was demonstrated to be mediated by DC-SIGN (24, Amraei, R., W. Yin, M. A. Napoleon, E. L. Suder. J. Berrigan, Q. Zhao, J. Olejnik, K. B. Chandler, C. Xia, J. Feldman, et al., manuscript posted on medRxiv, DOI: 10.1101/2020.06.22.165803, and Hoffmann, D., S. Mereiter, Y. Jin Oh, V. Monteil, R. Zhu, D. Canena, L. Hain, E. Laurent. C. Grünwald-Gruber, M. Novatchkova, et al., manuscript posted on medRxiv, DOI: 10.1101/2021.04.01.438087). An increased expression of this C-type lectin at the initial days of infection suggests a more efficient viral recognition and subsequent clearance, resulting in a good prognosis of the disease.

Discussion

Our results demonstrated for the first time, to our knowledge, that SARS-CoV-2 infection imposes a glycosylation reprogramming of adaptive immune cells, suggesting a specific glycan switch of T cells that may define their ability to successfully deal with infection. We identified a specific glycosylation signature of circulating T cells that is associated with their activity and function, distinguishing IF patients from non-IF individuals. Our results also suggest that the glycan switch imprinted in circulating T cells appear to be mediated by a serum extracellular factor(s), pinpointing IL-2 as a potential driver of T cells glycan switching. In fact, previous evidence from others and us demonstrated that the deficiency in branched *N*-glycan structures on T cells imposes a hyperreactive phenotype with a decreased threshold of T cell activation and increased T cell activity (13, 16). We, in this study, propose that the immune response against SARS-CoV-2 infection appears to be influenced by the glycosylation profile of circulating T cells, which defines their effective functions. In fact, a pronounced deficiency of complex branched and sialylated glycans on $CD8^+$ and γδ T cells were observed in asymptomatic COVID-19 patients. This dynamic and plastic glyco-immune modulation ("Glyco-Immune Alert" mechanism) may constitute a novel mechanism of host response, which contributes to further understanding the immunological differences among IF individuals (Supplemental Fig. 2).

Furthermore, we also showed another glycan-based mechanism of host response to SARS-CoV-2 driven by the upregulation of the viral glycans recognizing receptor DC-SIGN in monocytes, with prognostic application when detected at diagnosis.

This study unlocks the identification of a specific glycosignature of T cells as well as a prognostic biomarker in COVID-19. Further studies are needed to explore the cellular and molecular mechanisms underlying IL-2–mediated T cell glycan switch in infection and along disease course. This knowledge will be fundamental to pinpoint IL-2 as a critical serological factor that instructs the glyco-reprogramming of peripheral T cells, envisioning the identification of a potential novel biomarker and therapeutic target in COVID-19. Our study was conducted during the first wave of COVID-19 (March–July 2020) and had a limitation in terms of sample size, indicating the need to validate these promising observations in larger and well-characterized multicentric cohorts as well as analyzing the impact of other SARS-CoV-2 variants in T cell glycan switch.

This new evidence in COVID-19 paves the way to the identification of a specific blood glycosignature that is able to stratify patients at diagnosis according with their risk to evolve to worsened disease. This will certainly contribute to improved vaccination strategy and patient risk stratification, optimizing an effective allocation and management of health care resources such as ventilators and intensive care facilities.

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Disclosures

The authors have no financial conflicts of interest.

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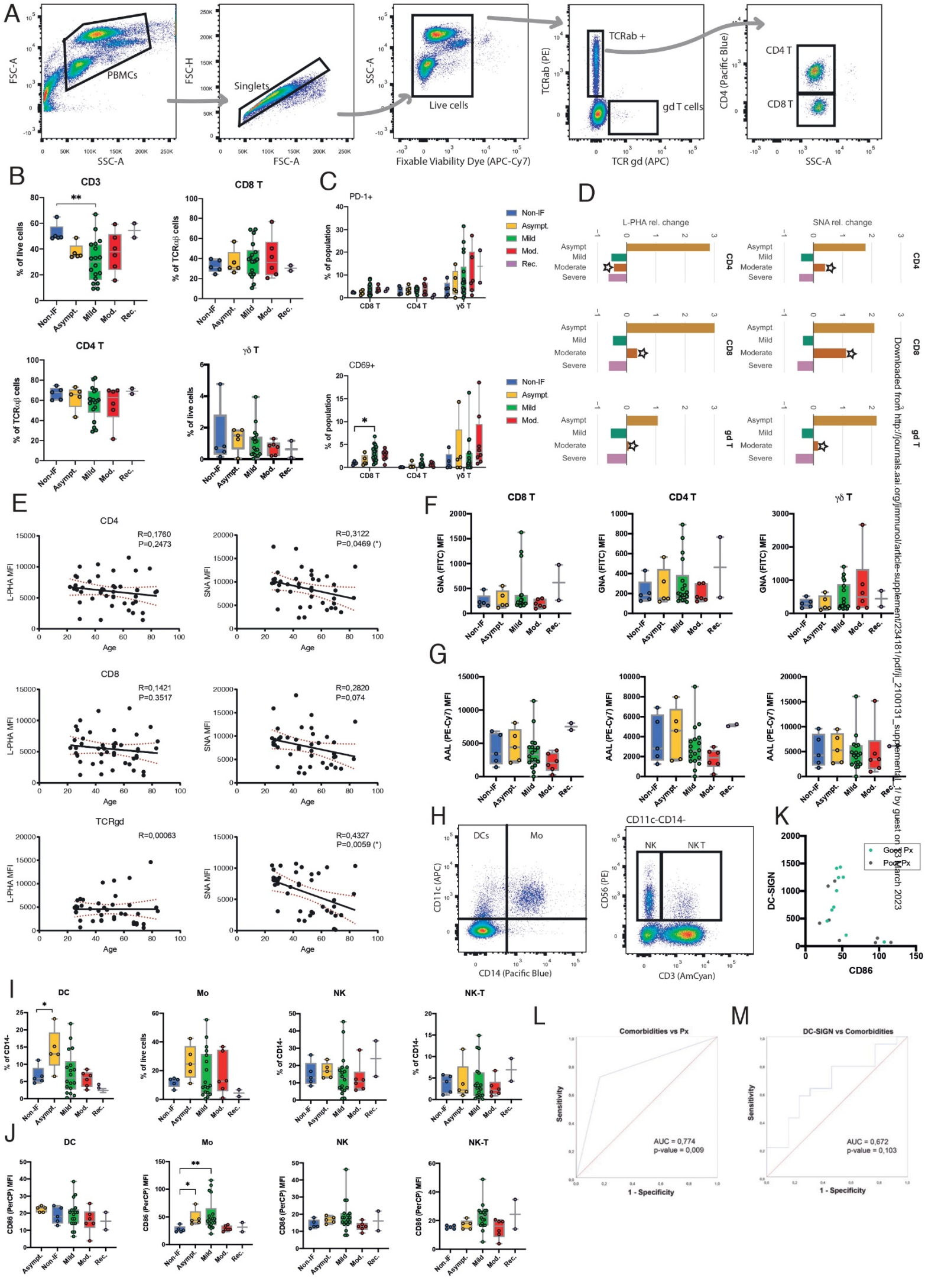
Supplementary information

Supplementary Table 1: Characterization of COVID-19 cohort in terms of clinical and epidemiological parameters. Data on age, gender and existence of comorbidities were obtained at diagnosis. Clustering of patients either by Good (asymptomatic who remained with no symptoms and amelioration of symptomatology, at day 14) or Poor (worsen severity at day 14) disease course.

Characteristics	Severity COVID-19			Disease Course	
	Asymptomatic (N=5)	Mild (N=19)	Moderate (N=6)	Good (N=19)	Poor (N=13)
Age (y)	57,4 ± 14,32	51,24 ± 14,18	63,17 ± 11,89	51,11±11,67	55,50±17,50
Age groups (y)					
22-65	3(60%)	16(84,21%)	4(66,67%)	15(78,95%)	9(69,23%)
>65	2(40%)	3(15,79%)	2(33,33%)	4(21,05%)	4(30,77%)
Gender					
Male	1(20%)	8(42,11%)	4(66,67%)	8(42,11%)	4(30,77%)
Female	4(80%)	11(57,89%)	2(33,33%)	11(57,89%)	9(69,23%)
Coexisting comorbidities					
Yes	1(20%)	6(31,58%)	5(83,33%)	4(21,05%)	10(76,92%)
No	4(80%)	13(68,42%)	1(16,67%)	15(78,95%)	3(23,08%)
Disease Progression					
Good (improved/asymp)	5(100%)	10(52,63%)	3(50%)	--	--
Bad (worsen severity)	0	9(47,37%)	3(50%)	--	--

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Supplemental Figure 1



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Supplemental Figure 1

(A) Gating strategy for multicolour flow cytometry analysis; (B) Frequency of CD3+, CD8+, CD4+ and γδT cells in peripheral blood from non-IF individuals and patients grouped according severity of disease. (C) PD1- and CD69-expressing T cell frequencies of peripheral CD4, CD8 and γδT cells from non-infected and healthy donors (Non-IF), and COVID-19 patients, at the time of diagnosis. (D) Relative change of L-PHA and SNA binding levels in several T cell subtypes at day 14 post-diagnosis, compared to the analysis at diagnosis, in N=1 patient/group (asymptomatic; mild and moderate); ☆ in the moderate group (orange bar) highlights a patient that has improved, with a recovery in disease severity at day 14 post-diagnosis, from moderate to mild disease, which was accompanied with a delayed T cell glycan switch. Patient with mild disease (green bar) maintained the disease status 14 days after diagnosis. (E) Correlation of the age from the cohort-included subjects and levels of L-PHA or SNA in the different T cell subsets analyzed, with respective P-value as well as correlation coefficient, R. (F) Levels of GNA binding (MFI) and (G) AAL binding in the non-infected donors (Non-IF), asymptomatic (Asympt.), mild and moderate patients in CD8+, CD4+ and γδT cells. (H) Gating strategy for multicolour flow cytometry analysis. (I) Frequency and activation of dendritic cells (DC), monocytes (Mo), natural killer cells (NK) and natural killer T cells (NK-T) of non-IF and COVID-19 patients at time of diagnosis. (J) Cellular activation measured by the expression levels of CD86 of respective innate immune cells of non-infected subjects (non-IF) and asymptomatic, mild, moderate and recovered COVID-19 patients. Each dot represents one patient. Mann-Whitney test was performed to evaluate statistically significance differences. * p-value < 0.05, ** < 0.005. (K) Correlation between CD86 and DC-SIGN levels in monocytes from patients with good or bad prognosis. (L) Receiver operating characteristic (ROC) curve plotted for the presence of comorbidities in association with COVID-19 prognosis and (M) DC-SIGN expression levels in monocytes in relationship with presence of comorbidities.

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CHAPTER 4

[RESEARCH PAPER]

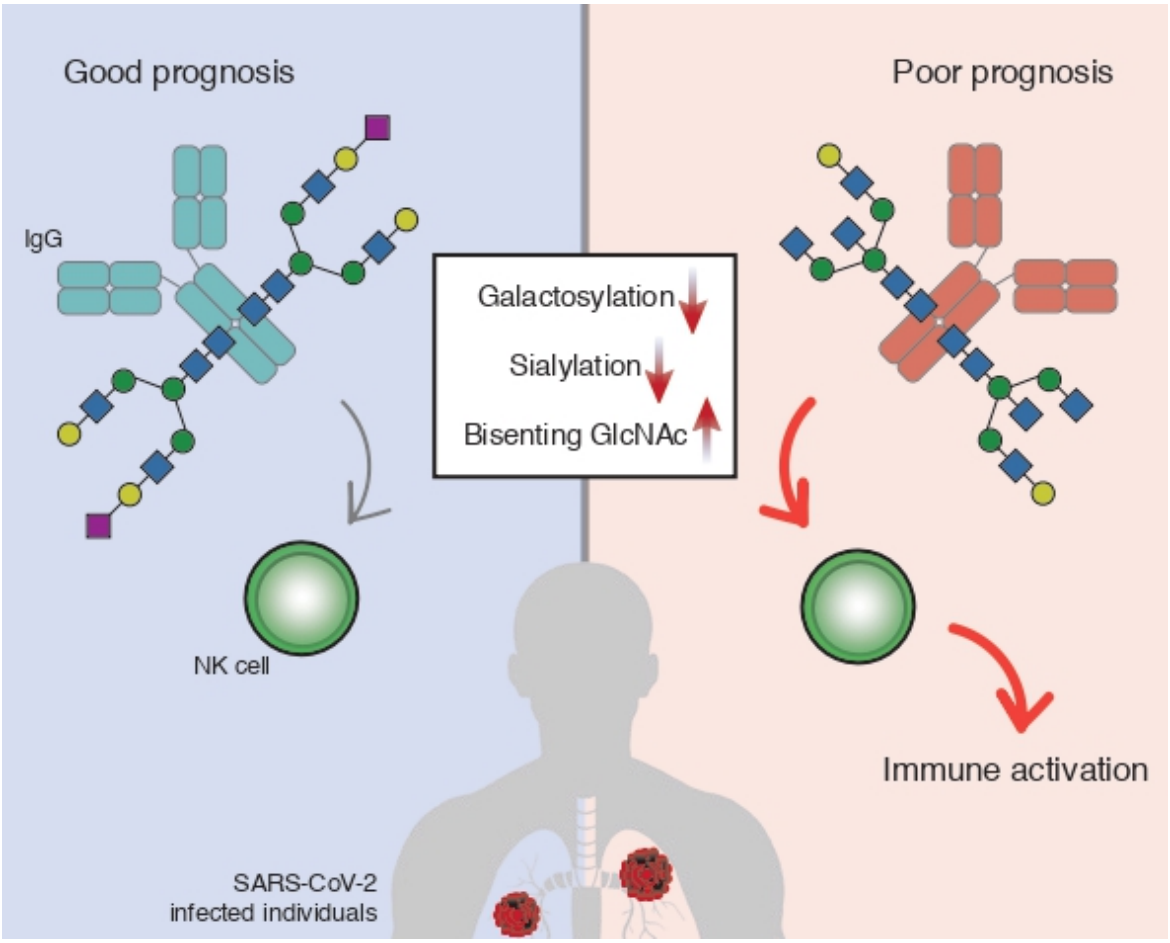
ALTERED IgG GLYCOSYLATION AT COVID-19 DIAGNOSIS PREDICTS DISEASE SEVERITY

Manuel M. Vicente*, Inês Alves*, Joana Gaifem, Cláudia S. Rodrigues, Ângela Fernandes, Ana M. Dias, Jerko Štambuk, Tea Petrovic, Pedro Oliveira, Frederico Ferreira-da-Silva, Adriana Soares, Nair Seixas, Tiago Teixeira, Luis Malheiro, Miguel M. Abreu, Gordon Lauc, Rui S. Castro, Salomé S. Pinho

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* These authors contributed equally

Graphical Abstract



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Clinical

European Journal of Immunology

Immunity to infection

Research Article

Altered IgG glycosylation at COVID-19 diagnosis predicts disease severity

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The nature of the immune responses associated with COVID-19 pathogenesis and disease severity, as well as the breadth of vaccine coverage and duration of immunity, is still unclear. Given the unpredictability for developing a severe/complicated disease, there is an urgent need in the field for predictive biomarkers of COVID-19. We have analyzed IgG Fc N-glycan traits of 82 SARS-CoV-2+ unvaccinated patients, at diagnosis, by nano-LC-ESI-MS. We determined the impact of IgG Fc glyco-variations in the induction of NK cells activation, further evaluating the association between IgG Fc N-glycans and disease severity/prognosis. We found that SARS-CoV-2+ individuals display, at diagnosis, variations in the glycans composition of circulating IgGs. Importantly, levels of galactose and sialic acid structures on IgGs are able to predict the development of a poor COVID-19 disease. Mechanistically, we demonstrated that a deficiency on galactose structures on IgG Fc in COVID-19 patients appears to induce NK cells activation associated with increased release of IFN-γ and TNF-α, which indicates the presence of pro-inflammatory immunoglobulins and higher immune activation, associated with a poor disease course. This study brings to light a novel blood biomarker based on IgG Fc glycome composition with capacity to stratify patients at diagnosis.

Keywords: Inflammation · agalactosylation · asialylation · COVID-19 · IgG Fc glycosylation · SARS-CoV-2

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

Coronavirus SARS-CoV-2 is the responsible agent for the global pandemic of Coronavirus disease 2019 (COVID-19), that, as of the March 8, 2022, has infected over 448 million people worldwide [1,2]. As it is a highly selective disease, only some SARS-CoV-2-infected individuals display symptoms and an unpredictable percentage of patients may develop a severe pathology. Over the last year, the knowledge regarding this disease has risen in an unprecedented rate. However, there is still an urgent need to improve the understanding of COVID-19's pathophysiology, foreseeing better clinical and therapeutic strategies for patients, as well as an optimized management of health care resources and vaccination strategy improvements. The available vaccines against SARS-CoV-2 pose a great promise for the resolution of the pandemic, but its long-term protection is still on trial, as well as its distribution strategies [3–5]. A complete and thorough understanding of immune responses to SARS-CoV-2 is still lacking. Therefore, there is an urgent unmet need to identify and characterize the immune responses associated with COVID-19 pathogenesis and severity, envisioning the identification of predictive/prognostic biomarkers of this disease.

SARS-CoV-2 infection most often results in a mild DC, associated with heterogeneous symptoms [6]. Research regarding peripheral blood mononuclear cells (PBMCs) immune phenotyping of infected patients consistently revealed an immunological dysregulation that characterizes the disease.

Antibodies play a central role in humoral immunity against pathogens, as they are able to mediate immune responses to viral infections, being Immunoglobulin G (IgG) the prevalent isotype taking part in systemic antiviral immunity being a key effector of the humoral immune system by triggering leukocyte activation and inflammation [7].

IgG isolated from human serum is composed of multiple glycoforms, owing to the addition of a diverse type of glycans structures in the IgG crystallizable fragment (Fc) and fragment antigen-binding (Fab) region. Fc domain is responsible for modulating cellular response through interaction with FcγRs in effector immune cells. All IgG Fc domains contain a single, highly conserved, glycosylation site in Asn297 that carries complex *N*-glycans [8].

A single complex biantennary *N*-linked glycan is attached to each heavy chain in the Fc portion, being required for Fc receptor binding. Although the IgG Fc *N*-glycans are restricted to two antennae, the structures attached to the Fc domain of IgG are known to be present in a multitude of forms, being essential for the regulation of antibody effector functions [9]. Over 30 different glycan variations have been detected on circulating IgG in healthy individuals [10], which reflects a tremendous heterogeneity in IgG Fc *N*-glycome. In fact, the type of glycan attached to IgGs Fc is critical for the proper effector functions of all IgGs, regulating the binding to FcγRs and instructing either a pro-inflammatory (through binding to activating FcγRs) or an anti-inflammatory (through binding to inhibitory FcγRs) response, associated with the pathogenesis of many inflammatory diseases. For instance, increased levels of IgG fucosylation have been observed in rheumatoid arthritis patients [11], which has been associated with the fact that IgG-containing *N*-glycans that lack core fucose have been implicated in enhanced antibody-dependent cell-mediated cytotoxicity due to increase affinity for FcγRIIIa [12]. Terminal sialylation of Fc glycans also modulates FcγR binding. The presence of α2,6 sialic acid on the Fc glycan significantly reduces FcγR binding affinity and is associated with anti-inflammatory activity [10]. Accordingly, the glycoengineering of intravenous immunoglobulin (IVIg) with increased sialylation of Fc showed a 100-fold increase in anti-inflammatory activity in a mouse model of arthritis [10], which further supports the immunoregulatory potential of IgG Fc glycan structures. The mechanistic role of specific glycans modifying Fc and effector functions of IgG either through FcγRs binding or through other mechanism are still controversial [13–15]. Recently, using the glycoengineering of monoclonal IgG1 antibodies, further insights into the regulatory role of IgG glycan moieties in the affinity for FcγRIIIa in more complex scenarios have been generated [16,17]. Moreover, NK cells also express glycan binding receptors, such as sialic acid-binding receptors (siglecs) and others, that can modulate their functions [18].

Loss of terminal galactose (agalactosylation) on IgG1 Fc glycans was also observed in patients suffering from rheumatoid arthritis and appearing in circulation preceding disease onset [12]. More recently, the glycosylation profile of IgG was found to be different between ulcerative colitis and Crohn's disease patients and was associated with clinical severity of the disease [19,20]. In systemic lupus erythematosus, IgG fucosylation was associated with kidney damage [21]. The variability of IgG glycome composition among human population appears to be influenced by genetic and environmental factors as well as age [22,23].

In summary, this compelling body of evidence showing a tremendous variation on IgG glycome among population that have been linked to differential disease severities led us to hypothesize that IgG glycome heterogeneity among SARS-CoV-2 infected patients can be used as a reliable and minimally invasive biomarker to early identify those at risk of serious illness from those who might be protected.

The glycoprofile of serum IgGs in COVID-19 patients has received some attention. Chakraborty et al. showed that afucosylated Fc *N*-glycans are enriched in anti-SARS-CoV-2 receptor binding domain IgG1 from severe patients, which appears to contribute to the enhancement in the production of pro-inflammatory cytokines [24]. Consistently, Larsen et al. found that afucosylated Fc *N*-glycans in SARS-CoV-2-spike protein-specific IgGs are more prevalent in critically ill patients [25]. This afucosylated anti-spike IgGs was demonstrated to induced a hyper-inflammatory profile of human macrophages [25,26]. Moreover, total IgG *N*-glycome (Fc and Fab glycans) analysis was also characterized by us in three European cohorts of plasma samples from COVID-19 patients, showing that IgG glycome composition is different when comparing mild *versus* severe disease patients [27]. However, whether variations in circulating IgG Fc glycome composition among SARS-CoV-2-infected individuals, at diagnosis, are

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able to predict the development of a severe/complicated disease remains unexplored. Therefore, in this study, the serum IgG *N*-glycome composition was characterized in a longitudinal and multicentric cohort of SARS-CoV-2-infected individuals, assessing whether a serum IgG antibody glycosignature, analyzed at diagnosis, confers prognostic value as a predictive biomarker for the development of a poor DC after 14 days postdiagnosis. In addition, we also demonstrated, at a mechanistic level, the biological effects of a differential IgG glycome composition in the activation of effector NK cells and in FcγIIIaR-Fc binding.

Results

Glycans variations in IgG Fc among COVID-19 patients at diagnosis: Loss of galactosylation and sialylation in total IgG Fc are observed in patients with a more severe disease at diagnosis

Taking into consideration that there is still a gap in the knowledge of humoral immune response in SARS-CoV-2 infection at early timepoints, we set out to characterize the glycosylation profile of serum total IgGs at time of diagnosis of SARS-CoV-2 infection (up to 3 days upon PCR^+ test) comparing with noninfected (non-IF) controls. A total of 82 patients with PCR^+ COVID-19 were selected for plasma collection at diagnosis. Patients were classified at diagnosis as asymptomatic ($n = 8$), mild disease ($n = 40$), and moderate/severe disease ($n = 34$), accordingly with WHO clinical guidelines [28]. As controls, four non-IF individuals were included (Supporting Information Table S1).

Total serum IgG was isolated and the glycosylation profile of the Fc portion was determined by mass spectrometry (MS). Glycan structures were grouped by their traits in terms of galactosylation, sialylation, bisecting GlcNAc (Bis-GlcNAc), and fucosylation (Fig. 1A). Specifically, total IgG Fc agalactosylation was found to be significantly increased in the moderate/severe disease patients when compared with non-IF and with mild ones (Fig. 1B), namely in IgG1 (only between moderate/severe and mild severities) and IgG4 isotypes (Fig. 1C). Given the fact that age might influence IgG glycome composition [22,23], the data were adjusted for age and the results showed that the increased IgG Fc agalactosylation observed in moderate/severe disease patients compared with mild disease was statistically significant after age correction (indicated with §). Accordingly, digalactosylation is significantly decreased in moderate disease patients compared with mild disease group and to non-IF subjects (Fig. 1B, Supporting Information Fig. S1B), specifically for IgG1 and IgG2&3 (only between moderate/severe and mild severities) (Fig. 1C). The decrease in total IgG Fc digalactosylation, and specifically in IgG2&3 sub-type, in moderate disease patients was statistically significant after age correction, when compared to the mild group (indicated with §). Moreover, asymptomatic patients present a decrease in IgG1 digalactosylation (G2) relatively to non-IF and mild disease group (Fig. 1C). Regarding terminal sialyation of IgGs, known to be widely associated with anti-inflammatory properties of IgG, we observed a significant decrease of this glycan trait, predominantly in IgG2&3 sub-type, at diagnosis in the moderate disease group, compared to the mild group (Fig. 1C), that remained statistically significant after age correction (indicated with §). Distinctively, the presence of Bis-GlcNAc is significantly increased in total IgG Fc in moderate patients when compared to mild patients, even after age correction (Fig. 1C). No differences were detected in total IgG monogalactosylation and fucosylation at diagnosis between groups (Supporting Information Fig. S1A). The glycan traits that showed an association with severity, independently of the age, have 24–26% power of explaining the overall differences between mild and moderate patients.

In order to exclude the eventual confounder effect of anti-SARS-CoV-2-specific IgGs in this analysis, we analyzed the levels of anti-Spike IgGs. We showed that seropositivity in our cohort is very low compared with vaccinated individuals, in which only 40% of mild patients and 33% of moderate/severe groups display presence of anti-Spike IgG with similar levels (Supporting Information Fig. S1C). This is in accordance with the fact that serum was collected as early as up to 72-h postdiagnosis and therefore a complete antibody response to the virus had not occurred yet in the majority of the patients. To rule out the contribution of anti-Spike IgG glycosylation in our findings, we performed an ELISA using the ECA lectin to determine if the galactosylation pattern of these antigen-specific IgGs was following the total IgG behavior. We observed that in symptomatic seropositive patients (mild, moderate, and severe) the galactosylation levels of anti-Spike IgG decreased when compared to asymptomatic ones, with no differences between mild, moderate, and severe (Supporting Information Fig. S1D). Moreover, total IgG ECA binding, from the same set of seropositive patients, followed the same decrease in terms of galactosylation levels (Supporting Information Fig. S1E), as previously observed in the bigger cohort of samples (Fig. 1B). Overall, these results suggest that the galactosylation pattern of anti-Spike IgG is not a confounder in this study.

Overall, our data show that total IgG glycome composition is correlated with disease severity at diagnosis. A pro-inflammatory IgG glycosignature characterized by loss of galactosylation and loss of sialylation was correlated with patients that exhibit a moderate disease at diagnosis when compared with those presenting a mild disease.

Serum IgG glycosylation profile at diagnosis predicts COVID-19 prognosis

In order to explore the prognostic potential of serum IgG glycosylation profile as a biomarker for COVID-19 disease course (DC) prediction, we have tested for a correlation between IgG Fc *N*-glycome composition at diagnosis and a worse DC at day 14 postdiagnosis. Briefly, patients were grouped, according to their disease severity, 14 days postdiagnosis in terms of the presence of symptoms (no symptoms > good prognosis; presence of at least one of the criteria: pneumonia and/or need for oxygen therapy > poor prognosis) (Supporting Information Table S2). Our

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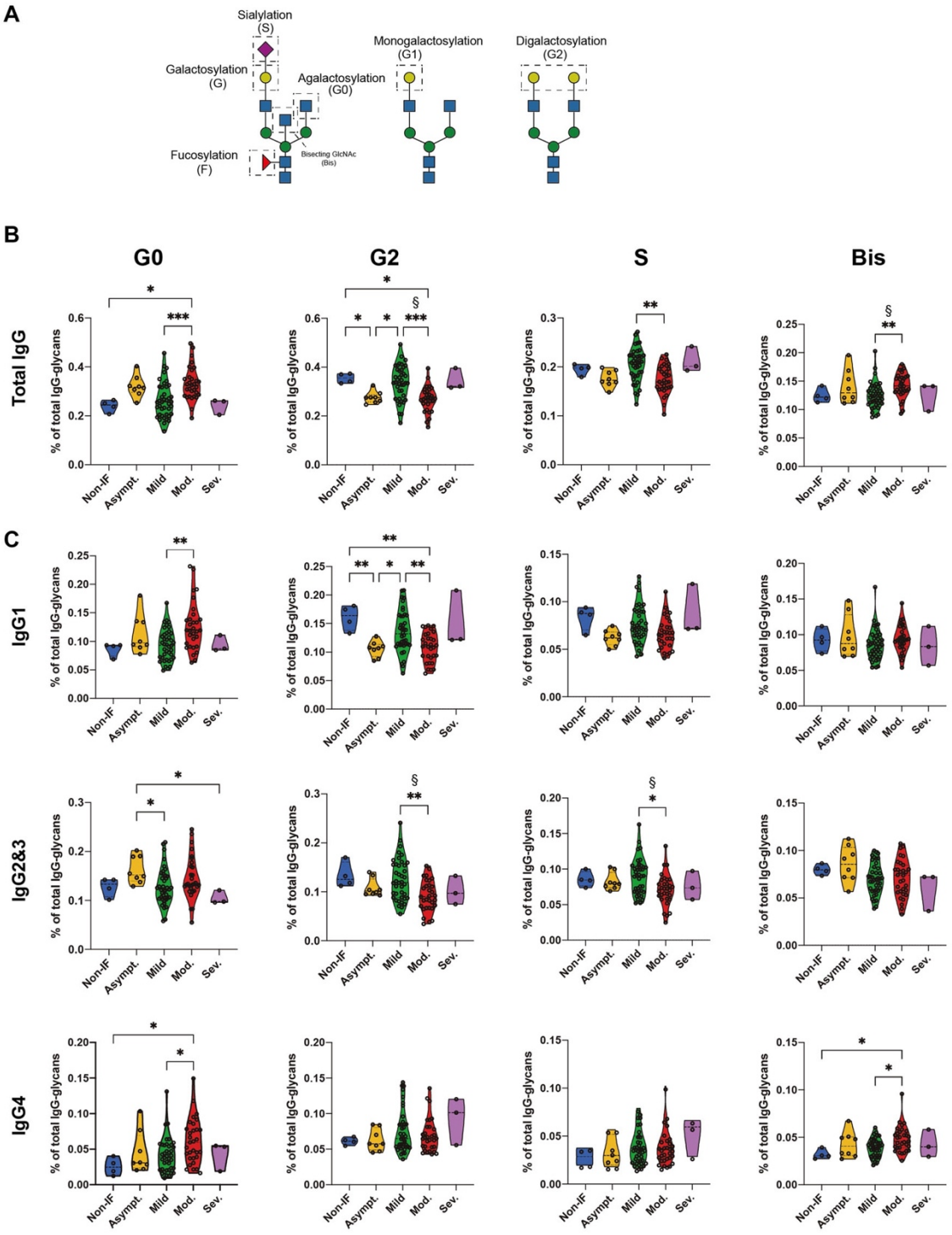


Figure 1. SARS-CoV-2 infection drives IgG Fc glycosylation alterations. (A) Schematic representation of analyzed IgG glycan traits. (B) Relative abundance of total IgG Fc agalactosylation (G0), digalatosylation (G2), sialylation (S), and Bis-GlcNAc (Bis) in different COVID-19 severities (asymptomatic $n = 8$, mild $n = 40$, moderate $n = 31$, and severe $n = 3$) and non-IF individuals ($n = 4$). (C) Relative abundance of isotype-specific IgG Fc glycan traits in different COVID-19 severities and non-IF individuals. Each data point represents the data from a single patient/subject isolated IgGs in a single LC-MS analysis (one replicate). Kruskal–Wallis test, $^{*}p$-value < 0.05; $^{**}p$-value < 0.005; $^{***}p$-value < 0.001. The data were corrected for age effect and linear regression model for age correction was used, $^{\S}p$-value < 0.05.

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data showed that patients with a poor DC were those who displayed a pro-inflammatory IgG glycoprofile at diagnosis. Specifically, patients that, at day 14 postdiagnosis, did not improve the symptomatology (having thereby a poor disease prognosis) exhibited, at diagnosis, an increased agalactosylation (mainly in IgG1) and Bis-GlcNAc in total IgGs, together with a decrease of digalactosylation (mainly in IgG2) and decreased sialylation (in IgG2), with no major differences in monogalactosylation and fucosylation (Fig. 2A and B, Supporting Information Fig. S2A). Considering the age of patients as a possible confounder effect, the results were adjusted for age (indicated with § in Fig. 2A and B). We showed that changes in IgG Fc galactosylation have survived, highlighting the biological relevance of total IgG Fc digalactosylation (mainly IgG2&3 subtypes) as well as IgG1 Fc agalactosylation as independent predictors of COVID-19 prognosis.

In order to further determine the predictive performance of serum IgG glycosignature in predicting COVID-19 prognosis, and to validate the predictive potential of serum IgG glycan traits in stratifying patients at diagnosis regarding good *versus* poor disease prognosis, receiver operating characteristic curve analysis was performed. Total IgG digalactosylation (AUC = 0.722; *p*-value = 0.002), IgG1 agalactosylation (AUC = 0.714; *p*-value = 0.002), and IgG2&3 digalactosylation (AUC = 0.705; *p*-value = 0.003) were found to, independently, be able to stratify patients according with prognosis. Remarkably, the combined values (age-corrected) of these IgG glycan traits (AUC = 0.729; *p*-value < 0.001) significantly improved the prediction capacity (at diagnosis) in terms of likelihood of developing a good *versus* a poor COVID-19 DC (Fig. 2C). As IgG Fc *N*-glycosylation has been associated with several health conditions, we tested their association with the presence of comorbidities in the patients of our cohort (Supporting Information Fig. S2B). We detected no significant associations, concluding that IgG Fc *N*-glycan traits are able to predict good and poor prognosis of COVID-19, independently of the presence of comorbidities.

Taken together, we demonstrate that the composition of glycans of total IgG, in terms of presence/absence of galactose, sialic acid, and Bis-GlcNAc structures in IgG Fc domain, analyzed at diagnosis of COVID-19, has prognostic capacity in predicting a poor DC (up to 14 days postdiagnosis).

Loss of galactosylation in total IgGs from SARS-CoV-2-infected patients promotes increased FcγRIIIa binding and NK cell activation associated with poor prognosis

In order to gain mechanistic insights on the impact of a differential IgG glycoprofile, detected at diagnosis, in immune cell activation and function, we evaluated the potential of isolated IgGs to activate NK cells in vitro. We started by assessing the binding capacity of isolated total IgGs to FcγRIIIa, one of the major targets for IgG-mediated NK cell activation; thus, we used a subset of patients and analyzed the binding potential by surface plasmon resonance (SPR). Our results showed a 30% increase in FcγRIIIa binding of the IgGs from SARS-CoV-2-infected subjects, compared to non-IF (Supporting Information Fig. S3A). Accordingly, patients that developed a poor prognosis revealed an increased binding of IgG Fc to FcγRIIIa when compared to patients with good prognosis (Supporting Information Fig. S3B).

NK cells recognize a multitude of targets with a vast repertoire of membrane-expressed receptors. Among them, there is the FcγRIIIa, as mentioned, but also glycan-binding receptors, such as siglecs. We asked if the increased binding between FcγRIIIa and patient-derived IgG, and/or their differential glycosylation profiles could have an impact in NK cell activation. To tackle this hypothesis, NK cells were cultured with purified IgGs from patients with different disease severities, and NK cells' activation were evaluated through the expression of the activation marker CD69 (Supporting Information Fig. S3C). Interestingly, we observed that NK cell activation was increased in infected and asymptomatic patients, when compared to non-IF and mild ones. Moreover, in symptomatic patients, we have observed that moderate patients-derived IgGs (low galactosylation and sialylation) induced increased CD69 expression when compared to mild ones (high galactosylation and sialylation) (Fig. 3A).

Importantly, from the clinical point of view, we found that IgG, isolated at diagnosis, from patients that developed a poor prognosis, enriched in agalactosylated and asialylated *N*-glycans, induced a higher activation of NK cells, when compared to IgG from patients with a good prognosis (Fig. 3B). To further characterize isolated IgG-mediated NK cell activation, we have analyzed the production of pro-inflammatory cytokines (IFN-γ and TNF-α), which showed increased production, in line with CD69 expression between the severity groups and prognosis (Fig. 3C). These evidence highlight the pro-inflammatory role of agalactosylated total IgG in COVID-19 disease.

To further validate the role of the IgGs Fc glycosylation composition, specifically the galactosylation levels, in defining immune cell activation and disease prognosis, we have glycoengineered the galactose levels of IgGs to assess its impact in NK cell activation. In fact, IgG agalactosylation has been widely described as imposing a pro-inflammatory function to IgGs in different immune-mediated diseases [20,29,30]. In accordance with our results on COVID-19, lack of galactosylation in the Fc portion has been shown to promote FcγR binding and activation of innate cellular response [31]. Total IgGs isolated from patients that displayed a good *versus* poor prognosis were digested with β-galactosidase (β-gal; which removes the terminal galactose residues). The removal of galactose from IgGs was confirmed by lectin blot analysis with *Erythrina cristagalli* agglutinin (ECA) lectin, which recognizes terminal galactose, showing a reduction in the reactivity to this lectin in IgG treated with β-gal (Fig. 3D). This reduction in ECA staining was more pronounced in IgGs from patients with a good prognosis compared to poor prognosis, as the latter have already low levels of galactosylation (Fig. 3D). The glycoengineered IgGs were tested for NK cell activation, where the removal of galactose from IgG isolated from patients who displayed a good DC (and high IgG Fc galactosylation levels) increased NK cells activation, which was not observed in patients

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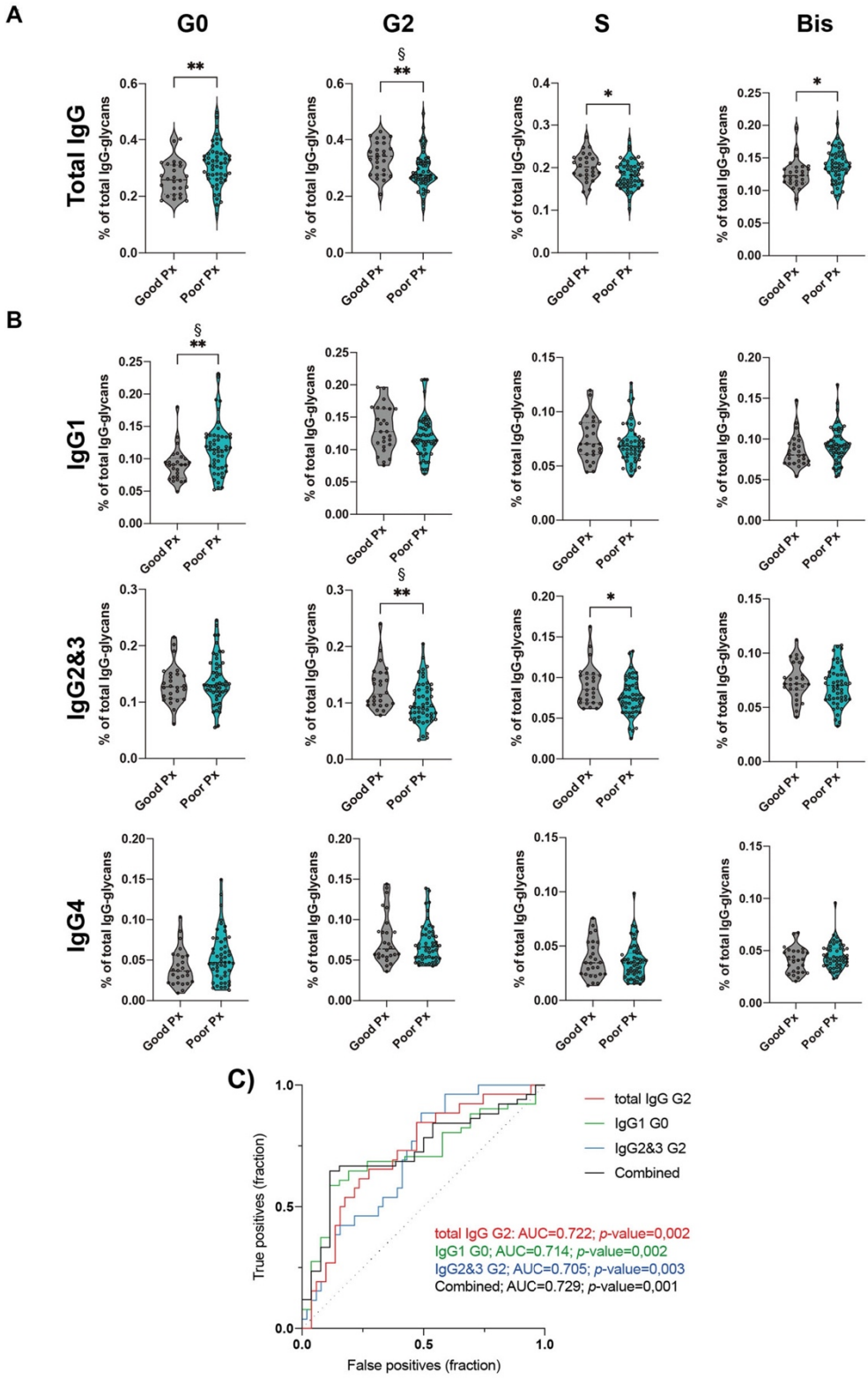


Figure 2. Specific glycosignature of IgG Fc associates with COVD-19 prognosis. (A) Relative abundance of total IgG Fc agalactosylation (G0), digalactosylation (G2), sialylation (S), and Bis-GlcNAc (Bis) in good (asymptomatic at day 14, $n = 51$) and poor (symptomatic at day 14, $n = 26$) prognosis (Px) of COVID-19 disease. (B) Relative abundance of isotype-specific IgG Fc glycan traits (GT) in good and poor prognosis. (C) Significant associations between IgG glycan traits and disease outcome (FDR < 0.1). (D) Receiver operating characteristic (ROC) curve plotted for the IgG Fc glycan traits levels of COVID-19 patients ($n = 77$), either separated or combined. Each data point represents the data from a single patient/subject isolated IgGs in a single LC-MS analysis (one replicate). Kruskal–Wallis test, $^{*}p$-value < 0.05; $^{**}p$-value < 0.005. Binomial logistic regression model for age correction, $^{\S}p$-value < 0.05.

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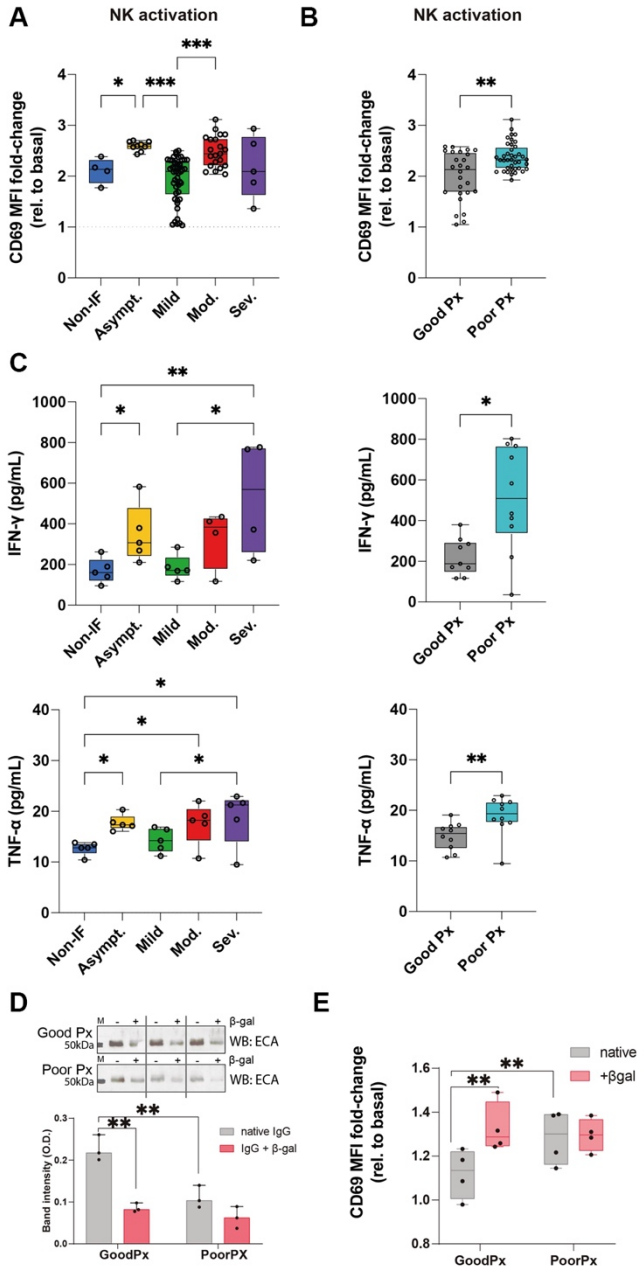


Figure 3. Loss of IgG galactosylation in patients with poor prognosis leads to increased NK cell activation. (A) NK cell activation quantified by the fold change of CD69 expression (compared to basal NK cells) after culture with coated IgGs from COVID-19 patients with different severities (asymptomatic $n = 9$; mild $n = 46$; moderate $n = 21$; severe $n = 5$) and (B) prognosis (good prognosis $n = 26$; poor prognosis $n = 39$). (C) Quantification of pro-inflammatory cytokine, IFN-γ and TNF-α, production by IgG-activated NK cells. (D) ECA lectin (recognizing terminal galactose) blot and band intensity quantification of different patients' IgGs (each patient is exhibited in paired lanes) upon β1-4-galactosidase S digestion (+βgal) or not (-βgal, native), $n = 8$. (E) NK cell activation quantified by the fold change of CD69 expression (compared to basal NK cells) after culture with coated IgGs digested with β-galactosidase S (+βgal) or not (native) from COVID-19 patients. Results shown from at least two independent experiments. Each data point represents data from a single patient/subject. Mann–Whitney *t*-test $^{*}p$-value < 0.05; $^{**}p$-value < 0.005.

with poor prognosis that already display low levels of IgG Fc galactosylation (Fig. 3E). These results support the specific role of IgG galactosylation as a chief glyco-determinant for the modulation of NK cells activation, associated with COVID-19 prognosis.

Discussion

In this study, we have demonstrated that IgGs Fc domain from SARS-CoV-2-infected patients display different glycosylation profiles (glycan variations) that can be detected at diagnosis and with potential to predict COVID-19 prognosis. We found that COVID-19 patients that display a poor DC at day 14 post-PCR^{+} test (exhibiting either pneumonia and/or need for oxygen therapy), had at diagnosis, IgGs with a deficiency in galactosylation and sialylation, and increased levels of Bis-GlcNAc structures. This specific plasma IgG glycosignature can be detected within the first 72 h upon diagnosis. Despite having a small fraction of seropositive patients (anti-Spike IgG) in our cohort, we have shown that the glycosylation pattern of anti-Spike IgG does not constitute a confounding issue. Some mechanisms have been pointed out as possible drivers of IgG glycosylation changes such as those occurring through the action of extracellular glycosidases/glycosyltransferases enzymes [32]. The presence of extracellular sialidases, responsible for the removal of terminal sialic acid, secreted by endothelial cells may also represent a player for the extracellular modulation of sialylation in circulating IgGs [33]. Recently, we also proposed that SARS-CoV-2 infection appears to impose a glycan switch on T cells associated with COVID-19 severity [34]. Moreover, age, gender, lifestyle, and pathologies may implicate in the steady state of an inherited IgG glycosylation profile [23]. Importantly, several studies have shown that afucosylation of SARS-CoV-2-specific IgGs was associated with overall poor prognosis, namely in hospitalized versus outpatients [24], infected subjects with or without acute respiratory distress syndrome [25], and patients that progressed to a severe disease [35]. Therefore, many causes have been proposed to explain glycan-variations of IgG Fc among population, however the mechanisms underlying the glycoalterations in IgGs associated with SARS-CoV-2 infection is a topic that needs to be further investigated.

Levels of IgG galactosylation are one of the most prominent glycosylation alteration observed in several chronic inflammatory and autoimmune diseases [36]. In fact, galactosylation of total IgGs has been described to affect activation thresholds of immune cells. Karsten et al. showed in mice that the anti-inflammatory property of high galactosylation of IgG immune complexes is due to the promotion of its association with FcγRIIB and dectin-1, which blocks the pro-inflammatory effector functions [37]. Therefore, this suggests that individuals with higher levels of immune activation would have more pro-inflammatory antibodies, as they have lower levels of IgG galactosylation/sialylation. In accordance with our study, a seminal report from Larsen et al., where the *N*-glycome of total IgG and SARS-CoV-2 specific IgGs were studied, from patients with or without acute respiratory distress syndrome,

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it was described that the levels of galactosylation and sialylation are decreased in the higher severity group [25]. Our results showing the loss of galactose in IgGs from COVID-19 patients can be thereby the reflection of a general pro-inflammatory humoral immune response that we found to be associated with a poor prognosis. This decrease of galactosylated IgGs was independent of the age of the subjects, and was accompanied by decreased sialylation. In fact, we demonstrated that patients with low galactosylation of IgGs display increased activation potential of NK cells, with production of pro-inflammatory cytokines such as IFN-γ and TNF-α. These basal pro-inflammatory antibodies defined by glycans composition appear to underlie an exaggerated immune response that may foster the "cytokine storm" associated with poor prognosis of COVID-19. The mechanism underlying NK cell activation by IgG Fc-glycosylation still needs further research, namely the involvement of other molecules besides FcγRs, such as the case of glycan binding receptors.

In accordance, previous studies on IgG Fc glycosylation demonstrated that terminal sialylation also reduces FcγR binding [10]. Our results suggest that loss of sialylation and decreased galactosylation in IgG Fc *N*-glycans from patients diagnosed with COVID-19 is consistent with an immunological misfiring, associated with NK cell activation, that defines severity and poor prognosis of COVID-19. These evidence suggest that the degree of plasma IgG galactosylation and sialyation can be used as a biomarker for immune activation and thus as a biomarker for COVID-19 prognosis. Moreover, we cannot exclude the potential contribution of increased Bis-GlcNAc in the immunological misfiring we propose, given that it has been suggested that Bis-GlcNAc in the Fc domain increases the affinity of IgG1 for FcγRIIIa binding, independent of fucosylation and galactosylation [17].

Our study was conducted during the first wave of COVID-19 (March–July 2020) and had a limitation in terms of sample size, undefined day 0 of infection, and limited biological material that explain the fact that differential FcγR binding between the groups did not present major differences, indicating the need to validate these promising observations in larger and well-characterized multicentric cohorts as well as analyzing the impact of other SARS-CoV-2 variants in total IgG *N*-glycosylation. Moreover, NK cell-IgG interactions are far from being fully understood, and IgG-glycans should be a major focus of research. Therefore, the identification of the precise molecular mechanism of IgG-glycan-mediated NK cell activation needs to be further explored.

One aspect that needs to be explored should be the potential effect of COVID-19 vaccination in the levels of total IgG galactosylation and in IgG glycome composition in general. Curiously, influenza and tetanus vaccination modulates the galactosylation of antigen-specific IgG1 associated with increase on antibody titers [38]. Whether COVID-19 vaccine modulates levels of galactosylation in total (non- and antigen-specific) IgGs as a way of assessing vaccine efficacy and development of neutralizing antibodies needs to be investigated.

Taken together, this study brings to light a novel risk screening system based on the serum IgG Fc *N*-glycome signature (as a minimally invasive blood biomarker) able to discriminate at diagnosis high-risk individuals of progressing to a severe/complicated disease from those with low/no risk. This new knowledge has the potential to improve and optimize the allocation and management of health care resources and vaccination strategies in COVID-19.

Materials and Methods

Cohort description and patient's selection criteria

The present study integrates a total of 82 patients diagnosed with SARS-CoV-2 between from three individual Portuguese cohorts (May to July 2020): 20 patients from Infectious Disease Department, Centro Hospitalar Universitário do Porto (CHUP), Porto, Portugal; 17 patients from Infectious Disease Department, Centro Hospitalar de Vila Nova de Gaia/Espinho (CHVNG), Vila Nova de Gaia, Portugal; and 45 patients from Internal Medicine Department of Hospital Beatriz Ângelo (HBA), Loures, Portugal. The total cohort includes patients of both genders and age between 19 and 99 years old. Blood was collected for plasma at the time of diagnosis, within 72 h of the PCR SARS-CoV-2$^+$ test. The eligibility criteria for inclusion in this study were a confirmed SARS-CoV-2 infection (by RT-PCR) in a nasopharyngeal swab, without evidence of previous infection, irrespectively of symptoms (asymptomatic or symptomatic) or disease severity (mild, moderate/severe). Asymptomatic patients ($n = 8$) were defined as positive for the SARS-CoV-2 PCR test, but with no signs of disease. The following criteria were used to stratify symptomatic SARS-CoV-2 patients in terms of severity, accordingly with WHO guidelines [28]; 1-MILD ($n = 40$): individuals without evidence of pneumonia; 2-MODERATE/SEVERE ($n = 34$): individuals with evidence of pneumonia and need of supplemental oxygen and/or individuals with need of invasive mechanical ventilation and of admission to hospital intensive care unit. Patients' demographic and relevant clinical data are summarized in Supporting Information Table S1. Additionally, disease progression was evaluated at day 14 post-diagnosis and patients were grouped according to their severity: presence of symptoms at day 14 postdiagnosis (poor prognosis) ($n = 51$) *versus* absence of symptoms (good prognosis) ($n = 26$) (Supporting Information Table S2). Diagnosis of pneumonia and need of oxygen therapy were the clinical criteria used to define presence/absence of symptomatology at day 14 postdiagnosis.

Healthy controls ($n = 7$) are represented by individuals with no history of recent or active infectious diseases.

Informed consent was obtained from all included patients and the research protocols were previously approved by the institutional ethics committee of CHUP, CHVNG, and HBA.

Isolation of IgG from human plasma

All human plasma samples (100 μL) were defrosted and centrifuged at 1620 g for 10 min. Afterwards, the plasma samples were diluted 8× with PBS (1×) and filtered through 0.45 μm

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Supor AcroPrep 96-well filter plates. Plasma samples were applied to the protein G plate and instantly washed three times with 10 column volume (CV)× PBS (1×) to remove unbound proteins. IgG was eluted from the protein G using 5 CV of 0.1 M formic acid (pH 2.5), into a 96-deep-well plate (Waters, Milford, MA) and immediately neutralized to pH 7.0 with 1 M ammonium bicarbonate to maintain the IgG stability. Preparation and analysis of Fc *N*-glycans is described in the Supporting Information.

Glycopeptide preparation

To 20 mg of IgG sample, 0.2 μg of TPCK-treated trypsin (Promega, Madison, WI) was added, followed by an overnight incubation at 37°C. Tryptic digests were purified using a solid-phase extraction on C18 beads (Chromabond, MACHEREY-NAGEL, Düren, Germany). Note that 10 μg of C18 beads was applied to each well of an OF1100 96-well polypropylene filter plate (Orochem, Naperville, IL). The RP stationary phase was activated three times with 200 μL of 80% ACN and conditioned with three times with 200 μL 0.1 % TFA (Sigma-Aldrich/Merck, Darmstadt, Germany). The IgG digests were diluted with 0.1% TFA (Sigma-Aldrich/Merck, Darmstadt, Germany), loaded onto the C18 beads, and washed with three times 200 μL 0.1% TFA. The entire procedure was performed on a vacuum manifold under pressure reduction of 2 inches of mercury. IgG glycopeptides were eluted into an ABgene PCR 96 well plate (Thermo, Waltham, MA) with 200 μL of 20% ACN by centrifugation at 105 g for 5 min. Eluates were dried by vacuum centrifugation and stored at –20°C until analysis by MS.

nano-LC-ESI-MS of IgG N-glycopeptides

Purified tryptic IgG glycopeptides were analyzed on Waters ACQUITY M-Class UPLC system (Waters, Milford, MA), consisting of binary pump, auxiliary pump, autosampler maintained at 10°C, and column oven compartment set at 30°C. Note that 10 mL of sample was applied to a PepMap 100 C18 (5 mm × 300 μm id, 5 μm; Thermo, Waltham, MA) solid-phase extraction trap column conditioned with 0.1% TFA (mobile phase A) for 1 min at 40 μL/min. After sample loading, the trap column was switched in-line with the gradient and C18 nano-LC column (150 mm x 100 μm id, 2.7 μm HALO fused core particles; Advanced Materials Technology, Wilmington, DE) for 9.5 min. Trap column was cleaned with two full loop injections containing 20 μL of 95% ACN and 50% ACN in IPA, respectively. C18 nano-LC column was equilibrated with 100% mobile phase A (0.1% TFA) for 2 min. IgG glycopeptides were reconstituted in 80 μL of ultrapure water before injection. Separation was achieved at 1 mL/min using the following gradient of mobile phase A and mobile phase B (80% ACN and 20% 0.1% TFA, respectively): 0.5 min 12% B, 0.5–4 min 12% B–17% B, and 4–5 min 17% B. The ACQUITY M-Class system was coupled with a Compact mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with Captive Spray ion source and nanoBooster for introduction of acetonitrile vapor into the source. A nitrogen was used as a drying gas (4 L/min) and nebulizer (0.4 bars). Quadrupole and collision energies were set to 4 eV. Spectra were recorded from *m/z* 800 to 2000 with two averaged scans at a frequency of 0.5 Hz. Acquity M-class UPLC system was operated under MassLynx software 4.1 while Bruker Compact Q-TOF-MS was operated under HyStar software version 4.1.

Glycan traits were grouped accordingly: agalactosylated—G0 (H3N4, H3N4F1, H3N5F1, H3N5), monogalactosylated—G1 (H4N4, H4N4F1, H4N4F1S1, H4N5F1, H4N5F1S1, H4N5), digalactosylated—G2 (H5N4, H5N4F1, H5N5F1, H5N5F1S1, H5N4F1S1, H5N4S1), sialylated—S (H4N4F1S1, H4N5F1S1, H5N5F1S1, H5N4F1S1, H5N4S1), bisecting—Bis (H3N5F1, H3N5, H4N5F1, H4N5F1S1, H4N5, H5N5F1, H5N5F1S1), and fucosylated—F (H3N4F1, H3N5F1, H4N4F1, H4N4F1S1, H4N5F1, H4N5F1S1, H5N4F1, H5N5F1, H5N5F1S1, H5N4F1S1).

NK cells isolation

Peripheral blood mononuclear cells (PMBCs) were obtained from buffy coats from healthy donors. The separation of PMBCs was achieved by density centrifugation using Lymphoprep (density 1.077 g/mL) (Stem Cell Technologies, Vancouver, Canada). NK cells were purified by negative selection from freshly PBMCs using EasySep Human NK Cell Isolation Kit, according to the manufacturer's instructions (Stem Cell Technologies). The purity (>94%) of NK cells was determined by flow cytometry analysis on FACSCanto II system (BD Biosciences, San Jose, CA). Monoclonal antibodies used for staining of NK cells were Pacific Blue anti-human CD3 (OKT3), PE anti-human CD56 (HCD56), and Brilliant Violet 421 anti-human CD16 (3G8) (all from BioLegend, San Diego, CA). Cells were cryopreserved in freeze medium (90% of fetal bovine serum [FBS] and 10% of dimethyl sulfoxide [DMSO] [Scienova, Jena, Thuringia, Germany]) at –80°C. After defrosting, cryo-preserved PBMCs and cryo-preserved NK cells were maintained in complete culture medium, RPMI 1640 Medium, GlutaMAX Supplement, HEPES (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% of FBS, 1% penicillin/streptomycin, and 0.1% gentamicin at 37°C in an incubator with a humidified atmosphere with 5% of CO_2, overnight in order to recover and settle.

SPR analysis for FcγRIIIa-IgG Fc binding analysis

Due to the limited amount of total IgG available, for each sample of patients and healthy controls, a traditional affinity assay was not feasible, hence we opted for the binding assay format.

SPR interaction analysis was performed on a Biacore X100 system (Cytiva). For interaction analysis of FcγRIIIa and the Fc fragment of IgG isolated from COVID-19 patients and healthy controls, FcγRIIIa (R&D Systems) was immobilized with standard amine coupling (Cytiva) on a CM5 chip (Cytiva) at a density of 233 resonance units (RU) using a solution of 10 μg/mL in sodium

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acetate buffer 10 mM at pH 4.5. IgG samples were diafiltered for buffer exchange into the SPR running buffer (HBS-EP buffer: 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% Tween-20) using Amicon Ultra-2 Centrifugal Filter Units (Merck) and then were injected at a concentration of 250 nM in triplicate and a flow rate of 30 μL/min for 240 s (association time). The dissociation phase was monitored for 600 s. Regeneration was carried out with two 30 s-injections of 3.6 M $MgCl_2$, 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.6. For the evaluation (Biacore X100 evaluation software 2.0.3), a report point has been set before injection of the analyte and another at the end of the association phase. The differences between these two report points were used for the evaluation of relative differences in binding.

Antibody-dependent NK activation

In order to evaluate the impact on IgG effector functions, the activation of NK cells was evaluated. Briefly, a pre-coating of 96-wells cell culture plate, flat bottom, tissue culture treated, polystyrene (Orange Scientific) was performed with 3 μg/mL of native IgGs and 3 μg/mL of IgGs treated with β-galactosidase. Coated 96-well plate was maintained at 37°C in an incubator with a humidified atmosphere with 5% of CO_2 overnight. Afterwards, purified NK cells were washed once with culture medium, suspended in complete RPMI medium supplemented with IL-2 (100 U/mL) and adjusted to a final concentration of 2×10^5 cells per well in a coated 96-well plate. Cells were maintained at 37°C in an incubator with a humidified atmosphere with 5% of CO_2. Following 24 h of incubation, cells were stained with PE anti-human CD69 (FN50) (Thermo Fisher Scientific), as marker of activated NK cells (Supporting Information Fig. S2). Posteriorly, cells were analyzed by flow cytometry on a FACSCanto II (BD, Biosciences) using the FACSDiva software (BD), compensated and analyzed in FlowJo v10.4. (Tree Star, Inc.). Data acquisition and analysis adhered to the general guidelines for flow cytometry [39].

Degalactosylation of human IgG

Total IgGs from each patient (1 μg) were incubated for 1 h at 37°C with 1 μL β1-4 Galactosidase S (New England BioLabs). The efficiency of sugar removal was confirmed by lectin blotting. Briefly, 100 ng of IgG was subjected to 10% SDS-PAGE electrophoresis and membranes were blocked with BSA 4% before incubation with *Erythrina cristagalli* (ECA) lectin, which recognizes terminal galactose residue. Bands were then visualized using the Vectorstain Elite ABC kit (Vector Labs) and the detection was performed using ECL reagent (GE Healthcare, Life Sciences).

Anti-Spike and total IgG ELISA and ECA lectin ELISA

Detection of lectin-binding motifs in the serum total IgG and anti-Spike IgG from COVID-19 seropositive patients was performed by a lectin ELISA adapted from Ref. [40]. Briefly, serum anti-Spike IgG was captured using SARS-CoV-2 ELISA kit plates (Immunostep). Blocking was performed with Carbo-free blocking solution (Vector Labs) for 1 h at RT. Plates were washed five times with PBS + 0.05% Tween 20 (PBST) before COVID-19 samples, diluted 1:50 in PBS containing 1% Carbo-free blocking solution (diluent solution) (Vector Labs), were added, and incubated by shaking at 200 rpm for 2 h at RT. After washing as described above, galactosylation by incubating captured IgG with biotinylated ECA lectin (Vector Labs) diluted 1:2000 in diluent solution (Vector Labs) and incubated for 1 h shaking at 200 rpm at RT. Bound lectin was detected using an HRP-conjugated streptavidin (DuoSet R&D Systems) incubated for 20 min, and tetramethylbenzidine substrate (DuoSet R&D Systems) was incubated for 20 min protected from dark. Reaction was stopped using H2SO4 and the amount of bound lectin was measured at 450 nm using a μQuant Microplate Reader (BioTek, Agilent).

Total IgG galactosylation was detected by coating microtiter plates (Maxisorp, Nunc) with 1:50 isolated IgGs (as described above) from seropositive patients. The procedure afterwards was performed as described above for anti-Spike IgG.

Serum IgG antibodies against recombinant SARS-CoV-2 spike protein (S) were analyzed from vaccinated healthy donors ($n = 4$), noninfected individuals ($n = 4$), asymptomatic-infected individuals ($n = 8$), mild disease ($n = 10$), and moderate/severe disease patients ($n = 12$) using a chemiluminescence method (Immunostep) according to the manufacturer's instructions. An antibody level of ≥1 signal was considered positive and a level of <0.8 was considered negative.

Cytokine quantification

Supernatants from 24-h-cultured NK cells were analyzed by flow cytometry using the BD Cytometric Bead Array Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences) following the manufacturer's instructions.

Statistical analysis

Data visualization and statistical analysis were done using GraphPad Prism 9 and SPSS software. For the age correction analysis between the association of IgG glycan traits and disease severity, logarithm of IgG glycan traits abundance was used in a linear regression model. For the age correction analysis between IgG glycan traits and prognosis, logarithm of IgG glycan traits abundance was used in a binary logistics regression model.

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Conflict of interest: The authors declare no conflict of interest.

Author contributions: MMV, IA, and SSP designed research. MMV, IA, JG, CSR, ÂF, AMD, JS, TP and FFS performed research. SSP and GL contributed new reagents/analytical tools. AS, NS, TT, LM, MMA, and RSC provided and characterized clinical samples. MMV and IA analyzed data. MMV, IA, and PO performed statistical adjustments. MMV, IA and SSP wrote the manuscript with contributions from all authors.

Ethics approvals: Informed consent was obtained from all included patients and the research protocols were previously approved by the institutional ethics committee of CHUP, CHVNG, and HBA, Portugal.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Abbreviations: **DC**: disease course · **ECA**: *Erythrina cristagalli* agglutinin · **SPR**: surface plasmon resonance

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Supplementary information

Supplemental Tables

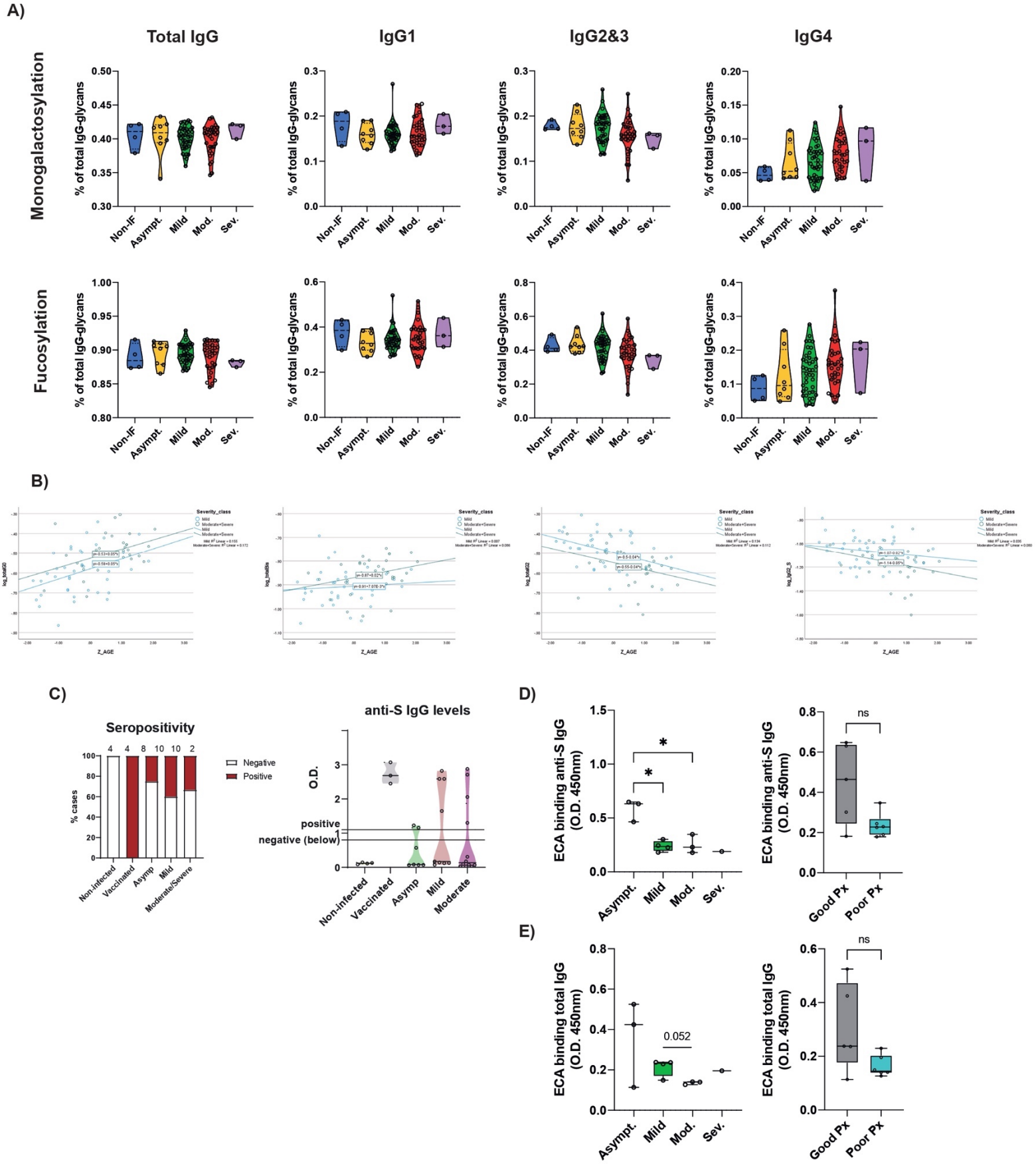
Supplemental Table 1 - Demographics of COVID-19 patients at time of sample collection. The following comorbidities were considered: heart disease (HD), obesity (Ob) or pulmonary disease (PD); other diseases (other) includes: diabetes, cancer and arterial hypertension.

	Asymptomatic n=8 (%)		**Mild** n=40 (%)		**Moderate/Severe** n=33 (%)	
Age						
21-30	1	(12.5)	5	(12.5)	1	(3.03)
31-40	1	(12.5)	5	(12.5)	1	(3.03)
41-50	1	(12.5)	10	(25.0)	2	(6.06)
51-60	2	(25.0)	6	(15.0)	4	(12.1)
61-70	2	(25.0)	8	(20.0)	8	(24.2)
71-80	1	(12.5)	3	(7.50)	7	(21.2)
81-100	0	(0)	3	(7.50)	10	(30.3)
Gender						
F	6	(75,0)	21	(52,5)	10	(30,3)
M	2	(25,0)	19	(47,5)	23	(69,7)
Comorbidities						
HD, Ob or PD	0	(0,0)	4	(10,0)	8	(24,2)
other	5	(62,5)	9	(22,5)	22	(66,7)
No	3	(37,5)	27	(67,5)	3	(9,1)

Supplemental Table 2 - Demographics of COVID-19 patients for the longitudinal analysis. The following comorbidities were considered: heart disease (HD), obesity (Ob) or pulmonary disease (PD); other diseases (other) includes: diabetes, cancer and arterial hypertension.

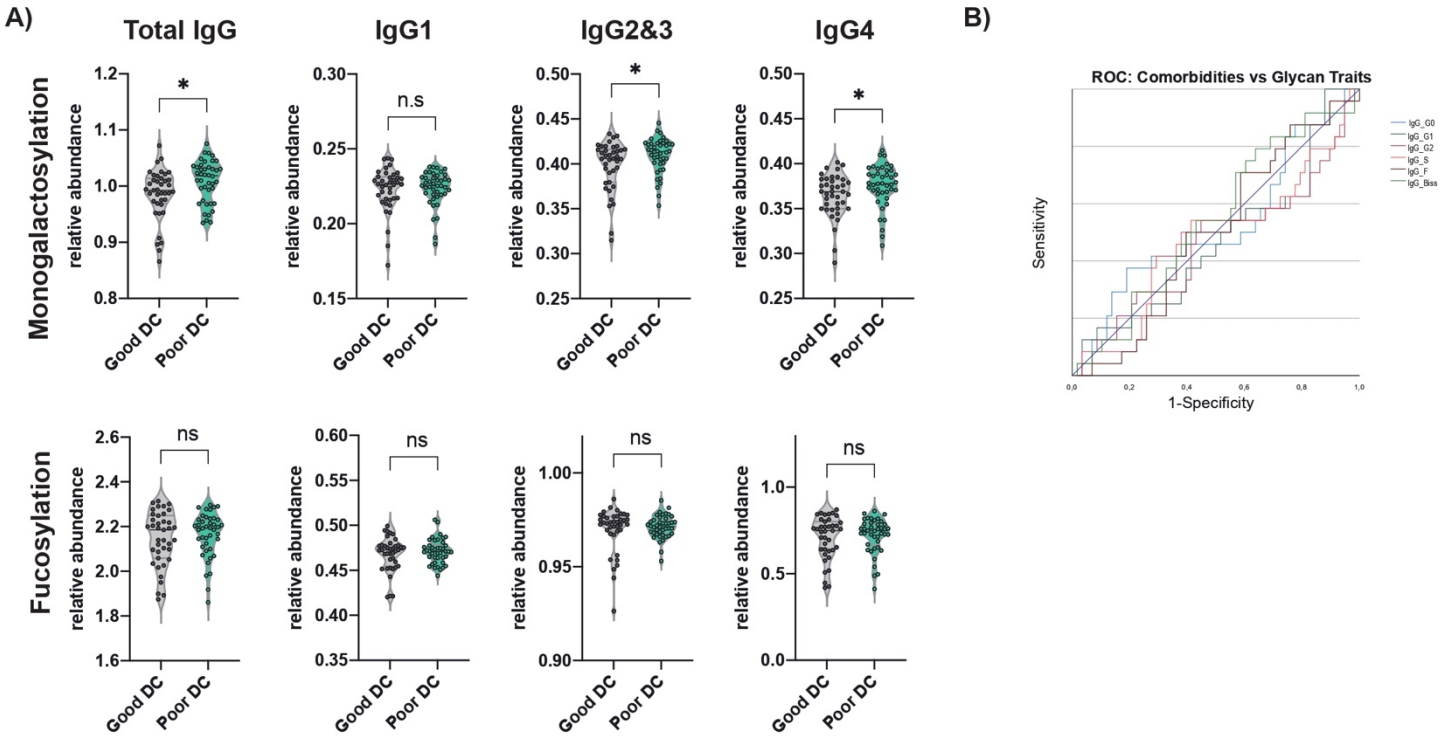
	Good Prognosis n=26 (%)	**Poor Prognosis** n=51 (%)
Age		
21-30	4 (15.4)	2 (3.92)
31-40	0 (0)	6 (11.8)
41-50	5 (19.2)	7 (13.7)
51-60	4 (15.4)	8 (15.7)
61-70	8 (30.8)	10 (19.6)
71-80	1 (3.85)	9 (17.6)
81-100	4 (15.4)	9 (17.6)
Gender		
F	14 (53,8)	21 (41,2)
M	12 (46,2)	30 (58,8)

Supplemental Figure 1



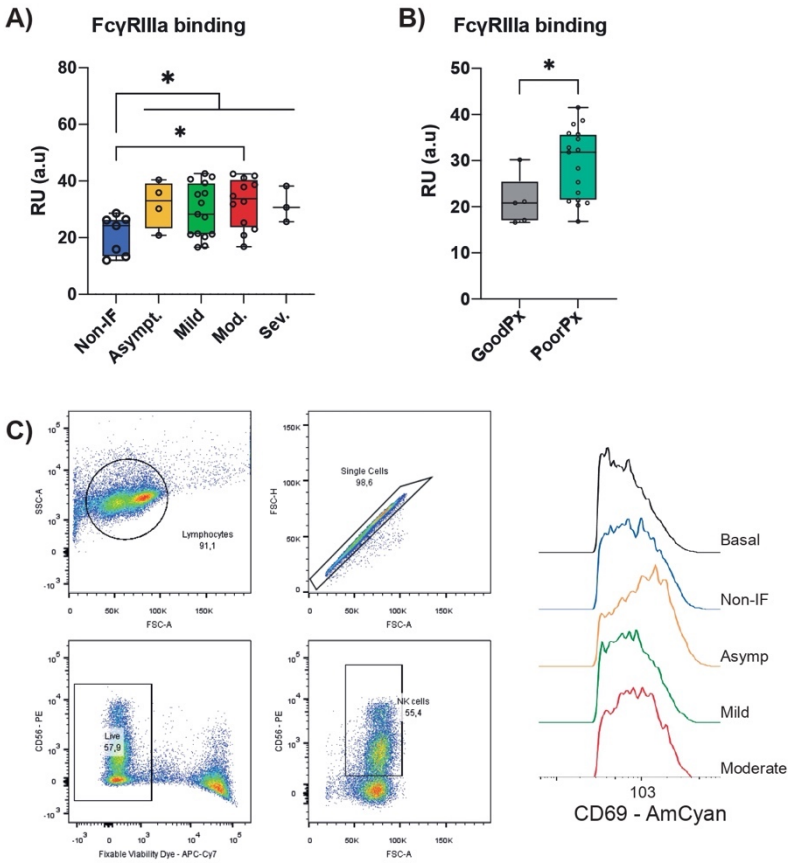
Supplemental Figure 1 - A) Relative abundance of total and isotypes IgG Fc monogalactosylation and fucosylation in different COVID-19 severities (asymptomatic n=8, mild n=40, moderate n=31, severe n=3) and non-IF individuals (n=4). B) Linear regression analysis for age and IgG glycan traits. C) ELISA relative quantification of anti-Spike IgG in serum from COVID-19 patients. D) Levels of terminal galactosylation of anti-Spike IgG, according to severity and prognosis of COVID-19 disease. E) Levels of terminal galactosylation of total IgG, according to severity and prognosis of COVID-19 disease. Each data point represents the data from a single patient/subject isolated IgG's in a single LC-MS analysis (one replicate).

Supplemental Figure 2



Supplemental Figure 2 – A) Relative abundance of total and isotypes IgG Fc monogalactosylation and fucosylation in good versus poor prognosis (good prognosis n=51; poor prognosis n=26) and non-IF individuals (n=4). B) Receiver Operating Characteristic (ROC) curve plotter for the IgG Fc N-glycan traits' levels of COVID-19 patients with comorbidities (heart disease, obesity and/or lung disease). Each data point represents the data from a single patient/subject isolated IgG's in a single LC-MS analysis (one replicate).

Supplemental Figure 3



Supplemental Figure 3 – A) SPR response unit (R.U) quantification of human recombinant FcγRIIIa binding to the IgGs from patients with COVID-19 disease severities (n=31) and (B) prognosis (n=22). C) Gating strategy for the identification of NK cells through the expression of marker CD56, and representative histograms showing the differences in the median intensity fluorescence (MFI) of activation marker CD69. Results shown from at least two independent experiments. Each point represent data from a single patient/subject.

CHAPTER 5

GENERAL DISCUSSION AND FINAL REMARKS

GENERAL DISCUSSION

Glycans in T cell development

The development of the several branches of immune system is crucial for effective responses during an organism's life. Specifically, T cell development is a key biological process that ensures the formation of a functional and diverse TCR repertoire of the T cell pool, required for homeostatic and perturbed immunity. The discovery of cellular developmental mediators of thymocyte maturation has deserved attention since the 1960´s, and have shaped modern immunology [1]. Unsurprisingly, there are still several avenues for further research in the molecular and cellular mechanisms required for T cell development, being the role of glycans a promising one. As discussed in **Chapter 1.1**, glycans have played an "omnipresent" role in the birth of modern immunology [2]. In terms of T cell development, glycans have been shown to influence several key receptors, such as NOTCH1 [3], CD4 and CD8 co-receptors and the TCR [4], and their related developmental checkpoints. In **Chapter 1.2**, we have gathered the relevant bodies of work to suggest that glycans are in fact developmentally regulated in T (and B) cell development, having many regulatory implications in immunity and susceptibility to immunological diseases. Furthermore, in **Chapter 2**, we have shown a dynamic nature of the glycome of developing thymocytes, specifically in terms of *N*-glycan branching and terminal sialylation. The glycome can be regulated by the transcription of glycosylation pathway-related genes (glycogenes), encoding glycosyltransferases, glycosidases or monosaccharide transporters. Further studies are needed to uncover the transcriptional regulation of glycogenes in thymocytes, with the use of single-cell RNA or ATAC (assay for transposase accessible chromatin, that measures chromatin condensation/relaxation, which is related to active transcription regulation) sequencing. In **Chapter 2** we have also found thymocyte glycome differences between human and mice, specifically in ß1,6-GlcNAc branching. Species-specific developmental processes may require different glycomes [5]. To the best of our knowledge, the manuscript that composes **Chapter 2** is the first report of simultaneous glycan structure detection in human and murine thymocytes. Regarding the detection of glycans, we have employed lectins as probes, in flow cytometry. Lectins are plant-derived proteins with specificity for glycan structures [6]. Despite being valuable tools for glycome determination, analytical methods, such as mass spectrometry (MS), would provide an added layer of information in the study of glycomes. The lectin-based approach confirmed the suggested dynamic feature of the glycome in T cell development, and future

work on MS-based *N*-glycome determination of sorted thymocyte populations should provide a more complete picture of this potential developmental regulator.

Having demonstrated the presence of high levels of complex *N*-glycans in the surface of human and murine thymocytes, specifically in DN stages, we generated two novel murine models of *N*-glycosylation deficiency in DN2 thymocytes. The genetically glycoengineered thymocyte analysis showed severe population alterations upon *Mgat1* deletion. These alterations were further explored, and we found highly mannosylated DN3 cells had decreased levels of CD127 (IL-7 receptor **α** chain) and CD25 (IL-2 receptor), compared to their control littermates. These two receptors play essential roles in ß-selection [8] and we observed defects in the generation of ß-selected DN3 thymocytes, in the conditional knockout of *Mgat1*. Accordingly, it was recently shown that DN2-specific deletion of the *Gfat1* gene increased the presence of mannosylated *N*-glycans and block the ß-selection developmental checkpoint [9]. The GFAT1 (glutamine-fructose-6-phosphate transaminase 1) enzyme catalyzes the rate-limiting reaction involved in the synthesis of UDP-GlcNAc, the substrate of GnT's. Furthermore, we observed striking deficiencies in DP selection in the absence of complex *N*-glycans, related to their role in regulating TCR thresholds for activation, a phenotype that had been previously observed [4], and further validated by us. In order to push forward the knowledge on the role of complex *N*-glycans in DP selection, we studied CD4/CD8 lineage commitment and found differences in *Mgat1*-deficient thymocytes, which agrees with previous knowledge on TCR signaling requirements for lineage specification [10]. Moreover, we found a novel impact of the absence of complex *N*-glycans in TCR repertoire formation, having demonstrated differences in TCRvß usage in *Mgat1*-deficient mature SP cells. We are currently evaluating this impact with next-generation sequencing, which should provide additional information into the characterization of the TCR repertoires. Finally, we observed the lack of Treg generation in the absence of *Mgat1*, which was also the first report of complex *N*-glycan requirements for Treg development, to our knowledge. This observation also agrees with the influence of complex *N*-glycans on TCR thresholds of activation, being the Treg pool derived of CD4 SPs which display high levels of TCR signaling in the thymus [11]. Interestingly, the analysis of *Mgat2*-deficient mice did not show severe phenotypes, which was surprising. Upon the lectin-based glycoprofiling of thymocytes from these mice, we observed a 10-fold increase in poly-LacNAc structures, which agrees with the previously suggested mechanism of *N*-glycan compensation. It was suggested that in the absence of additional antennae, hybrid *N*-glycans contain aberrant antenna elongation, thus generating "bioequivalent" structures [12].

The γδ T cell subset development is still not fully understood, being the subject of intense debate in the last few years [13]. Two models were suggested for the γδ T cell lineage commitment: the “stochastic” and the “signal strength” models. In the first, it was suggested that γδ lineage fate determination occurred prior to *Tcr* gene rearrangements, in DN2/3 thymocytes with high levels of IL-7 signaling [14]. In the second, nowadays more accepted, claims that high levels of TCR signaling in DN2/3 cells lead to commitment to the γδ lineage [15]. In this model, *Tcrb*, *Tcrg* and *Tcrd* loci are rearranged simultaneously. Interestingly, in the murine model of *Mgat1* deficiency, we observed no alterations in γδ T cell generation, in contrast with severe deficiencies in αß T cells. In fact, Mgat1-deficient γδ T cells displayed both in the thymus and the spleen high levels of cellular activation. Our results argue that the complex *N*-glycan mediated regulation of αß TCR clustering, can also occur in the γδTCR. In the thymus, *Mgat1*-deficient γδ T cell progenitors display increased signal strength, which can explain why this subset is not reduced in these mice.

The role of *N*-glycans in the regulation of peripheral mature T cell responses has been the focus of several bodies of work [16, 17]. We were interested in the peripheral consequences of such alterations in T cell development in *Mgat1* conditional knockout mice. Interestingly, we observed a spontaneous increase of colon and kidney inflammation, in these mice, when compared to littermate controls. This inflammatory phenotype agrees with the lack of TCR clustering control of T cells that lack complex *N*-glycans. Additionally, we observe increased representation of highly activated γδ T cells in the periphery, which may also be driving inflammation. When we infected *Mgat1*-deficient mice with the obligate intracellular protozoan *N. caninum* (as immune responses to this parasite were previously shown to be highly T-cell-dependent [18]) we observed a tendency of increased susceptibility to infection, that should be further explored in a chronic model Curiously, in the spleen of infected mice we observed a lack of naïve CD4 and CD8 T cells, being these pools composed of effector T cells ($CD44^+CD62L^-$). This finding deserves further attention, as it hints for a role of *N*-glycans in peripheral T cell differentiation, beyond TCR regulation. Altogether, the generation of the *Mgat1*-deficiency model highlighted the requirements of complex *N*-glycans, and the pathological role of mannosylated ones, in immune homeostasis and function.

T cell glycosylation in COVID-19

With the rise of SARS-CoV-2 infected subjects in early 2020, there was a pressing need to further increase the knowledge on the immune responses to the virus. Initially, COVID-19

patient cohorts were highly characterized in order to gain insights on the immediate immune responses to infection, so patient stratification and improved healthcare systems management could be done [19-21]. Differential cytokine profiles were observed in serum of patients with differential disease severity, indicating several degrees of the initial immune response to the infection [22]. In **Chapter 3** we hypothesized on whether such inter-individual differences could also be observed at the level of the glycome of circulating immune cells. Interestingly, we could observe decreased levels of ß1,6-GlcNAc *N*-glycan branching and α2,6-Sia specifically in circulating CD8 T cells from asymptomatic patients, compared to non-infected healthy individuals. These glycome alterations were not seen in circulating T cells from symptomatic patients. In a simple setup, we could drive the same T cell alterations (from healthy donor-derived PBMCs) by culturing these cells with serum from asymptomatic patients, which was not observed in the culture conditions with serum from non-infected or symptomatic subjects. Interestingly, we observed decreased levels of IL-2 in the serum of asymptomatic patients, when compared to non-infected controls and with symptomatic ones. Signaling driven by IL-2 was shown to increase the *MGAT1* expression levels in human T cells [23]. Counterintuitively, levels of branched *N*-glycans decrease upon MGAT1 upregulation, due to the striking difference in UDP-GlcNAc affinities from the diferent GnT's. In fact, GnT-I has a 250-fold higher affinity for its substrate that GnT-V [23]. Studies are still to be done on IL-2 mediated regulation of sialyltransferase genes expression. The glycome alterations found in asymptomatic patients were suggested to be related to more efficient responses, not found in symptomatic ones. Further work should be done in the direction on the role of circulating T cell glycome changes and tissue/lymphoid tissue migration.

IgG glycans in COVID-19

One of the major features of adaptive immunity is generation of antibodies against pathogens. Immunoglobulins compose the family of molecules that recognize pathogen-derived epitopes, being IgG's the major class responsible for systemic antiviral immunity. As it was mentioned in **Chapter 1.1**, the IgG Fc *N*-glycome composition is associated with several inflammatory diseases, including COVID-19. In **Chapter 4**, we tackled the connection of the differential dynamic responses of circulating IgG's glycan composition between disease prognosis. We characterized the glycosylation signature of plasma IgG isotypes, namely IgG1, IgG2&3 and IgG4, in healthy non-infected subjects COVID-19 patients, with different degrees of disease severity at diagnosis. Interestingly, we observed a pronounced pro-inflammatory IgG signature in patients with a moderate disease severity,

highlighted by decreased levels of galactosylation and sialylation, when compared to mild ones. These observations agree with other studies [24]. The potential of molecular determinants for COVID-19 prognosis prediction was at the time, and still is, a subject under exploration. In **Chapter 4**, we asked whether the glycan signature of circulating IgG's could be used as a prognosis predictor. Using the same longitudinal patient cohort we evaluated the disease course between diagnosis and day 14 post-diagnosis, and observed that patients with a worse disease course (pneumonia or need for oxygen therapy at day 14) had decreased levels of IgG galactosylation, sialylation and increased bisecting GlcNAc, all promoting a pro-inflammatory effector function [25], when compared to patients with a good disease course (no symptoms at day 14). Moreover, we identified three glycan traits with high predictive potential of COVID-19 diagnosis, namely total IgG digalactosylation, IgG1 agalactosylation and IgG2&3 digalactosylation. This set of results showed the potential of a glycan based screening system for the detection of individuals with a high-risk of progressing to a severe/complicated disease. In order to gain mechanistic insights of the differential IgG glycan signatures observed in the patients, we showed that the pool of circulating IgG's from patients with a worse disease course could induce higher NK cell activation, *in vitro*, with increased IFN-γ and TNF-α production levels, when compared to the IgG's of patients with a good disease course. Analysis of SARS-CoV-2 specific IgG from other studies highlight fucosylation as the main driver for Fc γ-receptor binding, and relationship with disease severity [24]. The experiments in **Chapter 4** were done with the whole pool of circulating IgG's, and these results should be more related to overall systemic responses. In summary, in **Chapter 4** we addressed a cutting-edge issue on how the IgG *N*-glycome impacts immune responses to infection, including the one of SARS-CoV-2. Our study was conducted during the first wave of the COVID-19 pandemic (March – July 2020), when there were no vaccination strategies and an urgent need to optimize the allocation and management of healthcare resources. In the era we are currently living, with high rates of vaccination in developed countries, we believe that the data presented may be relevant in viral infection initial responses.

FINAL REMARKS

Though generally understudied in life sciences, glycans form the outermost structure of cells and thus the essential communication interface with the microenvironment. Glycan biosynthesis is a template-free process including a multitude of consecutive and in part competing enzymatic steps. Each error in the glycan biosynthesis results in a 'gain of function' and may disturb the checks-and-balances system that guarantees immunological integrity. The work in this doctoral thesis was developed with the focus of understanding the role of glycans and the glycome in key aspects of adaptive and humoral immunity. The evidences generated highlight a vast library of biological processes where glycans could play a major role, namely in development and initial responses to viral infections and immunological disorders. Technological developments in the field of Glycomics should pave the way for a better understanding of glycans in Immunity, with the exciting prospect of generating advances in basic and translational research.

The work that constitutes this thesis pushed forward the understanding on how the glycome can influence such fundamental biological processes, such as T cell development and initial response to infection, as well as the IgG *N*-glycome involvement in COVID-19. This thesis also leaves some outstanding questions:

(1) What is the precise glycome composition of developing T and B cells? This should be addressed by the isolation cells in specific developing stages, followed by the MS-based glycome determination;
(2) What key T cell development-related receptors, such as the IL-7R or the TCR, are differentially glycosylated in thymocytes? The glyco-proteomic analysis of pulled down lysates from specific thymocyte populations should be used to investigate this question;
(3) Is human T cell development altered in Congenital Disorders of Glycosylation patients? Surprisingly, this has not been explored, to the best of our knowledge;
(4) Are glycans promoting/inhibiting leukemic transformation of early developmental stage thymocytes? The study of glycans in leukemic transformation has also not been a major focus of research;
(5) How does the glycome influence in T and B cell development translates in the susceptibility of immunological diseases? Here, the use of murine models of glycosylation alterations in T and B cell development should be done to evaluate disease susceptibility.

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APPENDIX

I GLYCANS AS KEY CHECKPOINTS OF T CELL ACTIVITY AND FUNCTION

Frontiers in Immunology. 2018;9

II GLYCANS AS SHAPERS OF TUMOUR MICROENVIRONMENT: A SWEET SRIVER OF T-CELL-MEDIATED ANTI-TUMOUR IMMUNE RESPONSE

Immunology. 2022;168(2):217-232

III COMPOSITION OF THE IMMUNOGLOBULIN G GLYCOME ASSOCIATES WITH THE SEVERITY OF COVID-19

Glycobiology. 2021;31(4):372-377

APPENDIX I

[REVIEW PAPER]

GLYCANS AS KEY CHECKPOINTS OF T CELL ACTIVITY AND FUNCTION

Márcia S. Pereira, Inês Alves, Manuel M. Vicente, Ana Campar, Mariana C. Silva, Nuno A. Padrão, Vanda Pinto, Ângela Fernandes, Ana M. Dias, Salomé S. Pinho

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Here, we revised the current knowledge on the roles of T cell glycans biology. My contribution focused on the revision of the evidences regarding T cell development.

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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

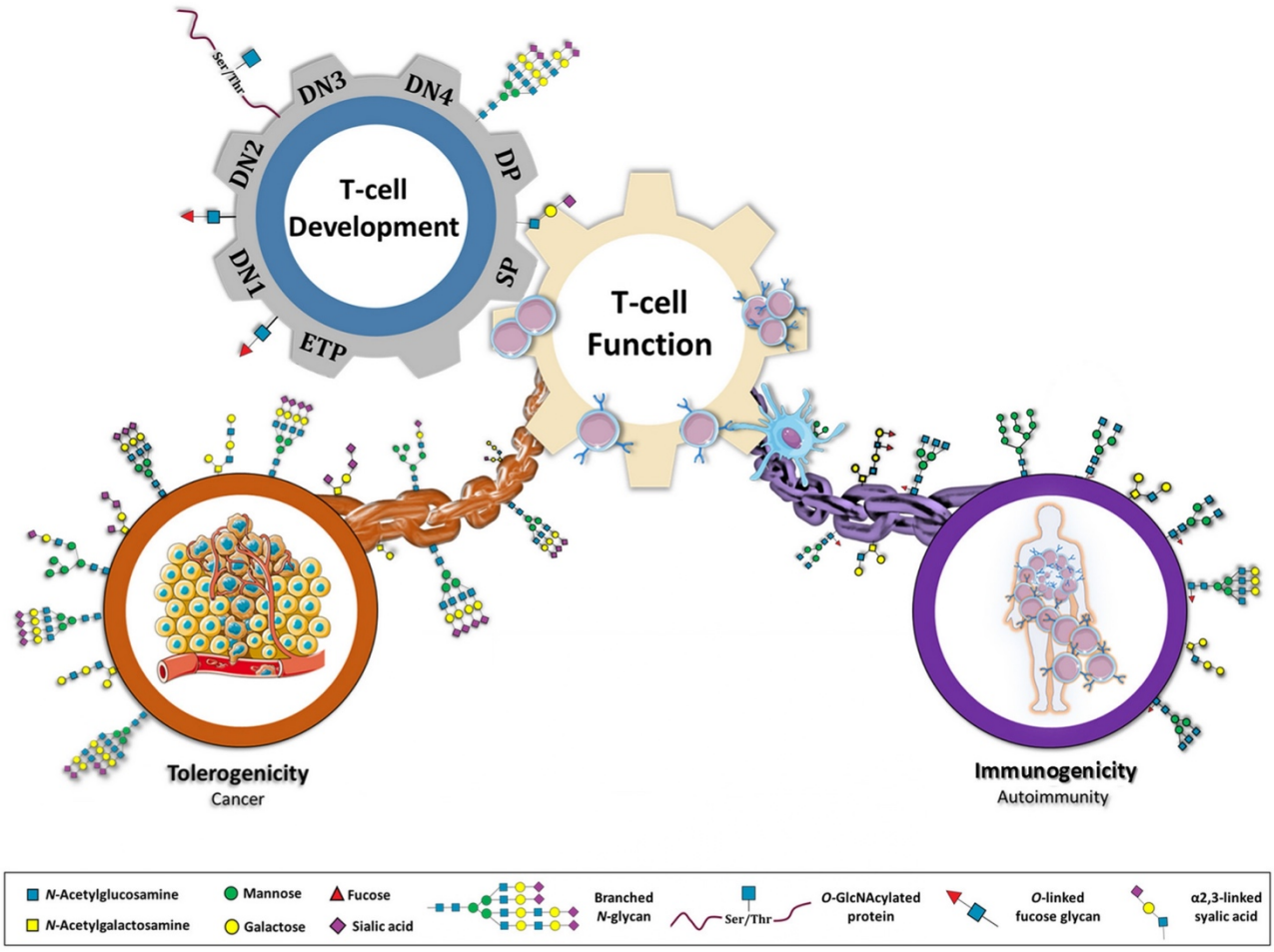


FIGURE 1 | Glycans as a major connective chain that controls T cell response in either a tolerogenic or immunostimulatory scenario. Glycosylation appears to be central in regulating several steps of a T cell's life. During T cell development, different population of T cells (ETP, early thymocyte progenitor; 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.

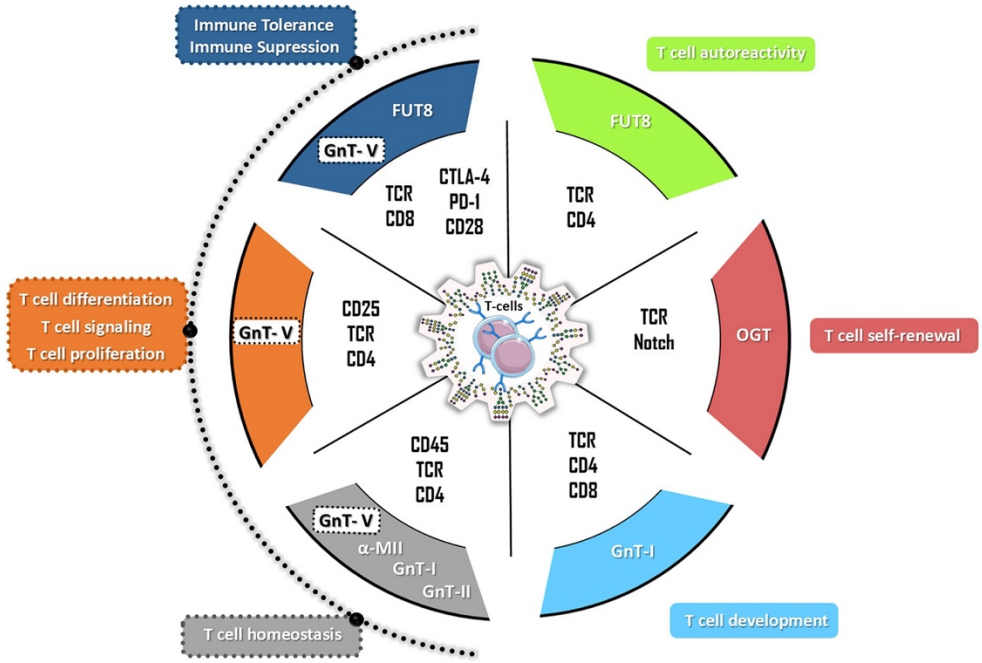


FIGURE 2 | The hallmarks of glycans in T cell biology. *N*-glycans have a broad effect on the multiple T cell functions with impact both in autoreactivity and in immune tolerance. Particularly, the complex branched *N*-glycans catalyzed by beta 1,6-*N*-acetylglucosaminyltransferase V (GnT-V) (encoded 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 II

[REVIEW PAPER]

GLYCANS AS SHAPERS OF TUMOUR MICROENVIRONMENT: A SWEET DRIVER OF T-CELL-MEDIATED ANTI-TUMOUR IMMUNE RESPONSE

Ângela Fernandes, Catarina M. Azevedo, Mariana C. Silva, Guilherme Faria, Carolina S Dantas, Manuel M. Vicente, Salomé S. Pinho

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Here, we revised the work on the role of T cell glycans in the cancer field. My contribution focused on addressing glycans in T cell development, the creation of the main figure, and the editing of the manuscript.

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REVIEW

immunology
Insights into immunity in health and disease

Glycans as shapers of tumour microenvironment: A sweet driver of T-cell-mediated anti-tumour immune response

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Abstract

Essentially all cells are covered with a dense coat of different glycan structures/sugar chains, giving rise to the so-called glycocalyx. Changes in cellular glycosylation are a hallmark of cancer, affecting most of the pathophysiological processes associated with malignant transformation, including tumour immune responses. Glycans are chief macromolecules that define T-cell development, differentiation, fate, activation and signalling. Thus, the diversity of glycans expressed at the surface of T cells constitutes a fundamental molecular interface with the microenvironment by regulating the bilateral interactions between T-cells and cancer cells, fine-tuning the anti-tumour immune response. In this review, we will introduce the power of glycans as orchestrators of T-cell-mediated immune response in physiological conditions and in cancer. We discuss how glycans modulate the glyco-metabolic landscape in the tumour microenvironment, and whether glycans can synergize with immunotherapy as a way of rewiring T-cell effector functions against cancer cells.

KEYWORDS
cancer immunology, cancer immunotherapy, glycosylation, T cells, tumour microenvironment

INTRODUCTION

One of the most widely occurring post-translational modifications of cells is glycosylation, an enzymatic process in which carbohydrate structures (glycans) are attached to proteins (or lipids), predominantly via nitrogen (*N*) and oxygen (*O*) linkages [1]. This pathway starts with an *en bloc* transfer to asparagine residues and entails a sequential addition/removal of carbohydrates, including N-acetylglucosamine (GlcNAc), glucose (Glc), fucose (Fuc), galactose (Gal), Mannose (Man), N-acetylgalactosamine (GalNAc), 5-N-acetylneuraminic acid (Neu5Ac or sialic acid), among others, by a variety of glycosyltransferases and glycosidases enzymes. This culminates in the generation of intricate glycan structures, with α- or β-linkages between monosaccharides. Glycans then cover the surface of essentially all cells, creating a dense coat of sugar chains, collectively known as glycocalyx. These carbohydrate structures act as key mediators for the

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control of several biological processes, including cell–cell adhesion, inflammation, host–pathogen interaction and immune recognition [2].

Alterations on the cellular glycan repertoire (glycome) are widely described as a hallmark of cancer [3, 4]. Specifically, aberrant expression of *N*-glycan structures in cancer cells have been associated with malignant transformation, invasiveness and metastatic potential of cancer cells [5–9]. Notably, this aberrant glycophenotype of malignant cells has a role in cancer immunosurveillance and immunoediting [10–12], which reinforces the major role of glycans in the interaction between cancer cells and immune system.

The immune system is known to be tightly regulated by glycosylation [13], with the development, trafficking, activation and overall function of T-cells being highly dependent on the glycosylation profile of their surface receptors [14]. Therefore, the glycan repertoire at T-cell surface constitutes an important player in the tumour microenvironment (TME) by regulating anti-tumour immunity. This highlights the functional relevance of T-cell glycome in neoplastic conditions, reinforcing the possibility of leveraging T-cell glycosylation as a new strategy to treat and prevent cancer.

In this review, we will discuss the role of T-cell glycosylation in physiological conditions and during malignant transformation, addressing how changes in T-cell glycosylation impact the effector functions of these cells against tumours. We will also highlight the latest advances concerning T-cell glycoreprogramming as a potential immunotherapeutic approach to enhance anti-tumour immune responses and to intercept cancer development and progression.

T-CELL GLYCOSYLATION: THE ROLE OF GLYCANS AS ORCHESTRATORS OF T-CELL DEVELOPMENT, FUNCTION AND ACTIVITY IN HOMEOSTASIS

The immune system is tightly regulated by glycosylation, in which glycans are vital tuners of the circuits that coordinate both innate and adaptive immune responses. Specifically, T-cell development, trafficking, antigen recognition and T-cell activation are highly dependent on the glycosylation profile of T-cell surface receptors [14, 15]. Disruption of this tightly regulated glycan-mediated system can lead to the formation of aberrant immunosuppressive networks that foster malignant transformation and cancer progression.

T-cell development dynamics are regulated by glycans

The development of T-cells occurs in the thymus. Thymic T-cell development is a major physiological process, in which the formation of a diverse repertoire of T-cell receptors (TCRs) and the establishment of central tolerance are the main goals, as they ensure proper T-cell responses [16]. This process is dependent on both cell-intrinsic and -extrinsic factors, which points to a major role of glycans (Figure 1) [14, 15].

The study of T-cell development has deserved a lot of attention in the last decades, having shaped modern immunology [17]. However, the role of glycans in this complex process is far from being completely understood. The development of T-cells has its initial step in the bone-marrow, where lymphoid progenitors migrate into the thymus, through the bloodstream. Evidence have been suggesting that glycans are essential determinants needed to assure a proper T-cell development [14, 18]. Specifically, we have shown that *N*-glycans are key molecules in the crucial steps of T-cell development, ensure proper stage transition dynamics [18]. In the sialyltransferase *St8SiaIV*$^{-/-}$ mouse model, deficient thymic seeding was observed, caused by inefficient progenitor bone-marrow egression [19]. The trafficking of thymus seeding progenitors (TSPs) requires the expression of P-selectin glycoprotein ligand-1 (PSGL-1) by those cells, and of its partner, P-selectin, by the thymic epithelium [20]. Being PSGL-1 a glycoprotein, it was observed that α1,3-fucosylation presence were required for its binding to P-selectin, and in its absence, by the genetic deletion of the *Fut4* and *Fut7* fucosyltransferases, TSPs homing was impaired [21]. Once TSPs enter the thymus, occurs their development into early thymocyte progenitors (ETPs), a subset of the CD4^{-}CD8^{-} double-negative 1 (DN1) population. These cells may give rise to multiple lymphoid lineages and their commitment to the T-cell lineage is dependent on Notch signalling [22]. The glycoprofile of Notch receptors was shown to regulate Notch-dependent intracellular signal transduction. Notch receptors are modified by the *O*-fucosyltransferase *Pofut1*, in the repeats of the consensus epidermal growth factor-like (EGF-like) sequences, present in the extracellular domain of Notch [23]. Mice lacking this enzyme in haematopoietic progenitors have severely decreased T lymphopoiesis [24]. These receptors are further modified by the lunatic, manic and radical Fringe glycosyltransferases, which transfer

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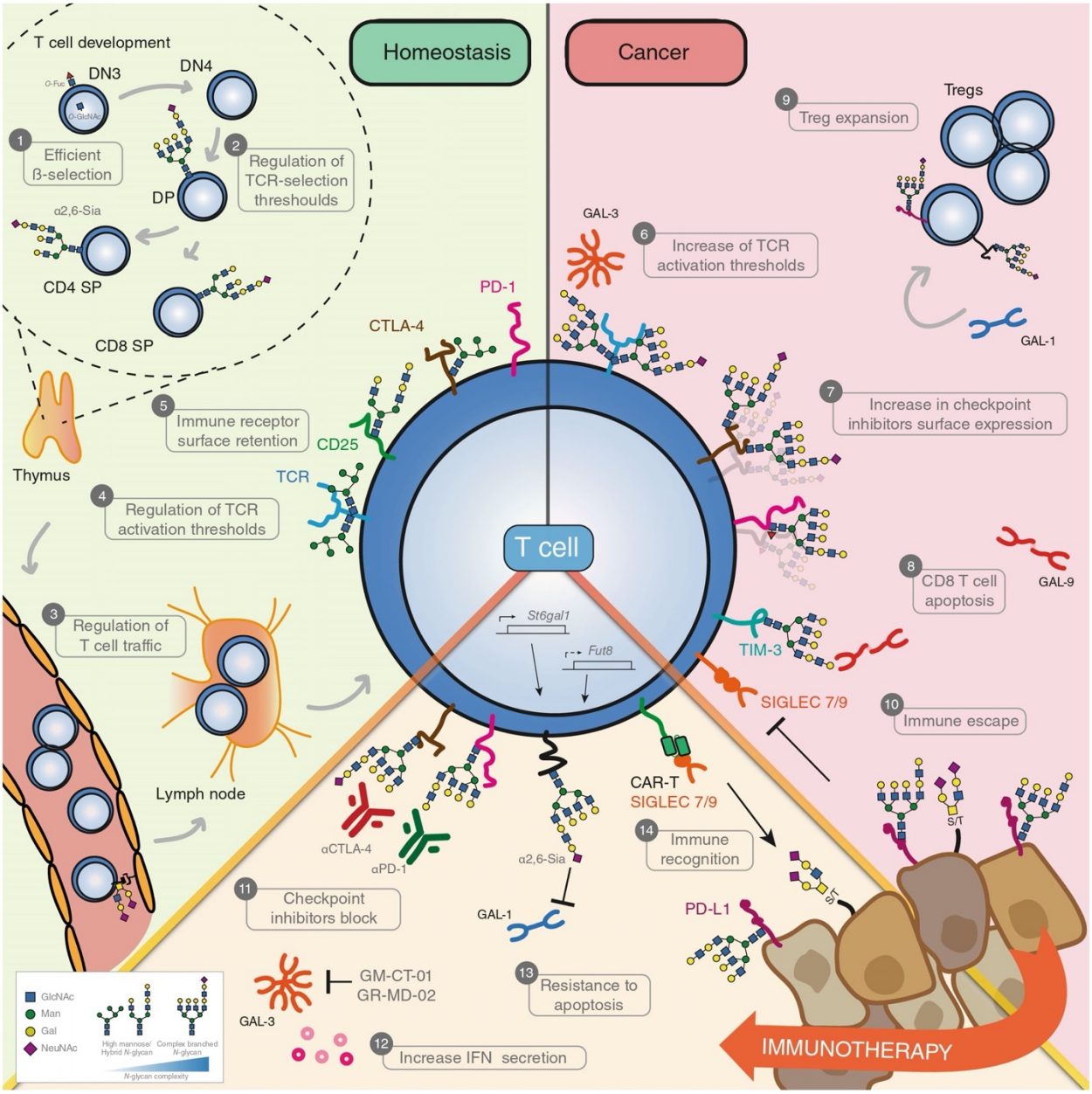


FIGURE 1 Glycans as chief molecules in the T-cell-mediated immune response both in physiological conditions and in cancer. Glycans are master regulators in T-cell function, having a key role in thymocyte developmental progression. In homeostasis, in the initial αß T-cell lineage commitment step, ß-selection of DN3 cells (1), O-GlcNAc and notch glycans are key determinants for normal development. On other hand, branched *N*-glycans regulate the thresholds of TCR-selection (2), occurring in the DP stage, as well as TCR repertoire diversity found in SP thymocytes. On T-cell activation, these migrate to the lymph nodes, via expression of core 1 *O*-glycans (3). At the periphery, activated T-cells express less complex *N*-glycans both on their TCR (4) and their co-receptors, such as CD25 (allowing its surface retention (5)) and CTLA-4, guaranteeing a controlled immune response and homeostasis. Nevertheless, changes in the cellular glycosylation pattern expression occur at the tumour microenvironment during the process of malignant transformation at the tumour microenvironment, contributing to cancer immunoediting. In fact, the overexpression of complex branched *N*-glycans that occurs in cancer, is described to increase the TCR activation thresholds (6) leading to increased surface expression of the inhibitory CTLA-4 and PD-1 (7). In addition, expression of galectins on the tumour microenvironment have profound effects on T-cell function, such as Gal-9 binding to TIM-3 (8), associated with the promotion of $CD8^+$ T-cell apoptosis as well as the expansion of Tregs by Gal-1 (9). Sialic acids expressed on tumour cells have been described to inhibit T-cell activity through siglec-9 binding (10). Importantly, from the clinical point of view, T-cell glycans and the correspondent glycan-binding proteins can be modulated to improve T-cell effector functions against tumour cells. Specifically, antibodies against glycosylated forms of CTLA-4, PD-1 (11) and anti-Gal-3 promote T-cell function with increased IFNγ secretion (12). Moreover, the increase of α2,6 sialic acid (and not α2,3 sialic acid) on T-cells abrogates Gal-1 binding, avoiding T-cell apoptosis (13). CAR-T-cells expressing siglecs-7/9 receptor exodomain also promote immune recognition of sialic acids on tumour cells (14). Overall, these biological processes highlight T-cell glycoengineering as a promising immunotherapeutic strategy for cancer treatment

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N-acetylglucosamine to *O*-linked fucose glycans [25]. In a mouse model of triple Fringe deficiency, the lack of such glycans in the Notch receptors led to reduced binding to Delta-like ligands (DLL), namely DLL4, altering the frequencies of several T-cell subsets in the thymus [26]. The DN3 thymocytes perform the decisive commitment to the T-cell lineage, by the generation of a pre-TCR signalling complex [27]. Together with Notch and interleukin (IL)-7, the pre-TCR signalling initiates ß-selection by inducing the transition to DN4 cells (Figure 1/1). We have shown that DN3 thymocytes lacking the *Mgat1* gene fail to efficiently transit to the DN4 stage [18]. These cells then begin a round of multiple divisions, giving rise to the most represented thymocyte population, the $CD4^+CD8^+$ double-positive (DP) cells. It was observed that intracellular O-GlcNAc is required for DN4 expansion, induced by Notch signalling [28].

In the DP stage, mature TCRs are screened by specificity and binding strength for the major histocompatibility complex (MHC) ligands presented by thymic epithelial cells [27, 29]. The initial step of selection is named 'positive selection', where the DP population is enriched for cells that express immunocompetent TCRs. Cells that exhibit high levels of activation, indicative of self-reactive potential, are targeted for apoptosis, a process called 'negative selection'. Finally, cells that survive these selection checkpoints 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 [30], and in fact, *ST3Gal1*$^{-/-}$ mice present significantly altered TCR repertoires, suggesting a role for sialylation in thymocyte selection [31]. Moreover, *ST6Gal1*$^{-/-}$ mice show rescued thymic cellularity, which may be a result of selection impairments [32]. It was also 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 (Figure 1/2). This was pointed out to result from decreased surface expression of CD4/CD8 co-receptors, in *Mgat1*- and *Mgat2*-deficient DP cells [33]. Finally, we have shown that *Mgat1*-deficient DP cells display an impairment on TCR repertoire diversity, suggesting that the regulation of selection thresholds by *N*-glycans is a major determinant on the overall goal of DP selection, the generation of TCR-diverse mature T-cells [18].

Once mature, SP thymocytes egress from the thymus to the periphery, up-regulating sphingosine-1-phosphate receptor-1 (S1P1), which is required for the cells to reach the bloodstream, where the concentration of S1P1 ligand is higher [34]. It was demonstrated that Cosmc-deficient thymocytes, containing only truncated *O*-glycans, are able to fulfil thymic developmental programs, but fail to populate the periphery, indicating a role for *O*-glycans in thymic egress [35].

It is worth noting that the majority of the findings regarding the role of glycans in T-cell development has relied on murine models, highlighting an urgent need for validation in human thymocytes. Nevertheless, the body of evidence in this section showcases glycans as master regulators in T-cell immunology, starting in the development of this key immune cell type.

Glycans as modulators of T-cell trafficking

Following their development and egress from the thymus, recent thymic emigrants need to undergo post-thymic maturation to enter the peripheral naïve T-cell pool. It was shown that the expression of sialyltransferase genes, regulated by the transcriptional repressor NFκB activating protein (NKAP) and the histone deacetylase 3 (HDAC3), was essential for overall sialylation of recent thymic emigrants. In the absence of such sialylated glycans, peripheral T-cells were bound by IgM and complement proteins, leading to their elimination [36, 37]. Once matured, the trafficking of lymphocytes to lymph nodes is mediated by L-selectin ligands, that are constitutively expressed in endothelial cells in high endothelial venules (HEVs) (Figure 1/3) [38]. The ligands for L-selectin, such as GlyCAM-1 and CD34, rely on the synthesis of sulfated core 1 *O*-glycans [39, 40]. Interestingly, it was found that L-selectin ligands could also be formed on complex *N*-glycans [41]. Although sulfated core 1 *O*-glycans are the primary ligands for L-selectin, in their absence, *N*-glycans decorated with sulfated sialyl Lewis X (sLeX) can promote the binding of T-cells on HEVs, as demonstrated by ß-1,3-N-acetilglucosaminil transferase 3 (β3GnT-3) deficiency and the blocking of sulfated core 1 *O*-glycans present on HEVs [41–43].

On activation, T-cells can synthesize core 2 *O*-glycans and this post-translational modification converts proteins, such as PSGL-1 and CD43, into P- and E-selectin ligands, which allows their trafficking into non-lymph nodes tissues during inflammatory processes (Figure 1) [44]. Different from recently activated effector T-cells, memory T-cells do not actively synthesize core 2 *O*-glycans, which limit their capacity to leave the circulation during homeostatic/non-inflammatory conditions. Memory $CD8^+$ T-cells, however, acquire the capacity of trafficking into sites of inflammation or tissue injury [45]. In fact, a study showed that memory $CD8^+$ T-cells that traffic into the lung or skin after an inflammatory event or a viral infection express core 2 *O*-glycans and the blocking of

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P- and E-selectin impaired the traffic of this population to the local of inflammation [46].

Glycans as modulators of T-cell activation and function

Glycosylation, particularly *N*-glycosylation of the TCR and co-receptors, is also involved in the regulation of T-cell activation/function (Figure 1). T-cell response starts by the recognition of the antigen presented in the context of MHC-I/II. On MHC-I, *N*-glycans are important for protein folding, trafficking to the cell surface and peptide loading [47–50]. In fact, it was demonstrated that blocking MHC-Ia *N*-glycosylation leads to a significant increase in intracellular misfolded protein along with a decrease in cell surface expression [51]. Interestingly, a single conserved site for *N*-glycosylation across all known alleles in the heavy chain of MHC-I was described [52]. Moreover, the knockout of the *Mgat2* gene, by compromising the synthesis of complex branched *N*-glycans, decreases antigen presentation by MHC-II and leads to loss of T-cell stimulatory activity [53].

N-glycans decorating TCRs play an important role in stabilizing molecules at the immunological synapse, protecting against the action of proteases and restricting nonspecific protein–protein interactions, helping to orient the interactions of the proteins in the central clusters [54]. We and others have demonstrated that T-cell activity is particularly regulated by β1,6-GlcNAc branched *N*-glycans (catalysed by *N*-acetylglucosaminyltransferase V—GnT-V enzyme; encoded by the gene *Mgat5*) on the TCR, by modulating the threshold of T-cell activation and signalling (Figure 1/4) [55, 56]. Additionally, these complex branched *N*-glycans on T-cells can be extended with poly-N-acetyllactosamine structures, which are ligands for galectins. Specifically, Galectin (Gal)-3 establishes a molecular lattice with TCR and the CD45 receptor, through the binding to these polylactosamine structures in branched *N*-glycans, preventing TCR clustering, and in this manner controlling T-cell activation and signalling [56, 57]. Additionally, it was demonstrated that the presence of β1,6-GlcNAc branched *N*-glycans on the TCR inhibits Th1 cell differentiation and promotes Th2 cells polarization [58]. Moreover, a decrease in UDP-GlcNAc and consequently on complex branched *N*-glycans promotes a pro-inflammatory T helper (Th)17 over anti-inflammatory-induced regulatory T-cell (iTreg) differentiation [59]. The anti-inflammatory cytokine IL-10 was also described to induce the expression of *Mgat5*, thus reducing the antigen recognition capacity of $CD8^+$ T-cells [60].

N-glycosylation also targets co-stimulatory and co-inhibitory receptors present on T-cells, such as CD28 and cytotoxic T-lymphocyte protein 4 (CTLA-4) respectively, highlighting its critical role in regulating T-cell activation/function [14]. Co-stimulation through the interaction of CD28 with CD80/86 and CD28-mediated T-cell adhesion can be negatively regulated by *N*-glycans on human CD28. In fact, mutation of all potential *N*-glycosylation sites of CD28 leads to enhanced binding to CD80 expressed on antigen-presenting cells (APCs) [61]. On the other hand, Lau et al. demonstrated that CD28 surface levels are not affected by the absence of branched *N*-glycans [62]. CTLA-4 also exhibits two *N*-glycosylation sites that are involved in the modulation of its cell surface retention on T-cells and consequently in its affinity to CD80/86 on APCs and function (further discussed in section 'Glycosylation of immune checkpoints at the cross-talk between tumour and immune cells') [63–65].

Other T-cell receptors are also highly dependent on *N*-glycosylation. For instance, CD45 is decorated with *N*-glycans that are involved in T-cell activation, differentiation, migration, signalling and apoptosis [66]. Of note, all of the different isoforms of CD45 present distinct glycosylation profiles [67, 68]. The branching *N*-glycosylation of the CD25 receptor also modulates its cell surface expression and retention, thus controlling T-cell differentiation with an impact in immune tolerance (Figure 1/5) [59, 61]. Moreover, when complex and hybrid *N*-glycans were depleted on CD25 through *Mgat1* silencing, a reduction on CD25 surface expression and on IL-2 signalling was observed [59, 69].

Taken together, these evidence highlight *N*-glycans as essential players in T-cell activation and functions in homeostatic conditions.

THE ROLE OF T-CELL GLYCOSYLATION IN ANTI-TUMOUR IMMUNE RESPONSE

Besides the well-known glycosylation alterations in cancer cells [4–6, 10, 70, 71], the glycocalyx of T-cells in the TME may also undergo glycosylation changes, with impact on how the immune system perceives and fights malignant transformation. In fact, alterations of T-cell glycosylation patterns are involved in immunosuppressive pathways and have crucial implications for tumour immune evasion.

T-cell-mediated anti-tumour immunity

To prevent tumour development, the immune system contains a vast library of processes involving different immune cell types. Among them, T-cell responses are crucial for a proper tumour immune surveillance [72].

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The successful recognition and elimination of tumours proceeds through the cancer-immunity cycle. The first step consists in the release and exposure of neoantigens/tumour-associated antigens to the intrinsically complex TME, as a consequence of the inherent genomic instability of cancer cells. These tumour-specific antigens are recognized by APCs, which activate T-cells in lymph nodes. Then, activated T-cells traffic to the tumour site and infiltrate the TME, eliminating tumour cells. On the other hand, $CD8^+$T-cells are also able to recognize tumour-antigens presented through MHC-I on the tumour cell surface, resulting in cytotoxic T-cell activation [73–76].

$CD8^+$ T lymphocytes are directly associated with tumour eradication, being involved in the elimination of cancer cells through granzyme-, TRAIL- and FAS-induced apoptosis, as well as through the cytokines release, including INFγ [73, 77]. $CD4^+$ T-cells can also participate in the elimination of tumour cells by modulating TME through the release of cytokines and chemokines [78]. Thus, successful T-cell-mediated cancer immune surveillance relies on the synergistic effect of $CD4^+$ and $CD8^+$ T-cell responses. However, despite this intricate well-oiled process, tumours can progress, as malignant cells acquire several mechanisms to evade immune recognition, hampering an effective anti-tumour immune response. This includes the expression of immune inhibitory checkpoints that, associated with T-cell dysfunction, lead to malignant transformation [79].

In fact, although T-cells recognize transformed cells, they are often rendered dysfunctional, leading to an immunosuppressive environment that fosters malignant transformation. Prolonged T-cell activation due to antigen exposure in nascent tumours—in combination with the changing cytokine milieu—leads to a progressive accumulation of exhausted $CD8^+$ T-cells. These cells exhibit reduced responsiveness, decreased cytotoxic potential and diminished production of key anti-tumoral cytokines, such as IFN-γ [80–84]. In fact, T-cell exhaustion has been shown to be a determinant of cancer progression and response to therapy with prognostic value for many cancer types [85–91]. Moreover, increased abundance of tumour-infiltrating regulatory T-cells (Tregs) is also known to favour tumour development, as Tregs contribute to immunosuppressive environments [92–96]. Furthermore, cancer-associated imbalances between Th populations have also been described [97–100].

Altogether, T-cell dysfunction is driven by various TME factors [82]. However, there is still a lack of knowledge on whether and how the glyco-metabolism on the TME contributes to renders T-cells dysfunctional associated with malignant transformation. The understanding of how glycosylation is implicated in such alterations and how it can be leveraged to rewire an effective T cell-mediated anti-tumour immunity may open up new possibilities for preventing and treating cancer.

The role of galectins and siglecs within the tumour microenvironment

Galectins, a family of glycan-binding proteins, are crucial for the regulation of the immune system by modulating TCR signalling and T-cell death, acting either as positive or negative regulators of T-cell function. In cancer context, galectins have a key role in the TME by controlling T-cell activity and inflammation (Figure 1) [101].

Gal-1 was described to be involved in T-cell function, controlling activation, differentiation, survival and cytokine production [102–104]. In cancer, Gal-1 has an immunosuppressive role [105], as its overexpression is inversely correlated with the number of tumour infiltrating lymphocytes (TILs) [106]. Additionally, in a breast cancer model, it was demonstrated that Gal-1 leads to the expansion of Tregs in the TME (Figure 1/9) [107]. In ovary cancer, Gal-1 secretion by γδ T-cells was described to promote malignant progression [108, 109]. Gal-1 was also associated with exclusion of T-cells from the tumour in head and neck squamous carcinoma [110]. This galectin can specifically exert its inhibitory function on Th1 and Th17 cells due to their differential glycosylation profile, rich in α2,3-sialic acids, which promotes the binding of Gal-1, inducing apoptosis [111]. In this line, Th2 cells are protected from the inhibitory function of Gal-1 because they express α2,6-sialic acids on their surface receptors, which inhibit Gal-1 binding [111].

Additionally, Gal-3 binding to glycoproteins can also have anti- or pro-apoptotic effects on T-cells, depending on its cellular localization. Intracellular Gal-3 blocks apoptosis while extracellular Gal-3 binds to glycosylated receptors on the T-cell surface, leading to activated T-cell inhibition [103, 112–114]. Gal-3 produced by activated T-cells interacts with glycoproteins associated with the TCR, negatively regulating T-cell activation through the destabilization of the immunological synapsis, precluding TCR clustering (Figure 1/6) [56, 115, 116]. In tumours, Gal-3 establishes glycan-dependent lattices with the TCR and CD8, impairing cytotoxic T-cell function and IFN-γ secretion [117]. With the use of galectin disaccharide ligands, such as *N*-acetyl-lactosamine (LacNAc), which inhibits Gal-3 binding to cellular glycans, this effect was abrogated, reversing the anergy of human TILs [117]. Furthermore, Gal-3 was described as capable of binding to *N*-glycans on immune checkpoints, such as CTLA-4, maintaining its inhibitory signal [62] and lymphocyte-Activation Gene 3 (LAG-3), suppressing cytotoxic T-cell function within the TME [114].

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Gal-9 is another galectin known to negatively regulate T-cell immune response through the binding to mucin-domain-containing molecule-3 (Tim-3), its preferential and more studied receptor [118, 119]. In accordance, through glycosylation-dependent binding to Tim-3, Gal-9 abolishes Th1 and Th17 cell development and activity, promoting apoptosis of tumour-infiltrating $CD8^+$ T-cells (Figure 1/8) [120–122]. Interestingly, it was described that the interaction of Gal-9 with Tim-3 on T-cells in the TME is involved in programmed cell death protein 1 (PD-1) immunotherapy resistance in patients with cancer, suggesting that this interaction may be targeted for improving effectiveness of immunotherapy [123] (further discussed in section 'Glycans as fine tuners of cellular immunotherapy in cancer: T-cells glycoengineering as a promising strategy to rewire effectiveness against cancer progression'). Additionally, Gal-9 was described to promote the stabilization and function of Tregs through its association with CD44 [124].

Sialic acid–binding immunoglobulin-type lectins (siglecs) are another family of glycans-recognizing receptors that also exert an immunosuppressive role in the TME, triggered by the abnormal expression of sialylated glycans on tumour cells (Figure 1/10) [125]. Particularly, Siglec-9 was demonstrated to be expressed on tumour infiltrating $CD8^+$ T-cells in several cancers, being associated with poor survival [126, 127]. Furthermore, these cells feature an effector memory phenotype and co-express a variety of inhibitory T-cell receptors, such as PD-1, CTLA-4 and Tim-3 [126, 127]. Surprisingly, Siglec-9^+ $CD8^+$ T-cells, albeit presenting an inhibitory phenotype, exhibit better functional responses on co-stimulation with anti-CD3 and anti-CD28 than Siglec-9^- $CD8^+$ T-cells, in vitro [126]. In addition to Siglec-9, increased Siglec-10 expression on T-cells was found to inhibit T-cell activity in the TME in human cancer patients [128].

Together, these evidence highlight the biological relevance of glycan-recognizing receptors on the TME, such as galectins and siglecs, as key players in the promotion of immunosuppressive networks that damper an effective anti-tumour immune response, and thus as potential targets for immunotherapy (Figure 1).

Glycosylation of immune checkpoints at the cross-talk between tumour and immune cells

Evasion of the immune system by cancer cells is facilitated by the expression of immune checkpoint inhibitors in the TME. T-cells express different immune checkpoint receptors at their surface, such as PD-1, CTLA-4, Tim-3, among others, that are regulated by glycosylation and responsible for providing inhibitory signals (Figure 1) [14, 129].

T-cell exhaustion mediated by PD-1/programmed cell death 1 ligand 1 (PD-L1) is a widely characterized mechanism by which the anti-tumour immune response is impaired. The primary functions of the PD-1/PD-L1 axis between immune cells and cancer cells are to maintain immune tolerance and to control T-cell activation [130]. However, tumours take advantage of this mechanism to escape from anti-tumour immune response by inhibiting T-cell proliferation, cytokine production and cytolytic activity [130]. It has been described that PD-1 relies on *N*-glycosylation to preserve its cell surface [131], namely with core-fucose *N*-glycans stability (Figure 1/7) [132]. Moreover, glycosylation of the PD-1 receptor was shown to be altered, with increased levels of poly-LacNAc and core fucose, on T-cell activation [131]. It was also described that PD-1 glycosylation is important in immunotherapy, for the binding of antibodies against PD-1, which improves anti-tumour immunity [133, 134]. Importantly, the glycosylation of PD-1 controls its interactions with its primary ligand PD-L1, which is also glycosylated to maintain its stability at the membrane [131, 135]. Likewise, PD-L1 glycosylation also determines the binding to its receptor PD-1, influencing its immunosuppressive activity [135–137]. The β3GnT-3 was identified as a crucial glycosyltransferase in PD-L1 *N*-glycosylation and in the interactions with PD-1. Indeed, down-regulation of *B3GNT3* improves cytotoxic T-cell-mediated anti-tumour immunity [138]. Thus, targeting the glycosylated forms of PD-1/PD-L1 could provide an effective immunotherapeutic strategy.

CTLA-4, another immune checkpoint molecule, is also regulated by *N*-glycosylation [65]. Indeed, on TCR signalling, there is an increase in branched *N*-glycans in CTLA-4, resulting in its retention at the cell surface, with a consequent suppression of T-cell function (Figure 1/7) [62, 139–141]. In accordance, it was demonstrated that in *Mgat5*$^{-/-}$ T-cells the expression levels of CTLA-4 on the T-cell surface decrease on low TCR stimulation [62]. Moreover, *N*-acetylglucosamine (GlcNAc; a precursor of UDP-GlcNAc and the substrate for GnT-V enzyme) supplementation was shown to increase CTLA-4 surface levels, leading to immunosuppression by inhibiting T-cell activation [142, 143]. Together, these evidence highlight the importance of the CTLA-4 glycosylation in the regulation of its inhibitory functions in cancer and thus a potential target for improving immunotherapy.

The Tim-3 receptor was also described to be *N*-glycosylated with core-fucose [132, 144]. Interestingly, treatment of Tim-3 with PNGase F, which cleaves *N*-linked glycans from glycoproteins, impairs the binding

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of this immune checkpoint molecule to Gal-9, which can also regulate the regulatory function of Tim-3 [120].

Regarding Treg function, the expression of tri- and tetra-antennary *N*-glycans was described to be correlated with the expression of immunosuppressive molecules on these cells, including Glucocorticoid-Induced TNFR-Related (GITR), PD-1, PD-L1, CD73, CTLA-4 and inducible T-cell Co-stimulator (ICOS). This was associated with an improved in vitro Treg immunosuppressive function [145].

Altogether, these data pinpoint the importance of immune checkpoints glycosylation, which may contributes to the development of resistance to an effective anti-tumour immune response, leading to a suppressive TME.

GLYCANS AS FINE TUNERS OF CELLULAR IMMUNOTHERAPY IN CANCER: T-CELLS GLYCOENGINEERING AS A PROMISING STRATEGY TO REWIRE EFFECTIVENESS AGAINST CANCER PROGRESSION

The manipulation of the immune system has revolutionized cancer treatment. Indeed, the basis for the 2018 Nobel Prize to Allison and Honjo showed how different strategies for inhibiting the brakes on the immune system can be used in the treatment of cancer [146, 147]. Among the different strategies to boost the immune response, the ability to remodel glycans at the T-cell surface can constitute an appealing strategy to improve the efficacy of cancer immunotherapy, an issue that remains largely unexplored [148]. In this line, different engineering approaches have been applied for the T or chimeric antigen receptor (CAR)- T-cell glycodesign, showing promising results on the bolstering T-cell glycosylation to improve its effector function in the TME (Table 1; Figure 1) [159, 160]. In fact, the utilization of CAR-T-cells has been a major emerging strategy in the field of cancer immunotherapy.

Glycan-binding proteins as potential therapeutic targets in the tumour microenvironment

The potential of CAR-T-cells to target specific tumour-associated glycans has been suggested in several studies. The use of CAR-T-cells against glycosylated cancer-associated antigens has been described, including the recognition of Lewis Y antigen or Tn and sialyl(S)-Tn antigens on Mucin 1 (MUC1) [161, 162]. Different ongoing clinical trials are focused on the efficacy of CAR-T-cells to recognize these abnormal glycoforms (NCT03633773; NCT03525782), highlighting its potential for a future cancer immunotherapy [163, 164]. Importantly, glycoengineered-CAR-T-cells are also beginning to be studied as a promising approach to enhance its efficacy in vivo. However, one of the major challenges in CAR-T-cells therapy is the poor trafficking and reduced infiltration of these cells to tumour sites [165]. As previously mentioned, the E-selectin plays a key role in T-cell extravasation from circulation to the TME. In fact, when E-selectin ligands (sLeX antigens) are increased, by the addition of a soluble fucosyl transferase on CAR-T-cells, their homing to the tumour site in vivo is improved, thus reducing the required cell dosage and tumour immune burden [166]. This mechanism was first demonstrated in vivo in Tregs in the context of inflammation, with an increase of Tregs number in inflamed sites [149], representing a promising strategy for the use of glycoengineered-CAR-T-cells in cancer treatment.

As above-described, galectins and siglecs play a fundamental role in the creation of immunosuppressive networks in the TME, which advances the inhibition of these lectins as another potential therapeutic strategy.

The metabolic inhibition of Gal-1 binding motifs, through treatment of T-cells with peracetylated 4-fluoro-glucosamine (4-F-GlcNAc), an inhibitor of *N*-acetyllactosamine (LacNAc) biosynthesis, leads to an improvement of the anti-tumour immune response in melanoma- and lymphoma-bearing mice models, attenuating tumour growth by increasing tumour-specific $CD8^+$ T-cells and IFN-γ production [167]. In a similar way, in an orthotopic cancer model, intratumoral treatment with thiodigalactoside, a non-metabolizable disaccharide, was shown to prevent Gal-1 binding to T-cells, promoting $CD8^+$ T-cell-mediated anti-tumour immune response [150]. Interestingly, genetic deletion of the Gal-1 gene (*Lgals1*) in a model of pancreatic cancer promotes tumour T-cell infiltration, increasing mice survival and reducing metastasis [151]. Accordingly, targeting Gal-1 in a melanoma model leads to the improvement of Th1 and cytotoxic T lymphocytes responses, enhancing tumour killing [168]. The overexpression of the ST6Gal1 sialyltransferase on T-cells, and consequently the increase of α-2-6 sialylated *N*-glycans expression on the CD45 receptor, was also demonstrated to be efficient in impairing Gal-1 binding, preventing T-cell death, which can represent an effective anti-tumoral strategy (Figure 1/13) [169]. An anti-Gal-1 drug (OTX008) was also tested as an immunotherapeutic agent, but the results from the clinical trial were inconclusive [152], a thus further research is still needed.

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TABLE 1 T-cell glycosylation as a potential target for immunotherapy: Molecular mechanism and effects on anti-tumour immune response

Target	Therapeutic approach	Molecular mechanism	Anti-tumour immune response	References
E-Selectin	sLeX-CAR-T-cells—soluble fucosyl transferase treatment	Increase of sLeX antigens and E-Selectin binding	Improves CAR-T-cell homing to TME and its function	[149]
Gal-1	LacNAc biosynthesis inhibitor (4-F-GlcNAc)	Reduction of Poly-LacNAc structures and Gal-1 binding	Increases tumour-specific $CD8^+$ T-cells and IFN-γ production	[150]
	Thiodigalactoside	Binds to Gal-1 and blocks its binding to T-cells	Increases levels of tumour infiltrated $CD8^+$ T-cells	[151]
	ST6Gal1 overexpressing	Increase of α2,6-sialylated *N*-glycans on CD45 receptor, impairing Gal-1 binding	Avoids T-cell death	[152]
Gal-3	Galactomannan (GM-CT-01)	Binds to Gal-3 and blocks its binding to T-cells	Improves anti-tumour $CD8^+$ T-cell response, through increased IFN-γ secretion	[153]
Gal-9/Tim-3	Anti-Tim-3 + anti-PD-1 antibodies	Impairs Gal-9 and PD-L1 binding	Reverses $CD8^+$ T-cell exhaustion	[154]
Siglec-7 and -9	Siglec-7/9-based CAR-T-cells—with siglec-7/9 exodomain	Increase of T-cell recognition against tumour sialylated glycans	Improves CAR-T-cell efficacy (enhanced tumour recognition)	[155]
PD-1	Gene editing of N74 residue of *PDCD1*	Reduction of PD-1 *N*-glycosylation and its expression on CAR-T-cell surface	Prevents CAR-T-cell exhaustion	[156]
	Knockout of *FUT8*	Abolishes core fucosylation on PD-1	Decreases PD-1 expression surface and enhances T-cell activation	[133]
	Anti-glycosylated-PD-1 antibody (STM418)	Impairs PD-1/PD-L1 interaction	Avoids T-cell exhaustion	[132]
CTLA-4	Anti-glycosylated CTLA-4 antibody (mAb146)	Blocks CTLA-4-CD80/CD86 binding	Enhances T-cell function	[157]
IL-2R	2-deoxy-D-glucose (2DG), glucose analog	Impairs *N*-glycosylation, increasing IL-2R retention and reducing Gal-3 binding	Higher production of perforin/granzyme	[158]

The in vitro treatment of patient-derived TILs with galactomannan, GM-CT-01, an inhibitor of Gal-3, was also described to be efficient in improving the anti-tumour $CD8^+$ T-cell response, through increased IFN-γ secretion (Figure 1/12) [170]. Notably, the disruption of lectin-T-cell binding has been studied as a possible adjuvant therapy of chemo- and/or immunotherapeutic strategies. Studies using Gal-3 inhibitor as an adjuvant of the chemotherapeutic drug 5-fluorouracil were performed against different advanced/metastatic solid tumours [153]. Unfortunately, the results were unfavourable and some of them were withdrawn [153]. Interestingly, Gal-3 inhibition (using GR-MD-02), combined with antibodies against immune checkpoint inhibitors, including anti-Tim-3, anti-PD1 or/and anti-CTLA-4, appears to be a promising strategy with ongoing clinical trials for several solid tumours (e.g., NCT02575404; NCT02117362) [153].

The use of an antibody against Gal-9 was also described as a promising immunotherapy in a tumour mouse model, since this galectin is associated with T-cell death and consequently with poor prognosis in several human cancers [171]. Gal-9 is the main ligand of Tim-3 on T-cells, leading to T-cell exhaustion. In fact, T-cells which co-express Tim-3 and PD-1 are highly exhausted and represent the most abundant TILs population in solid tumours [172, 173]. Interestingly, it was demonstrated that PD-1 had the capacity to interact with Gal-9 and Tim-3, in $PD\text{-}1^+Tim\text{-}3^+$ exhausted T-cells, forming Gal-9/Tim-3/PD-1 molecular lattices, which protect this population from Gal-9/Tim-3-induced apoptosis [171].

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Regarding siglecs, Siglec-7 and -9 have been also highlighted as promising targets in cancer immunotherapy. The use of blocking antibodies to Siglec-7 and -9 leads to a significant reduction of lung and ovarian tumour burden in mice [154, 174]. In addition, taking advantage of the glycan-binding capacity of siglecs, CAR-T-cells composed by the exodomain of human Siglec-7 and -9 have been demonstrated as efficient in the recognition of sialylated glycans on tumour cells, both in vitro and in vivo, consequently improving anti-tumour CAR-T-cell target function (Figure 1/14) [175]. Moreover, similarly to anti-galectin agents, siglec blockers are now gaining momentum as novel adjuvant therapies. In fact, the co-inhibition of siglecs, including Siglec-7 and -9, and immune checkpoint molecules (Tim-3) was also proposed as a promising treatment for patients with glioma [155]. On the other hand, alterations in ligands of siglecs on tumour cells could also be a strategy to impair siglec–glycans interactions. For instance, the treatment of tumours with sialic acid analogues reduces cell surface sialylated glycans, precluding siglecs recognition and improving the activation of intratumoral T-cells [176].

All of these evidence highlight the major roles of selectins, galectins and siglecs in TME as promising therapeutic targets, single or in combination with other immunotherapeutic strategies, to enhance an effective anti-tumour immune response and to improve cancer patients' survival.

Reprogramming the glycosylation of T-cells in cancer immunotherapy

PD-1 expression in CAR-T-cells has been described as a major inhibitory molecule, constituting a hurdle for effective anti-tumour surveillance [177]. The removal of PD-1 *N*-glycosylation, by editing the coding sequence of the N74 residue of *PDCD1* gene, was shown to reduce PD-1 expression at the cell surface, improving overall survival and increasing anti-tumour activity in vivo [178]. In this line, the abolition of core fucose in T-cells was also able to decrease surface PD-1 expression and enhance T-cell activation, leading to more efficient tumour eradication in a melanoma murine model [132]. Importantly, it was highlighted that some therapeutic antibodies are only functional against the glycosylated form of PD-1 (Figure 1/11) [133]. The monoclonal antibody STM418 (an anti-PD1 antibody) was specifically developed to recognize the *N*58-glycan present in this molecule, inducing an improved anti-tumour effect when compared to other anti-PD-1 antibodies [131]. Moreover, the use of an antibody targeting glycosylated PD-L1 was described to promote the internalization and degradation of this molecule, resulting in T-cell induced tumour cell death [138]. Additionally, antibodies against glycosylated epitopes of CTLA-4 (mbAb146) are also being considered to increase its therapeutic efficacy against tumours (Figure 1/11) [156].

As mentioned above, T-cell signalling is highly regulated by branched glycosylation, in which the abolishment of *N*-glycans on the TCR, by removing the conserved *N*-glycosylation sites on TCR α and β chains, was associated with increased TCR activation [157]. In fact, when branched *N*-glycans are absent, on *Mgat5*$^{-/-}$ mice, an increased TCR clustering and T-cell activation was observed [56]. Moreover, a deficiency on T-cell branched glycosylation was detected in patients with inflammatory bowel disease and multiple sclerosis with an active pro-inflammatory activity [55, 143, 179].These evidence highlight the implications of *Mgat5*-derived *N*-glycan depletion specifically on T-cells, as a potential strategy to enhance anti-tumour immune response [14].

In addition, the treatment of T-cells with 2-deoxy-D-glucose (2DG), an analogue of glucose, has also been studied as a possible anti-tumour therapy. 2DG is able to regulate the *N*-glycosylation pathway, once it is converted to 2-deoxy-D-mannose and blocks *N*-glycan biosynthesis [180]. An in vitro study showed that alterations on *N*-glycosylation patterns of human T-cells, promoted by 2DG, enhance its anti-tumour immune activity through the retention of cell surface IL-2R, increasing the production of perforin/granzyme and the prevention of Gal-3 binding [181]. On the other hand, deglycosylation of PD-L1 induced by 2DG, in conjugation with other inhibitors of tumour immune evasion, was shown to promote anti-tumour immune response in breast cancer models [158, 182].

Taken together, these evidence highlight the power of glycans on T-cells and the correspondent glycan-binding proteins as attractive targets to improve T-cell effector functions against tumour cells, an issue that deserves further exploitation. The data gathered herein foster great hope in T-cell glycoengineering as a promising immunotherapeutic strategy to be considered as next generation of glycan-based immunotherapeutic approaches for cancer prevention and treatment (Figure 1).

CONCLUSION

Glycans are pivotal biological components that regulate each pathophysiological step of malignant transformation and progression. Dynamic alterations on the cellular glycosylation pattern of T-cells can lead to changes in the cross-talk between tumour cells and the immune system, with critical consequences for cancer development and progression.

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Despite the clinical success of cancer immunotherapies focused on immune checkpoint inhibitors, a large number of cancer patients fails to respond or develop resistance to this therapeutic modality. The identification of new immune modulators in the TME is therefore crucial for the improvement of the clinical and therapeutic management of patients with cancer. Here, we provide a novel perspective on T-cell glycosylation and T-cell glycoreprogramming as a next-generation strategy to improve effectiveness of cancer immunotherapy.

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CONFLICT OF INTEREST

Authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

Data openly available in a public repository that issues datasets with DOIs.

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APPENDIX III

[COMMUNICATION]

COMPOSITION OF THE IMMUNOGLOBULIN G GLYCOME ASSOCIATES WITH THE SEVERITY OF COVID-19

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Here, we analyzed the IgG *N*-glycome signature of COVID-19 patients, having discovered specific glycan structures associated with severity. My contribution was focused on the design of the study.

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Communication

OXFORD

Immunology

Composition of the immunoglobulin G glycome associates with the severity of COVID-19

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Abstract

A large variation in the severity of disease symptoms is one of the key open questions in coronavirus disease 2019 (COVID-19) pandemics. The fact that only a small subset of people infected with severe acute respiratory syndrome coronavirus 2 develops severe disease suggests that there have to be some predisposing factors, but biomarkers that reliably predict disease severity have

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not been found so far. Since overactivation of the immune system is implicated in a severe form of COVID-19 and the immunoglobulin G (IgG) glycosylation is known to be involved in the regulation of different immune processes, we evaluated the association of interindividual variation in IgG N-glycome composition with the severity of COVID-19. The analysis of 166 severe and 167 mild cases from hospitals in Spain, Italy and Portugal revealed statistically significant differences in the composition of the IgG N-glycome. The most notable difference was the decrease in bisecting *N*-acetylglucosamine in severe patients from all three cohorts. IgG galactosylation was also lower in severe cases in all cohorts, but the difference in galactosylation was not statistically significant after correction for multiple testing.

Key words: bisecting GlcNAc, COVID-19, disease biomarkers, galactosylation, IgG glycosylation

Introduction

The knowledge about severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus and coronavirus disease 2019 (COVID-19) increased tremendously in the last few months, but it is still unclear why some infected patients have a very mild disease or even no symptoms whereas others develop the serious disease with considerable mortality. Over a million COVID-19 related deaths have been reported so far, but a significant number of people (even over 80% in some populations) infected with SARS-CoV-2 manage to contain infection only to their upper respiratory tract and despite being positive for the virus do not develop any visible symptoms (Oran and Topol, 2020). Many factors have been raised as clinical predictors of worse disease progression, such as obesity, diabetes, hypertension, kidney injury and in general, the previous cardiovascular disease burden of the patient (Williamson et al., 2020). Undoubtedly, age is the most important factor predicting severity with 100-fold difference in mortality risk in different age groups (Sehra et al., 2020). Several independent studies suggested that environmental factors (temperature and humidity) play an important role for viral transmission, but also for severity of the disease (Kifer et al., 2020; Lauc et al., 2020).

Immunoglobulin G (IgG) glycome composition is an essential component of the immune system that regulates inflammation at multiple levels (Nimmerjahn and Ravetch, 2008; Seeling, Brückner, and Nimmerjahn, 2017), and is considered to be one of the important drivers of inflammaging (Franceschi et al., 2018). Many observational and molecular studies of the IgG glycome identified and confirmed its role of both a biomarker and a functional effector of inflammation that contributes to the development of different inflammatory diseases (Lauc et al., 2016). Moreover, it was shown that COVID-19 patients had substantial differences in antiglycan antibodies, IgG and IgM, as well as unusual antibodies to self-glycans, e.g. *N*-glycans, LacNAc-containing glycans, blood group H, gangliosides and sialyl Lewis X, compared with healthy controls (Butler and Gildersleeve, 2020). IgG glycome composition has not yet been thoroughly addressed in COVID-19 infection, especially in the context of disease severity. A recent (unreviewed) small study suggested that the generation of afucosylated antigen-specific IgG may be an important element in the defense against SARS-CoV-2 and other enveloped viruses (Larsen et al., 2020). In another unreviewed study antigen-specific IgG Fc fucosylation in polymerase chain reaction (PCR)-diagnosed COVID-19 patients was reduced compared with SARS-CoV-2-seropositive children and relative to adults with symptomatic influenza virus infections (Chakraborty et al., 2020). To address this question, we analyzed the total IgG N-glycome composition in three independent cohorts of COVID-19 patients.

Results

IgG glycome composition was analyzed in 167 patients with mild (without need of admission to hospital intensive care unit and mechanical ventilation) and 166 patients with severe form (with need of admission to hospital intensive care unit and mechanical ventilation, or those who deceased during hospitalization) of COVID-19 from three independent cohorts. The descriptive information about included patients is presented in Table I. Total IgG N-glycome (combined Fc and Fab glycans) composition was determined by ultra-high-performance liquid chromatography (UHPLC) analysis of glycans labeled with 2-aminobenzamide (2-AB) as described in the Materials and methods section.

Statistical analysis was performed on main summary features of the IgG glycome composition (G0—glycans without galactose, G1—glycans with one galactose, G2—glycans with two galactoses, S—percentage of all glycans with sialic acid, F—percentage of fucosylated glycans and B—percentage of glycans with bisecting GlcNAc). The analysis of differences between severe and mild cases was performed using a regression model with sex and age included as additional covariates. Significant difference in the IgG glycome composition in severe and mild COVID-19 patients was observed (Table II, Figure 1). Consistent decrease in the level of bisecting *N*-acetylglucosamine (GlcNAc) in severe cases was observed in all cohorts (meta-analysis effect = −0.34; adjusted meta-analysis $P = 0.009$). Galactosylation was also consistently decreased in severe cases in all three cohorts, but the statistical significance of this difference was observed only for monogalactosylation in Barcelona cohort (effect = −0.34; $P = 0.016$). Consistent changes in the levels of sialylated and fucosylated IgG glycan structures between mild and severe COVID-19 cases were not detected.

Discussion

In this study, we found consistent differences in IgG glycome composition between patients with mild and severe COVID-19. The level of bisecting GlcNAc was decreased in severe patients (effect = −0.34), replicated in all three cohorts. Despite the small size of each cohort, this change was statistically significant even after adjusting for multiple testing (adjusted meta-analysis $P = 0.009$). Higher levels of bisecting GlcNAc on IgG are often associated with increased FcγRIII binding and enhanced antibody-dependent cell cytotoxicity, explaining a more proinflammatory effector functions of IgGs (Irvine

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Table I. Descriptive information about COVID-19 patients included in the study

	Italy ($n = 107$)		Portugal ($n = 77$)		Spain ($n = 152$)	
Disease severity	Mild ($n = 40$)	Severe ($n = 64$)	Mild ($n = 52$)	Severe ($n = 25$)	Mild ($n = 75$)	Severe ($n = 77$)
Sex (male/female)	25/15	45/19	26/26	18/7	44/31	44/33
Age (median [IQR])	70 years (57–76)	66 years (57–73)	56 years (44–70)	64 years (54–81)	63 years (58–70)	63 years (55–72)

Table II. IgG glycome composition in severe and mild COVID-19 patients

Glycan	Italy effect	Italy *P* value	Portugal effect	Portugal *P* value	Spain effect	Spain *P* value	Meta effect	Meta *P* value	Meta *P* adjusted
B total	-0.27	0.174	-0.31	0.190	-0.39	0.009	-0.34	0.002	0.009
G0 total	0.14	0.437	0.12	0.547	0.21	0.101	0.17	0.065	0.112
G1 total	-0.06	0.756	-0.22	0.383	-0.34	0.016	-0.24	0.023	0.069
G2 total	-0.12	0.526	-0.10	0.592	-0.21	0.089	-0.17	0.075	0.112
S total	-0.17	0.369	0.01	0.670	-0.15	0.327	-0.10	0.341	0.409
F total	-0.13	0.498	-0.25	0.324	0.29	0.070	0.05	0.654	0.654

Notes: B—bisecting GlcNAc, G0—agalactosylation, G1—monogalactosylation, G2—digalactosylation, S—sialylation, F—fucosylation.

and Alter, 2020; Umana et al., 1999). If this study did not have a cross-sectional design, it would allow us to distinguish whether the observed associations reflect a pre-existing risk factor, or rapid changes in IgG glycosylation that occurred during the disease. This question will be addressed in a longitudinal study that we are currently setting up. A recent small study on antigen-specific antibodies found a decrease in bisecting GlcNAc specifically on anti-SARS-CoV2 antibodies (Larsen et al., 2020), which is consistent with changes in the total IgG glycome observed in our study. Interestingly, in the same study anti-SARS-CoV2 antibodies had higher galactosylation and sialylation (relative to the total IgG glycome composition), which is the opposite of what we have observed in this study. This difference suggests that the observed differences in IgG glycosylation between mild and severe cases may not be a simple reflection of the increased proportion of newly synthetized anti-SARS-CoV2 antibodies in the total IgG pool but also of a pre-existing risk factor for more severe COVID-19. However, another study found that IgG1 against the receptor binding domain of the SARS-CoV-2 spike protein from COVID-19 patients had significantly lower core fucosylation, galactosylation and bisection when compared with total IgG1 from healthy adult controls (Chakraborty et al., 2020), suggesting that these changes/differences may be very individual and that additional studies are needed.

"Cytokine storm" and overactivation of the immune system have been suggested key features of severe COVID-19 (Catanzaro et al., 2020), but this significantly svaries from patient to patient (Calfee et al., 2014) and increased levels of proinflammatory cytokines are not present in all severe patients (Kox et al., 2020). Interindividual differences in IgG glycosylation are large and reflect both genetic and environmental contributing factors (Klarić et al., 2020; Pučić et al., 2011). IgG glycans are known effectors of the immune system and composition of the IgG glycome associates with different diseases (Gudelj, Lauc, and Pezer, 2018). It has been shown that age and excess adiposity is a risk factor for severe disease and mortality in people with SARS-CoV-2 infection (Seidu et al., 2020). Furthermore, age (Krištić et al., 2014) and adiposity (Russell et al., 2019) are the main environmental factors that drive the decrease in IgG glycosylation implicating that the observed changes may be individual and partially depending on these and other factors. Outliers in our study also support the notion that interindividual variability in IgG glycosylation cannot be disregarded. Considering the previous research on IgG glycosylation and the cross-sectional design of the study, whether the observed IgG glycosylation changes reflect a pre-existing genetic or environmental risk factor, and/or rapid changes in IgG glycosylation during COVID-19 still remains an open question. The observed differences are not large enough to suggest the use of IgG glycome as a predictor of COVID-19 severity, but it is intriguing to hypothesize that changes in the IgG glycome that lead to the loss of its immunosuppressive potential may be one of the molecular mechanisms behind these two environmental risk factors for severe COVID-19. IgG glycome composition strongly associates with age, so it is very hard to exclude confounding factors with some other age-related changes, but the fact that people with severe COVID-19 had "older" IgG glycome composition suggests the need for further research in this direction.

Materials and methods

Studied cohorts

Biological samples were obtained from hospitals in Spain, Italy and Portugal. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and the study was approved by ethical committees of ASST Papa Giovanni XXIII° Hospital in Bergamo, Hospital del Mar in Barcelona and by the institutional ethics committee of Centro Hospitalar Universitário do Porto (CHUP), Centro Hospitalar de Vila Nova de Gaia/Espinho (CHVNG) and Hospital Beatriz Angelo (HBA), Loures, Lisbon. All participants gave informed consent.

Spain: Parc de Salut MAR Biobank (MARBiobanc) Barcelona. Patients with PCR confirmed SARS-CoV-2 infection admitted at the Hospital del Mar in Barcelona during the months of March–May were included in this study We classified the patients in severe or mild COVID, considered severe if they needed invasive or noninvasive

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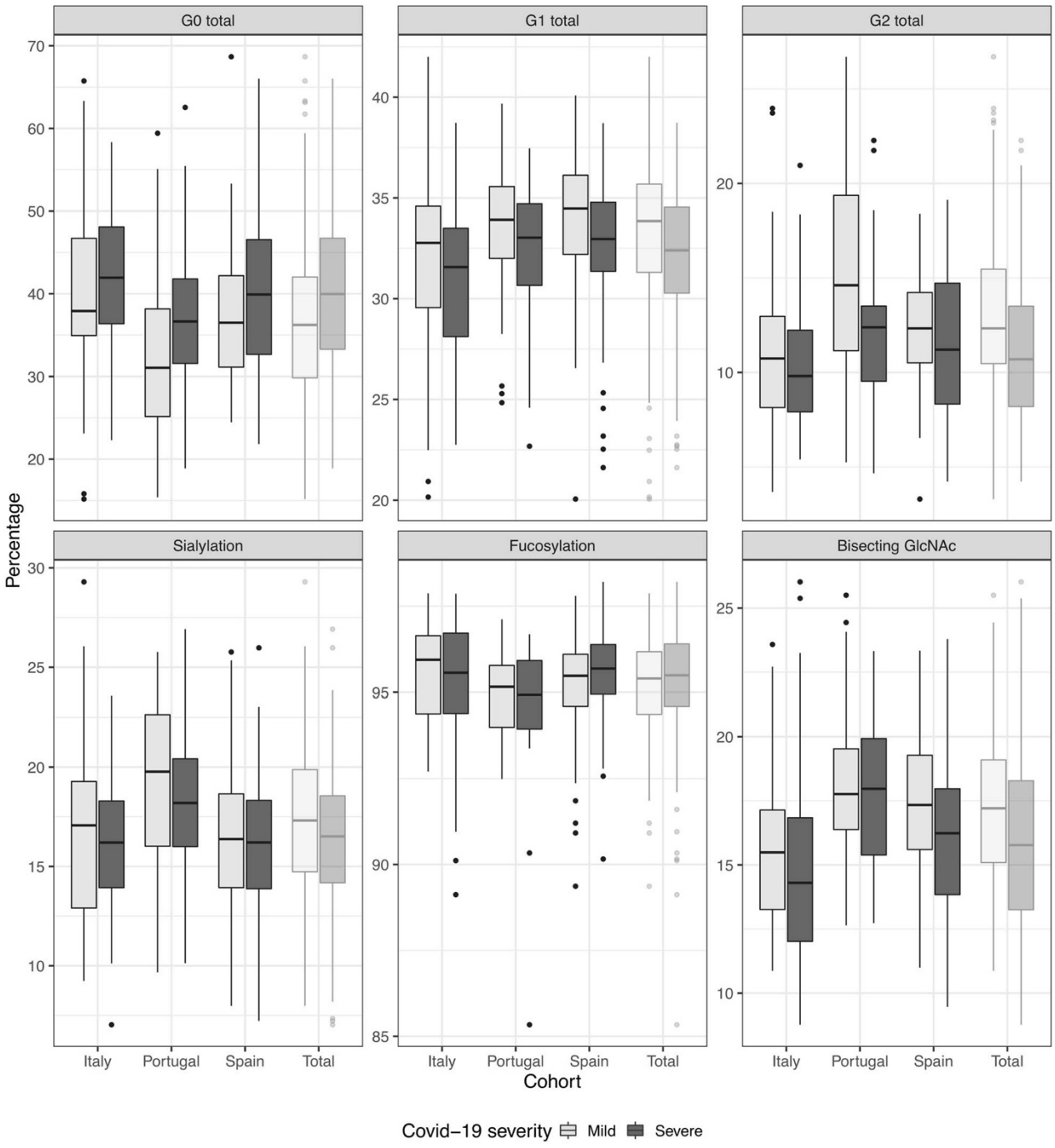


Fig. 1. Relative abundance of main IgG glycome features in severe and mild COVID-19 cases from three cohorts. G0—agalactosylation, G1—monogalactosylation, G2—digalactosylation. Boxes represent the 25th and 75th percentiles. Lines inside the box represent the median.

mechanical ventilation or intensive care unit, or deceased during the hospitalization.

Italy: ASST Papa Giovanni XXIII° Hospital, Bergamo. Hospitalized patients for acute respiratory failure due to SARS-CoV-2 infection were included in this study. They were divided in two categories: (a) *severe* disease: All patients requiring an ICU admission for respiratory failure, and treated with either mechanical ventilation or continuous positive airways pressure and (b) *mild* disease: All patients who required oxygen support by mask or no support at all.

Portugal: Patients from Infectious Disease Department of CHUPCHVNG and HBA were included. Blood was collected for plasma at the time of diagnosis. SARS-CoV-2 positive patients with different levels of severity (mild, moderate and severe) were included. The World Health Organization's criteria were used to stratify symptomatic SARS-CoV-2 patients by the disease severity into mild: Individuals with no evidences of pneumonia; moderate: Individuals with evidence of pneumonia, however without need of invasive mechanical ventilation and without need of admission to

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hospital intensive care unit; severe: Individuals with need of invasive mechanical ventilation and with need of admission to hospital intensive care unit. For this study moderate and severe patients were merged into a single group.

Isolation of IgG from human plasma

IgG was isolated from plasma using a 96-well protein G monolithic plate (BIA Separations, Slovenia) (Pučić et al., 2011), as previously described (Trbojević-Akmačić, Ugrina, and Lauc, 2017). After IgG isolation, IgG eluates were heated at 65 °C for 30 min to reduce risk from any potential residual virus in the IgG eluate, and 300 µL of each eluate was dried in a vacuum concentrator.

Glycan release, labeling and clean up

Glycans were released, fluorescently labeled and cleaned up as previously described (Trbojević-Akmačić et al., 2017), with a modification of using 0.2 µm Supor AcroPrep filter plate (Pall Corporation, 25 Harbor Park Drive Port Washington, NY, USA) as a stationary phase.

Hydrophilic interaction liquid chromatography-UHPLC

Fluorescently labeled *N*-glycans were separated by hydrophilic interaction chromatography on a Waters Acquity UHPLC instrument (Milford, MA). The instrument consists of a sample manager, a quaternary solvent manager and a fluorescence (FLR) detector. Released and labeled *N*-glycans were chromatographically profiled by hydrophilic interaction liquid chromatography (HILIC) using an amide-bonded sub 2-µm stationary phase column built from a novel type of column hardware (ACQUITY PREMIER Glycan BEH Amide 130 Å, 1.7 µm 2.1 × 100 mm Column, Waters Corporation, Milford, MA). Unlike traditional, metallic hardware, this column was constructed from components manufactured to have a barrier layer of hybrid organic inorganic silica to improve its inertness and to mitigate problematic analyte to metal adsorption. Having a chemical composition that is highly similar to ethylene-bridged siloxane (BEH) particles (Wyndham et al., 2003); this barrier layer is considerably more inert than fused silica surfaces and significantly less hydrophobic than polyether ether ketone (PEEK). That it is comprised of highly crosslinked ethylene-bridged siloxane groups also means that it is resilient to chemical and pH stress and amenable to usage between pH 1 and pH 12 mobile phase conditions.

Separation method used a linear gradient of 75–62% acetonitrile (v/v) at a flow rate of 0.4 mL/min in a 29-min analytical run. Separation temperature was 60°C, and samples were maintained at 10 °C before injection. Hydrolysed and 2-AB labeled glucose oligomers were used as external standard, from which the retention times for the individual glycans were converted to glucose units. The chromatograms were separated into 24 chromatographic peaks and the amount of glycans in each peak was expressed as percentage of total integrated chromatogram area (%Area).

Statistical analysis

In order to remove experimental variation from measurements, normalization and batch correction were performed on UHPLC glycan data. To make measurements across samples comparable, normalization by total area was performed where the peak area of each of 24 glycan structures was divided by the total area of the corresponding chromatogram. Prior to batch correction, normalized glycan measurements were log transformed due to right-skewness of their distributions and the multiplicative nature of batch effects. Batch correction was performed on log-transformed measurements using ComBat method (R package sva), where the technical source of variation (which sample was analyzed on which plate) was modeled as a batch covariate. To get measurements corrected for experimental noise, estimated batch effects were subtracted from log-transformed measurements. In addition to 24 directly measured glycan structures, six derived traits were calculated from the directly measured glycans. These derived traits average particular glycosylation features across different individual glycan structures and consequently they are more closely related to individual enzymatic activities and underlying genetic polymorphisms.

Association analyses between disease severity status and glycan traits were performed using a regression model with age and gender included as additional covariates. Analyses were firstly performed for each cohort separately and then combined using inverse-variance weighted meta-analysis approach (R package metafor). Prior to analyses, glycan variables were all transformed to standard Normal distribution (mean = 0, SD = 1) by inverse transformation of ranks to Normality (R package "GenABEL", function rntransform). Using rank transformed variables in analyses makes estimated effects of different glycans in different cohorts comparable as transformed glycan variables have the same standardized variance. False discovery rate was controlled using Benjamini–Hochberg procedure. Data were analyzed and visualized using R programming language (version 3.0.1).

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Abbreviations

2-AB, 2-aminobenzamide; B, percentage of glycans with bisecting GlcNAcBEH = ethylene-bridged siloxane; F, percentage of fucosylated glycans; G0, percentage of glycans without galactose; G1, percentage of glycans with one galactose; G2, percentage of glycans with two galactoses; GlcNAc, *N*-acetylglucosamine; HILIC, hydrophilic interaction liquid chromatography; IgG, immunoglobulin G; PEEK, polyether ether ketone; S, percentage of all glycans with sialic acid; UHPLC, ultra-high-performance liquid chromatography

Conflict of interest statement

GL is the founder and CEO of Genos, a private research organization that specializes in high-throughput glycomic analysis and has several patents in the field. TP, ITA and FV are employees of Genos. Other authors declare no competing financial interests.

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