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Unraveling the glycophenotype of T Cell Acute Lymphoblastic Leukemia (T-ALL): metabolic reprogramming as a novel avenue for treatment.

Eduarda Forte Rodrigues

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Unraveling the glycophenotype of T Cell Acute Lymphoblastic Leukemia (T-ALL): metabolic reprogramming as a novel avenue for treatment.

Dissertação de Candidatura ao grau de Mestre em Oncologia – Oncologia Laboratorial submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientador – Doutora Salomé Soares Pinho

Categoria – Principal Investigator / Group leader

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Abstract

Glycosylation is an important posttranslational modification for cellular homeostasis, and changes in this cellular pathway are associated with tumor development and progression.

The proper functions of T-cells, namely in development and in immune activation are modulated by two major posttranslational modifications: *N*-glycosylation and *O*-linked *N*-acetylglucosamine (*O*-GlcNAcylation). Changes in the expression of either *N*-glycans and *O*-GlcNAc structures promote cellular hyperactivation and malignant transformation, which can lead to the development of T-cell acute lymphoblastic leukemia.

The glycophenotyping of leukemic cells has not been done in an extensive manner, which reveals a missing gap in research, that this Master's thesis aims to fill. Throughout this dissertation, different types of T cell leukemias were glycoprofiled, revealing a distinct leukemic glycosignature, in which complex *N*- and *O*-GlcNAc glycans play a predominant role. When the levels of these carbohydrates were modulated on malignant T cells, either by *in vitro* metabolic supplementation with monosaccharides or by using glycoengineered mice models, an impact in apoptosis was observed associated with delayed leukemic progression. These evidences highlight the therapeutic potential of glycosylation modulation in hematological tumors.

Key words: *N*-glycosylation; *O*-GlcNAcylation; T-ALL; biomarker; cell death

Resumo

A glicosilação é uma modificação pós-traducional importante para a homeostasia celular, e alterações nessa via estão associadas à progressão e crescimento tumoral.

As funções adequada das células T, nomeadamente no seu desenvolvimento e na ativação imunológica são moduladas por duas modificações pós-traducionais principais: *N*-glicosilação e O-*linked* N-*acetylglucosamine* (*O*-GlcNAcilação). Mudanças na expressão das estruturas de *N*-glicanos e *O*-GlcNAc promovem hiperativação celular e transformação maligna, o que pode levar ao desenvolvimento de leucemia linfoblástica aguda de células T.

O glico-fenótipo de células leucémicas ainda não foi extensamente caracterizado, o que revela uma lacuna no conhecimento, que esta dissertação de mestrado tenta preencher. Ao longo desta dissertação, diferentes tipos de leucemias de células T foram caracterizadas em relação ao seu glico-perfil, revelando uma distinta glico-assinatura leucémica, na qual os *N*-glicanos complexos e *O*-GlcNAc desempenham um papel predominante. Quando os níveis desses carboidratos foram modulados em células T malignas, seja pela suplementação metabólica *in vitro* com monossacáridos ou modelos de ratinhos com deficiências na via de glicosilação, foi observado um impacto na apoptose associado a progressão leucémica retardada. Tais evidências destacam o potencial terapêutico da modulação da glicosilação em tumores hematológicos.

Palavras-chave: *N*-glicosilação; *O*-GlcNAcilação; LLA-T; biomarcador; morte celular

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List of Abbreviations

ALL	Acute lymphoblastic leukemia
AML	Acute myelogenous leucemia
APC	Antigen – presenting cells
Asn	Asparagine
B-ALL	B-cell acute lymphoblastic leukemia
BSA	Bovine serum albumin
CML	Chronic myeloid leukemia
ConA	Concavalin A
CTLA-4	Cytotoxic-T-lymphobcytes-associeted antigen-4
DN	Double negative
Dol-P	Dolichol-phosphate
DP	Double positive
EGFR	Epithelial growth factor receptor
EMT	Epithelial – mesenchymal transition
E-PHA	*Phaseolus vulgaris* erthroagglutinin
ETP	Early thymic precursors
FACS	Fluorescence – Activated Cell Sorting
FBS	Fetal bovine serum
Fuc	Fucose
FUT4	α-1,3-fucosyltransferase
Gal	Galactose
GalNAc	N-acetylgalactosamine
GANAB	α-glucosidases II
GFPT	Glutamine-fructose-6-phosphate transaminase
Glc	Glucose
GlcA	Gluconic acid
GlcNAc	N-acetylglucosamine
GLUT-4	Glucose-4 transporter
GNA	*Galantus nivalis* agglutinin
GnT or MGAT	N-acetylglucosaminyltransferases
HIF-1α	Hypoxia - inductor factor-1α
IdoA	Iduronic acid

IL-2R	Interleukin-2 receptor
IL-7	Interleukin-7
IL-7R	Interleukin-7 receptor
ILCs	Innate lymphoid cells
LB	Loading buffer
LEL	*Lycopersicon esculentum* agglutinin
L-PHA	*Phaseolus vulgaris* leukoagglutinin
MAL-II	*Maackia amurensis* agglutinin
Man	Mannose
MAN1B1	α-mannosidases I
MAN2A or MAN2B	α-Mannosidases II
MGL	Macrophage galactose - type lectin
MHC-I	Major histocompatibility complex class I
MOGS	α-glucosidases I
Neu5AC	5-N-acetylneurominic or Sialic acid
NFAT	Transcription factor associated with T-cell activation
NK	Natural Killer
NK-κB	Nuclear factor κB
O- β- GlcNAc	O-linked β-linked N-acetylglucosamine
OGA	O-GlcNAcase
O-GlcNAc	O-GlcNAcylation
OGT	O-GlcNActransferase
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate – buffered saline
PFK1	Phosphofructokinase 1
PI	Propidium Iodate
PI(3)K	Phosphatidylinositol-3 kinase
RB	Retinoblastoma
Ser	Serine
SLe	Sialyl Lewis
SNA	*Sambucus nigra* agglutinin
SP	Single positive
STn	Sialyl Tn
TAIL-7	T-cell acute-leukemia IL-7-dependent cell line
T-ALL	T-cells acute lymphoblastic leukemia

TBS	Tris – buffered saline
TCD	T-cell development
TCR	T-cell receptor
TEC	Thymic epithelial cells
Thr	Threonine
TJ2	TEL-JAK2
TN	Triple negative
Xyl	Xylose
γc	γ - chain

Chapter One

Introduction

In this chapter, the different theoretic subjects related with this dissertation will be addressed. The biological context of leukemic transformation and one of its major factors, T-cell development and interleukin-7 signaling, will be detailed. A comprehensive perspective on Glycosylation will be presented, through healthy and carcinogenic views, and finally, its relationship with a malignant transformation in T-cells.

1.1 Immune System

The immune system is responsible for defending organisms against harmful factors, that may have different origins, both exogenous and endogenous [1]. Thus, for the organism to have its defense assured, the immune system has at its disposal a diversity of mechanisms that allow it to detect and neutralize sources of danger [1, 2]. This important system can be divided into two distinct groups according to the speed of reaction and specificity, forming the innate immune system and adaptive immune system [3, 4]. The generation of immune cells depend on several cellular programs, including genetic recombination, which may have oncogenic potential [5].

1.1.1 Innate Immune System

The innate immune system is the first barrier of organism defense against pathogens [3, 4]. Showing a prompt answer [3], innate cells can recognize several ligands widely expressed in pathogens' surface, which allow the initiation of the immune response [2].

Innate immune cell population is composed of dendritic cells, natural killer (NK), macrophages, neutrophils, and others subsets [4]. Being part of one of the major group of innate cells, the NKs correspond to 5% - 10% of the peripheral lymphocytes [1] and release perforins after antigen interaction promoting their cytotoxic activity [3]. These cells do not have a specific receptor in their cell surface but can recognize the lack of major histocompatibility complex (MHC) class I (MHC-I), a characteristic of tumor cells that make them a target for NK [1, 3].

Neutrophils are the leukocytes that can be found in higher quantities in circulatory blood and their number can be increased upon inflammation [1]. Attracted by cytokines, when neutrophils arrive at the infection site, are able to recruit other myeloid cells [1].

Macrophages are differentiated monocytes that migrated to the damaged tissue and have phagocytic capacity helping in immune response, having, also, the property to present antigens to T-cells [1].

Dendritic cells can recognize different antigens in different tissues, process them, and then present the antigens to the naïve T-cells in lymphoid organs [1].

1.1.2 Adaptive Immune System

The adaptive immune system is composed by T and B lymphocytes that possess antigen specificity, promoting a targeted immune response. This response, although being chronologically later than the innate one, can result in immunologic memory, promoting a faster and effective response in case of a new invasion of the organism by the same or

similar pathogens, or other sources of danger signals [3, 4]. This immunity is activated in two steps, being the first one the presentation and recognition of the antigen by the T and B cells, and the second one T-cells activation and antibodies release by the B-cells [3].

B-cells are responsible for the release of antibodies and the presentation of an antigen to the T-cells [3]. These cells also can neutralize the toxins of the pathogen and unleash an antigen-dependent immune response [3].

T-cells can be divided into two different groups according to their T-Cell Receptor (TCR), forming the γδT-cells and αβT-cells. The latter subset can the further divided into cytotoxic CD8 T-cells and helper CD4 T-cells [3, 6], that recognize antigens presented by major histocompatibility complex (MHC) class I (MHC-I) or II (MHC-II), respectively [3]. The CD8 T-cells can be involved in antitumoral activity while the CD4 T cells recognize the pathogen and summon other immune cells to fight the pathogen [3].

1.2 T-cell development

T-cells develop in the thymus [7] and have a central role in the immune system, taking a significant part in the adaptive department [8, 9]. The thymus is divided in four compartments (subcapsular zone, cortex, medulla, and corticomedullary junction) that are important for correct T-cell development (TCD) [10]. The cortex and medulla are occupied by thymic epithelial cells (TEC), cTEC and mTEC, respectively, apart from thymocytes [10]. The different thymic regions and different constitutions provide the perfect setup for TCD [8, 10], an environment self-tolerant and self-restrictive that supports and guides allowing a vast repertoire of the T-cells [7]. Mature T-cells leave the thymus and migrate for the periphery, displaying have intermediate TCR signaling levels and self-tolerance. In other words, these thymocytes have basal signaling for a given antigen presented by MHC [9] and self-tolerance to prevent autoimmune diseases [10].

Common lymphocyte progenitors derive from hematopoietic stem cells and leave the bone marrow to initiate their migration to the thymus. There they become early thymic precursors (ETP), and begin the path towards the generation of mature and functional T-cells [7]. Once in the thymus, the cells become double-negative (DN) as the ETPs block the CD4 and CD8 expression [7, 9-12] (these cells can be called off the triple-negative (TN) taking into account the CD3/TCR absence) [7]. The DN stage can be divided into different substages according to CD25 and CD44 expression [7, 9, 10, 12, 13]. DN1 stage ($CD44^+$ $CD25^-$) cells can originate different cells of the immune system such as dendritic cells, macrophages, and different subtypes of T-cells [7, 9]. The cells in this phase are found in the corticomedullary junction and have their proliferative capacity increased [10, 11]. In this phase, the Notch signaling pathway is important for T-cell specification, inhibiting B-cell differentiation [10, 11].

At the end of the DN1 stage, cells migrate to the thymic cortex and continue their development passing to the DN2 stage ($CD44^+$ $CD25^+$) [7, 10]. In this phase it is possible to observe Interleukin–2 receptors (IL-2R) and Interleukin–7 receptors (IL-7R) expression in thymocytes [13]. The Notch and IL-7 trigger *Tcrb* rearrangements [7, 10, 14], mediated by RAG1 and RAG2, which generate random variable receptor chains [7].

In DN3 ($CD44^-$ $CD25^+$), thymocytes express a pre-TCR [13] (complex formed by a rearranged β-chain and an invariant pre-TCR-α-chain), and undergo β-selection [7, 10, 14, 15]. This selection is an important checkpoint that allows to select the pre-TCR with a functional TCR β-chain [10]. Cells positively selected continue to the αβ lineage in the subcapsular region of the thymus, when the TCRα chain rearrangements begin [9]. The Notch signaling in this stage is important for promoting cell survival by the phosphatidylinositol-3 kinase [PI(3)K] and Akt activation, however, Notch overexpression

promotes oncogenic properties [10]. In the final phase of this stage, the Notch and pre-TCR signaling promote thymocytes' self-renewal [16].

While on DN4 ($CD44^-$ $CD25^-$), thymocytes proliferate and attempt functional TCR-α gene recombination [13], which triggers the expression of CD4 and CD8 simultaneously, passing to double-positive (DP) stage [7, 10]. DP stage is marked by a successful *Tcrα* rearrangement, giving rise to a mature αβ-TCR complex [7, 17]. Afterwards, another selection process takes place, which DP cells are considered by the strength of the interaction between αβ-TCR and peptide-MHC-I or -MHC-II complexes [7, 11, 17]. Thymocytes expressing TCRs which show low levels of signaling die by neglect. On the other hand, when TCR signaling is high upon peptide-MHC interaction, these cells are eliminated by negative selection [9, 17], decreasing the risk for the generation of mature self-reactive T-cells [10, 17]. The cells with a range within intermediate TCR signaling strengths are positively selected and escape negative selection. These thymocytes pass to the single-positive (SP) stage, expressing only CD4 (Helper T-cells) or CD8 (cytotoxic T-cells) co-receptors, and exit the thymus **(Figure 1)** [7, 9]. During DP stage, IL-7 leads to STAT5 phosphorylation [18] and an increase of anti-apoptotic Bcl-2 [19, 20] protecting the thymocytes of the negative selection [18, 19].

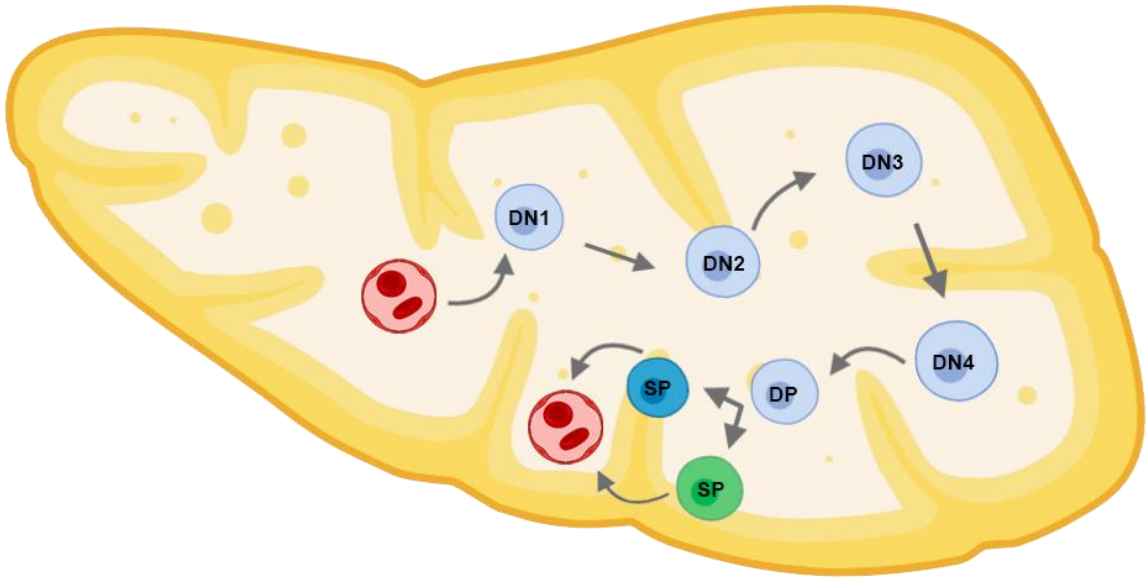

Figure 1 *- Scheme of T-cell development in Thymus. Adapted from [10].*

The IL-7 signaling pathway is very important for TCD being present in all stages, showing a crucial role in proliferation, survival, and differentiation in DN [7] and DP stages of thymocytes [18, 19] Alterations in this signaling can promote leukemia, namely the T- cell Acute Lymphoblastic Leukemia (T-ALL) [12].

1.3 Interleukin-7

Produced by the bone marrow cells and thymus stroma [21, 22] and some secondary lymphoid organs [12], IL–7 is an important interleukin for the cellular functions of lymphoid cells [12, 21, 22]. IL-7 is an essential cytokine to cell development and survival, having several functions in different cell mechanisms [16], namely in TCD stages [12, 23, 24], T-cell homeostasis [12, 24, 25] and innate lymphoid cells (ILCs) development [12, 25]. The IL-7 binding to IL-7R, that is composed by IL-7Rα [12, 23, 26-28] and γ-chain (γc) [12, 23, 26-28] triggers signaling cascades. The IL-7Rα is responsible for sensitivity for the ligand (IL-7) while γc mediates functional activity, corresponding to CD127 and CD132, respectively [23, 28, 29].

The γc present in the cytokine receptor is common to several interleukins, like IL–2, IL–4, IL-7, IL-9, IL-15, and IL-21 [12, 28, 30], with crucial functions in cell proliferation and survival [24, 30]. Relatively to CD127, which expression is regulated by NOTCH 1 [29]. The IL-7Rα glycosylation is important for STAT 5, PI3K, and JAK activation [31], however, IL-7Rα phosphorylation of the tyrosine 449 is crucial for IL-7 signaling [24, 29, 31]. Mutations in one of the receptor chains are responsible for severe combined immunodeficiency [29]. In this way, IL-7 can be implicated in alterations of the several molecules that are involved in signaling, metabolism, and cell growth [30].

When IL-7 binds to IL-7R, the signaling cascade begins and interfere with extracellular matrix modulation and homeostasis in lymphocytes [26]. Also, it influences the β-selection of the thymocytes [16]. The trimeric complex (IL–7, IL–7Rα and γc) activate the JAK 1 and JAK 3 that phosphorylate serine and threonine residues, activating the PI3K signaling (PI3K / AKT / mTOR), STAT 5 [12, 21, 28, 32] and MEK / Erk signaling [12, 21], responsible for stimulation of glucose metabolism and cell proliferation [33] and survival, are important in normal and malignant T-cells [29]. However, the IL-7 availability in the environment for T-cells is restricted by ILCs, since they consume more cytokine than other lymphocytes [25].

In TCD, the IL-7 signaling promotes the TCR rearrangement in DN2 and DN3, and in the DN4 stage promote the cell self-renewal by Bcl-6 repression [16]. In the DN2 stage, IL-7 and Notch signaling promote cell proliferation [16, 24] and cell survival, as well as in DN3 [24]. Alterations in IL–7 signaling, however, lead to the malignant transformation of these cells [34]. On the other hand, the IL-7 signaling block in DP thymocytes promotes apoptosis [31], blocking the TCD [29], evidence that shows the crucial role in survival and proliferation of the β-selected cells [31].

The abnormal IL-7 and IL-7R levels in cells are associated with immunopathology, being involved in autoimmunity, chronic inflammatory disease [12, 29], and hematological [12] and solid tumors [12, 29]. High cytokine and receptor levels, in colon mucosa, are associated with Crohn´s disease and ulcerative colitis [12]. IL-7 showing, also, resistance against anti-TNF in Crohn´s disease, mTOR inhibitor therapies in T-ALL, and when in presence of aberrant MYC activation, in pre-leukemic cells, is benefited by IL-7 [12]. In addition to the indirect contribution to the neoplasia progression, IL-7 also has a direct influence in tumor progression, promoting cell proliferation, epithelial-mesenchymal transition (EMT) and metastasis in breast cancer. In prostate and bladder cancer, IL-7 helps in cell migration, invasion, and EMT [12] **(Figure 2)**. IL-7 signaling can be affected by mutations, for example, Cysteine insertion in the extracellular or transmembrane domain of the cytokine receptor, that promotes cell survival and proliferation, leading to different hematological malignancies, namely Acute Lymphoblastic Leukemias (ALL) such as ETP-ALL, T-ALL and B-cell ALL [24, 29], changing the JAK / STAT 5 and PI3K / Akt / mTOR signaling [29].

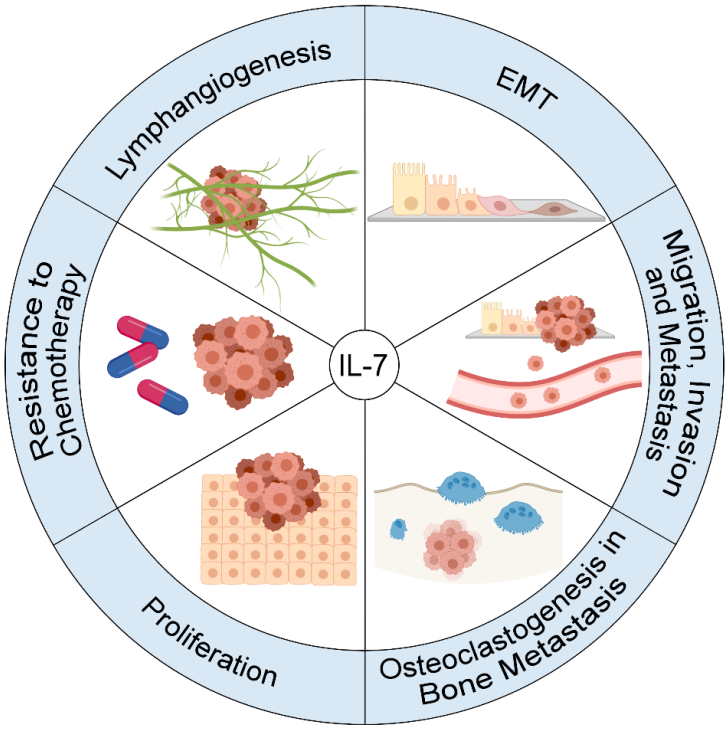

Figure 2 *- IL-7 functions in tumor progression. Adapted from [12].*

The IL-7 signaling is important for immune system, particularly by regulating the immune cells functions through *N*-glycosylation modulation [30]. Having a central role in TCD, IL-7 has a dual function in T cells, either by promoting a decreased complex *N*-glycans expression in resting T-cells and an increased expression of branched N-glycans in activated T-cells [35]. Moreover, IL-7R glycosylation can also modulate IL-7 affinity for the receptor. In fact, IL-7 have more affinity for glycosylated IL-7R [23].

1.4 Protein Glycosylation in homeostasis

Glycobiology is the science that studies the structure, biology, biosynthesis, and evolution of carbohydrates, or glycans [36, 37]. This subject has become important in the first part of the 20th century, being relevant for all fields of Biology and Medicine, in basic research, biomedicine and biotechnology [36, 37].

Glycosylation is a process that occurs in all free-live cells and multicellular organisms and is very important for their biological functions since it allows cell variability and recognition [36, 38]. This modification corresponds to an addition of carbohydrates (*N*-acetylglucosamine (GlcNAc), glucose (Glc), fucose (Fuc), galactose (Gal), Mannose (Man), Xylose (Xyl), *N*-acetylgalactosamine (GalNAc), gluconic acid (GlcA), iduronic acid (IdoA) and 5-*N*-acetylneuraminic acid (Neu5Ac or sialic acid)) [11, 36-42]. This alteration is a sequential enzymatic process that occurs at the post-translational level in proteins (glycoproteins) or lipids (glycolipids) [11, 36-42] that can occur by α- or β-linked enabling different connections leading the different three-dimensional structures promoting different biological functions [43]. These glycans have an important role in the innate and adaptive immune system, during development, differentiation, activation, and apoptosis of immune cells [44].

Glycans constitute one of the major features of organism's homeostasis, possessing structural and modulatory properties (protection, stabilization, organization, and barrier function), specific recognition of other molecules (glycan-binding proteins) and mimicry host glycan (the pathogen express in your cell surface glycans typical of the host, allowing the pathogen to evade the immune system) [36, 37, 42, 45]. The glycosylation process requires glycosyltransferases (responsible for the addition of glycans) and glycosidases (that perform the hydrolyzation of specific glycans), being their function vital for normal glycan biosynthesis, as well as the substrate availability [37].

In protein glycosylation, glycans are covalently bound to proteins by *N*- or *O*-linkage, forming *N*- or *O*-glycans, respectively **(Figure 3)** [36, 37, 46], using several substrate donors [33]. For 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 **(Figure 4)** [36, 37, 46]. The *O*-glycosylation occurs in oxygen atoms of serine(Ser)/threonine(Thr) residues (*O*-linked pathway) forming two *O*-glycans types: *O*-GalNAc, when a GalNAc monosaccharide is added (*O*-GalNAcylation), or *O*-GlcNAc, when it is added a GlcNAc molecule (*O*-GlcNAcylation) [36, 37, 47, 48].

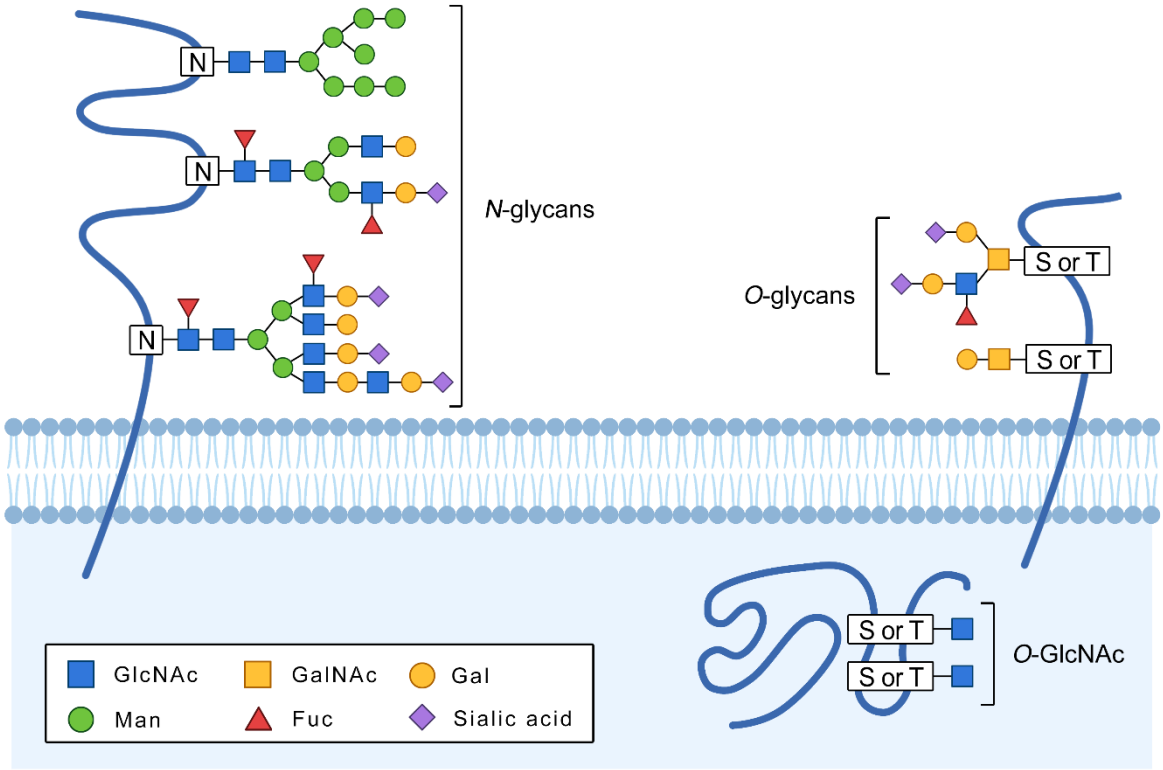

Figure 3 *- Protein glycosylation,* N- *and* O*-Glycans. Adapted from [38, 40].*

Afterwards, the *N*-Glycans and *O*-Glycans (more specifically, O-GalNAc glycans) can be elongated with different monosaccharide combinations, and terminated with a sialic acid, the Neu5Ac in humans [49]. The sialic acid termination plays several roles in cellular function, such as self-recognition [49], cell adhesion, through selectin binding, and signaling [38, 49]. The sialic acid termination on pathogens can be recognized by the immune system, which facilitates pathogen evasion and disease development. However some pathologies, such as cancer, are characterized by an increase in sialic acid, being associated with poor prognosis, promoting cancer progression [38].

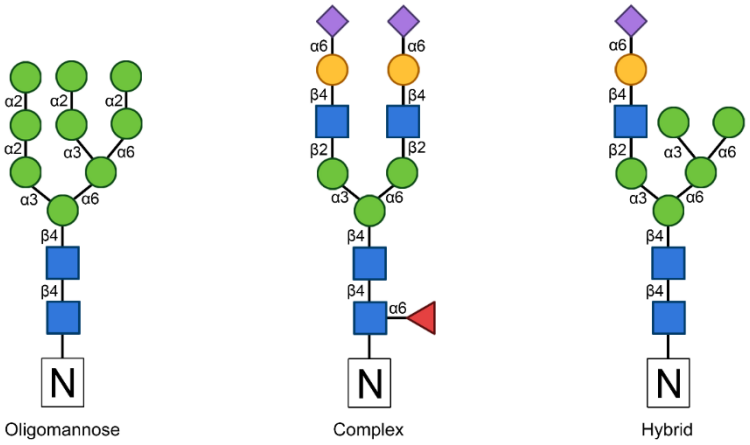

Figure 4 *- Types of N-Glycans. Adapted from [46].*

1.4.1 *N*-Glycosylation in health

N-glycosylation is an important post-translational modification for protein conformation, solubility, antigenicity, activity, and recognition. The *N*-glycans are present in about 90% of the mammalian proteins and are essential for cell biology. In this way, their defects translate into different diseases [33, 38, 39, 46].

The process of *N*-glycosylation is mediated by different enzymes (glycosyltransferases and glycosidases) such as the α-mannosidases and *N*-acetylglucosaminyltransferases I, II, IV, and V (GnT-I,II,II,IV, V; encoded by *MGAT1,2,4* and *5* genes), responsible for Man removal and GlcNAc antennae addition, respectively [35]. It can also depend on the substrate availability, as the different glycosyltransferases have different affinities for their substrate [33]. For instance, the affinity of *N*-acetylglucosamine to the substrate varies: the protein encoded by *MGAT1* has more affinity for the substrate than any other enzyme, so *MGAT1* activity limits the substrate for other enzymes [33].

The first step of *N*-glycosylation is the synthesis of dolichol-phosphate (Dol-P) in the ER cytoplasm. When this precursor passes to the ER lumen $Glc_3Man_9GlcNAc_2$ is formed. Afterwards, it is transferred for an Asn residue producing $Glc_3Man_9GlcNAc_2$Asn [33, 39, 40, 46]. The process begins in the ER and continues in the Golgi. At the ER, the α-glucosidases I (MOGS) and II (GANAB) remove glucose (Glc) of the $Glc_3Man_9GlcNAc_2$Asn and, before this, α-mannosidases I (MAN1B1) remove the α1-2Man residue of the central branching of $Man_9GlcNAc_2$, passing to $Man_8GlcNAc_2$ [33, 39, 40, 46]. The *N*-glycan biosynthesis, in the Golgi, begins with the elimination of the mannose terminals and addition of GlcNAc in C-2, mediated by *MGAT1* in α1-3 Man in the $Man_5GlcNAc_2$ followed by α-Mannosidases II (MAN2A or MAN2B) that remove terminal α1-3MAN and α1-6MAN from $GlcNACMan_5GlcNAc_2$ to $GlcNACMan_3GlcNAc_2$ [33, 39, 40, 46]. The next step corresponds to the additions of GlcNAc in C-2 of the α1-6MAN by *MGAT2* and the other GlcNAc is added in C-4 of the α1-3MAN by MGAT4 and in α1-6MAN by *MGAT5*, forming try- and tetra-antennary glycans **(Figure 5)** [33, 39, 40, 46].

The *N*-glycosylation pathway is very important for cell signaling and different levels of glycosylation lead to different cell responses, depending on the glycosylated receptor. For example, in T-cells the glycosylation promote cell arrest through the cytotoxic-T-lymphocytes-associated antigen-4 (CTLA-4) receptor retention in cell surface [33, 50], and by the increase of TCR activation threshold [50].

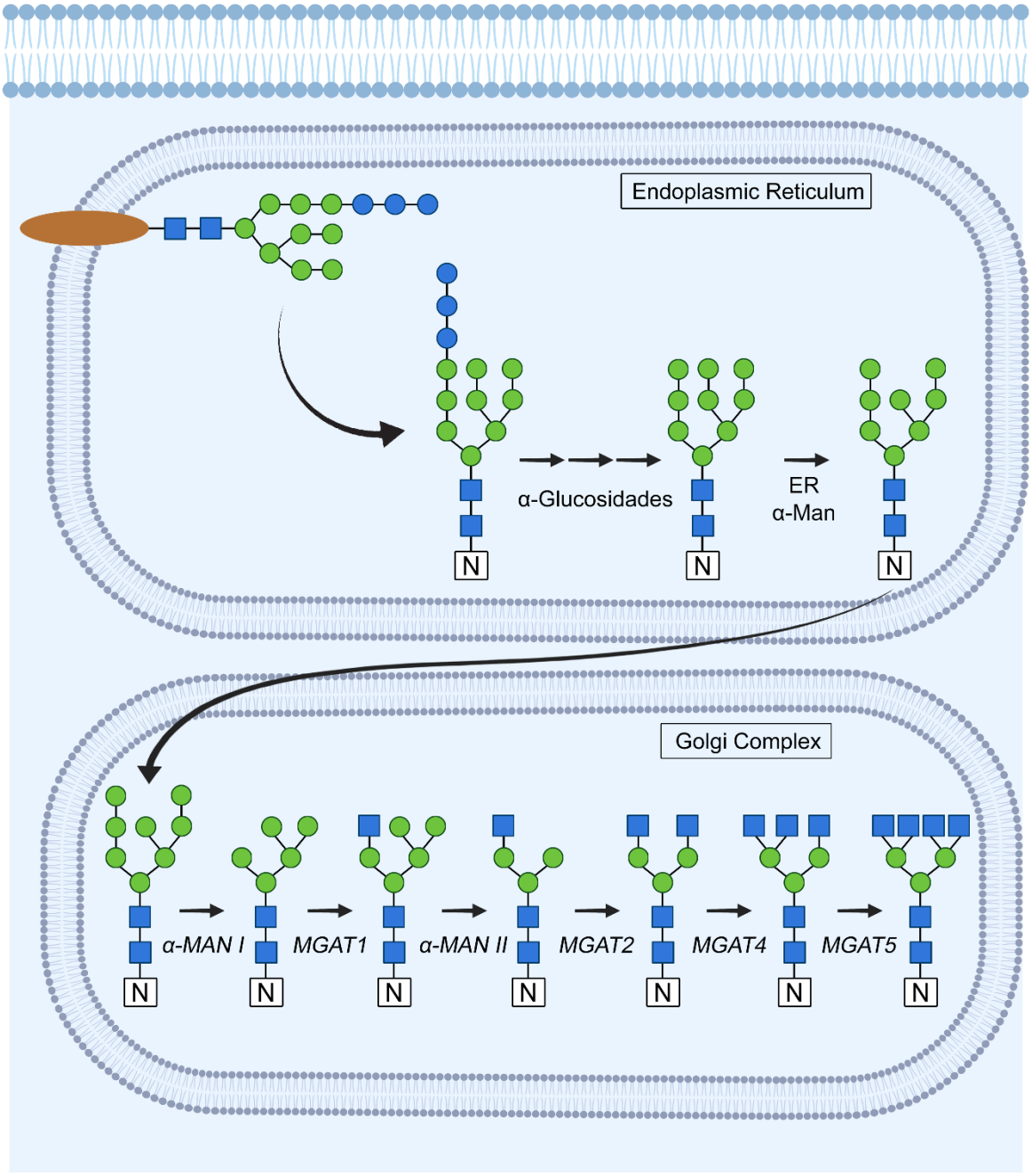

Figure 5 *- Biosynthesis of N-Glycans. Adapted from [40, 46].*

One of the cellular types that are regulated by *N*-glycans are T-cells, being involved in the development and functions of these lymphocytes [9, 11]. The number of branches/antennae's of these N-glycans can help regulate T-cell function, by regulating TCR clustering, through the interaction between galectin 3 and TCR-glycans [9], or the CTLA-4 surface half-life, through the control of endocytosis receptor rate [33]. The *N*-acetylglucosaminyltransferase enzyme, responsible for the formation of complex tetra-antennary *N*-glycans (β1,6 GlcNAc-branch), is encoded by the *MGAT5* gene and it is important for the regulation of T-cell activity by galectin-glycan interaction.

In T-cells, GnT-V activity changes during their development [9]. The expression variability of the glycan this enzyme generates can be observed in the thymocyte subsets [9]. *MGAT5* activity increases 12-fold when lymphocytes pass from DN4 to DP. After that, when the T-cells pass to SP, the enzyme activity decreases 3-fold [9]. Once formed, mature thymocytes migrate to the periphery and a decrease of the 6-fold in *MGAT5* activity is observed [9].

In T lymphocytes, IL-7 can influence the *N*-glycosylation, regulating the TCR clustering and the activation thresholds [30]. Indeed, in peripheral T lymphocytes, when the IL-7 levels increase, it is observed increased activation of T-cells by TCR signaling due to *N*-glycan branching decrease [30]. After T-cell activation, the IL-7 signaling decreases [12, 29], and when expressed, again, the IL-7 slows down the T-cells death, promoting their survival comparing the other T-cells without IL-7 re-expression, leading to several inflammatory diseases [12].

In peripheral T-cells, the low activity of the *MGAT5* enzyme decreases the level of complex glycans [9, 33]. This alteration reduces galectin-glycan interaction leading to an increment of TCR clustering and signaling for an immune response [9, 11, 33]. However, these levels can be reestablished by GlcNAc, uridine, and glutamine supplementation [33]. The different levels of glycosylation are important for the regulation of the immune response, since they allow a rapid response to a pathogen and its elimination [9, 33].

1.4.2 O-GlcNAcylation in Health

The *O*-glycosylation post-translational modification can occur in an intra or extracellular environment. When this binding occurs by *O*-linked β-linked *N*-acetylgalactosamine (*O*-β-GalNAc), or simply *O*-GlcNAc (*O*-linked β-*N*-acetylglucosamine), it is called *O*-GlcNAcylation. It is present in intracellular proteins [37, 47, 51-53] (nuclear, cytoplasmatic and mitochondrial proteins [51]), and is the only type of intracellular glycosylation [54]. *O*-GlcNAcylation competes with phosphorylation by Ser/Thr residues, with the potential of modifying cell signaling [37, 47, 55]. These modifications are mutually exclusive [56], however, both have a regulatory function [54]. *O*-GlcNAcylation is important for different mechanisms in cellular physiology, being involved in epigenetics, protein degradation, signaling transduction, cell cycle, mitochondrial bioenergetic **(Figure 6)** [51], morphogens and glucose metabolism [52]. Glycosylation in serine/threonine residues can occur in the nucleus, mitochondria, and cytoplasmic compartments by the action of *O*-GlcNActransferase (OGT) and *O*-GlcNAcase (OGA) enzymes **(Figure 6)** [51, 55]. These enzymes are essential for survival, as *Ogt* deletion leads to embryonic lethality, and removal of *Oga* leads to prenatal death [54, 57]. The OGT enzyme is responsible for the GlcNAc addition from the UDP-GlcNAc donor molecule [54, 58, 59]. Its activity is related to substrate

concentration in the cytoplasm, being associated with glucose levels [54, 56], with different tissue, cell differentiation, inflammation and substrate specificity (nucleotide sugar concentration-dependent) [59]. In situations with high glucose concentration in the extracellular environment, the UDP-GlcNAc levels will be increased. This leads to a hyperglycemic state, being possible to observe an increase in *O*-GlcNAc levels and showing the power of nutritional sensor of the O-GlcNAcylation [59].

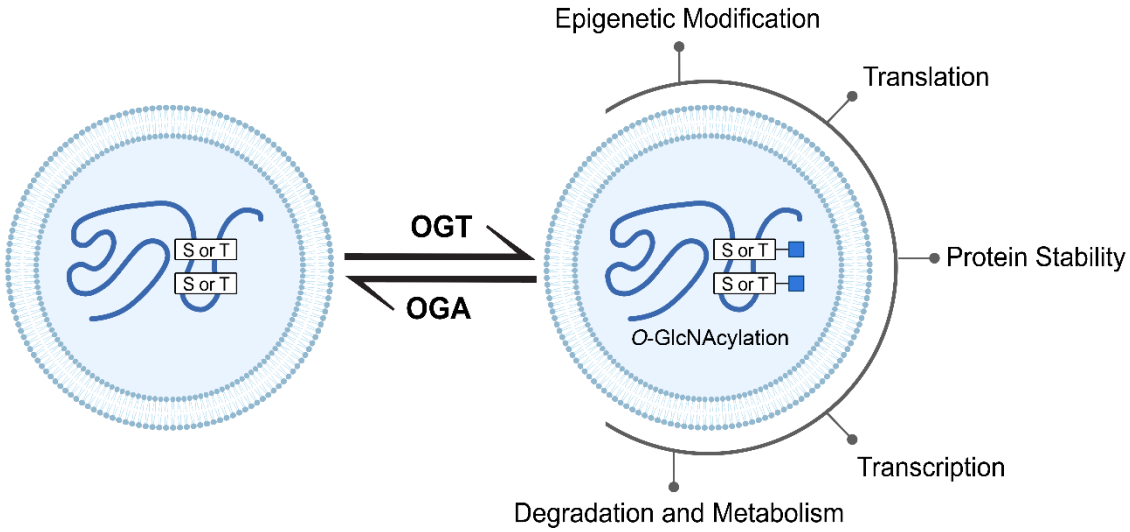

Figure 6 *- Enzymes involved in O-GlcNAcylation (OGT) and deacylation (OGA) and functions of O-GlcNAc in healthy cells. Adapted from [57, 59].*

O-GlcNAcylation has an important role in cell growth and progression and, in cooperation with phosphorylation, can regulate the cell cycle through the glycosylation and phosphorylation of the H3 [56]. High *O*-GlcNAc levels in H3 indicates that the cell is in interphase (inhibiting G2 to M transition). On the other hand, when this histone is phosphorylated, the cell is in mitosis [56], regulating the mitosis processes [58]. However in the interphase stage, composed by G1, S, and G2 phases of the cell cycle, a variation in *O*-GlcNAc levels can be observed: in G1 and G2 the levels of glycosylation are increased and the contrary at S phase, that shows lower levels [59].

When cells are under stress conditions, glucose levels increase [52] and, consequently, *O*-GlcNAc levels are elevated, which promotes cell survival [51, 60]. This survival is promoted by induction of the heat shock protein (HSP)70 and HSP40, leading to cell tolerance in stress conditions [52].

In T-cells, *O*-GlcNAcylation impose different functions depending on the activation and developmental stage of T-cells [61] [57]. During T-cell development, *O*-GlcNAcylation levels increase after β-selection, promoting cell proliferation and differentiation between DN3 and DN4 stages. *Ogt* T-cell conditional deletion leads to failures in β-selection

decreasing the number of mature T-cells [11, 53, 57] and the deficit in clonal expansion in DN4 stage. Moreover, the increase in *O*-GlcNAc levels is associated with malignant transformation of T-cells [11]. **In inactive T lymphocytes,** OGT also has an important role as when this enzyme is blocked, the T cell activation is incomplete or improper, and the number of the peripheral CD4 T-cells decreases with an increase of the apoptosis levels [61]. After T-cell activation, the *O*-GlcNAc levels increase, promoting a fast cell division and proliferation [53, 54]. **In active T lymphocytes**, there is a decrease in the *O*-GlcNAc levels in cytoplasmic proteins but an increase in nuclear proteins [51, 54, 60]. In the latter ones, *O*-GlcNAc is related to transcriptional machinery such as RNA polymerase II, getting involved in the cell cycle [51, 60]. In dying T-cells, the *O*-GlcNAc levels are increased [51, 60].

1.5 Alterations of Protein Glycosylation in Cancer

Changes in cellular Glycosylation is an hallmark of cancer cells [48, 62-64]. This post-translational modification promotes cancer progression [38, 41, 64] influencing the tumor environment and regulating growth, metastasis [64, 65] invasion, and angiogenesis [39, 62, 64] **(Figure 7)**.

Tumor cells are characterized by DNA damages and genomic instability, that can be regulated by glycosylation of the cMYC and kinase cascades [48]. Different changes in glycosylation are related to different tumor characteristics. The aberrant tumor glycans expression can lead to inflammatory reactions promoting an immune-suppression environment and interactions between cancer cells and endogenous lectins influence tumor and metastasis environment [48, 66].

In cancer cells, the misregulation of the glycosylation pathway is associated with an invasive behavior, like cell proliferation, metastasis, and can even be related with angiogenesis [38, 39, 48, 63, 66]. Malignant transformation is associated with the presence of oncofetal genes normally only expressed in embryonic phase [38, 39, 48, 63, 66], but also with an alteration in glycosidases and glycosyltransferases genes expression [48, 65], substrate donor availability and their localization [39, 65].

The changes in glycosylation can occur through two mechanisms: incomplete synthesis and neo-synthesis, being involved in different phases of the neoplastic development [38]. In the early phase, the most common is the incomplete synthesis process, which lead to truncated structures, like sialyl Tn (STn) [38]. Those structures are related to neoplastic progression and metastasis [41, 67], being good markers in human cancer [41]. In late stages, when cancer is more advanced, is possible to observe the neo-

synthesis process-induced neo-antigens [63], such as sialyl Lewis a (SLe^a) and SLe^x [38], that are related to transformation and neoplastic progression [41, 48].

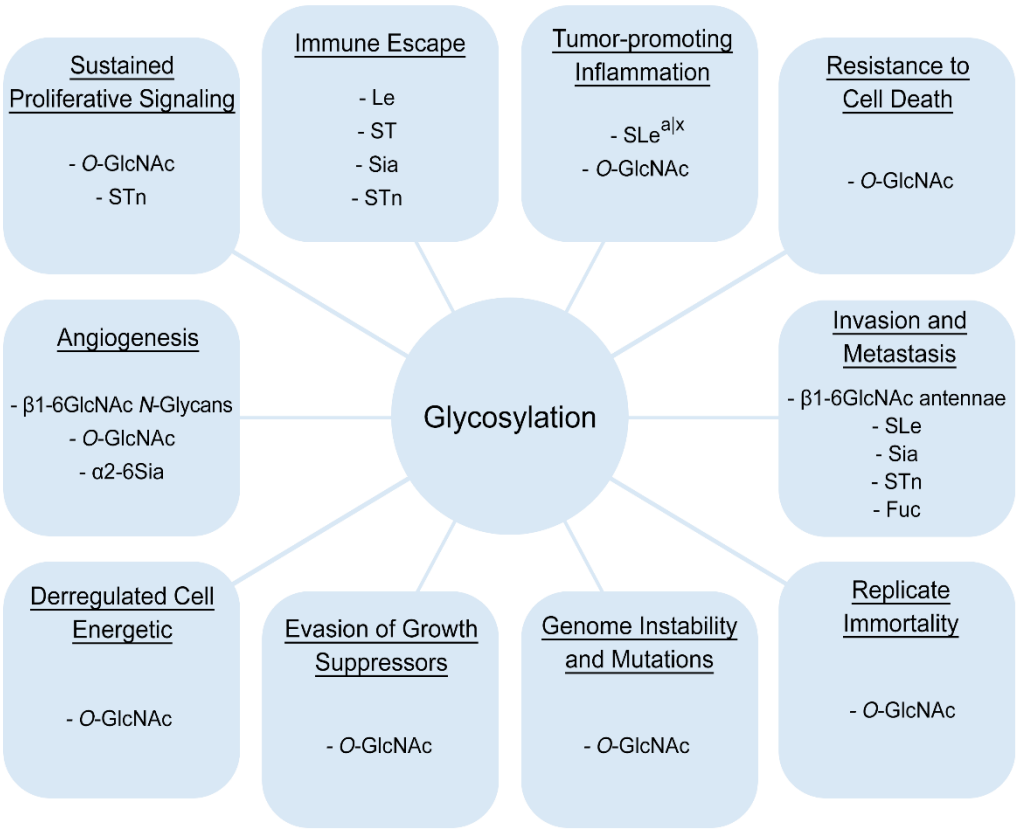

Figure 7 *- Summary of all contribution of different types of Glycosylation in cancer promotion. Adapted from [48].*

An important process in tumor progression is the immune scape. The tumor-associated proteins can be recognized by the immune system, however, neoplastic cells can evade this surveillance through the secretion of cytokines and chemokines [68]. As galectins are expressed by healthy and neoplastic tissues, they can modulate the inflammatory immune defense or immune evasion [64, 68]. Tumor cell surface expresses SLe^x and SL^a [68] (α2,3-linked sialic acid [38, 39, 50]), which are associated with malignancy and metastatic lesions, allow monitoring the effectiveness of the therapy and recurrence [68]. Besides, the high levels of sialylation in tumor cells promote the self-recognition by the immune system, inhibiting the immune response [64].

1.5.1 *N*-Glycosylation in Cancer

Altered *N*-glycans are related to different tumor characteristics and different cancer types [38, 39, 69]. There are several receptors influenced by the degree of glycosylation in asparagine residues. Receptors that influence proliferation, growth, and oncogenesis (epithelial growth factor receptor (EGFR) and integrins) present a high number of *N*-glycans

sites while the receptors responsible for growth arrest and differentiation show a low number of *N*-glycans sites [38, 39, 69]. The *N*-glycans inhibition leads to abnormal protein folding, having consequences on tyrosine kinase receptors [39].

In cancer, the RAS-RAF-MAPK signaling is activated, which leads to a higher expression of *MGAT*5, leading to an increase of β1,6-branching *N*-glycans [38, 63]. This branching is associated with increased cellular motility, contributing for tumor metastasis in mice [38, 63]. Besides, it promotes tumor invasion and metastasis in breast carcinoma in murine models [38], and its inhibition leads to metastasis suppression [38, 48, 63, 66, 70]. Indeed, low cell-cell and cell-matrix adhesion promote these malignant properties [48, 69, 70]. These malignant characteristics of complex *N*-glycans also can be founded in colorectal cancer, promoting the cancer development and progression [71]. However, *MGAT*3 (synthesis β1,4-branching) also is important in cancer progression displaying an essential role in metastasis suppression [63]. *MGAT*3 is responsible for the bisected *N*-glycans, important for cell adhesion, controlling E-cadherin adhesive function [38, 48, 62, 70]. E-cadherin activity is regulated by *MGAT*3 and *MGAT*5-related glycans, as it is glycosylated with bisecting *N*-glycans in a healthy tissue and by tetra-antennary *N*-glycans upon malignant transformation. In fact, the E-cadherin glycosylation profile given by GnT-V activity promotes the translocation of E-cadherin to the cytoplasm, which consequently decreases cell adhesion and promote tumor invasion [39, 48, 69, 72].

This type of glycans are important for the interaction with galectins (1 and 3) [39, 48], that create structures that allow the regulation of the expression of some receptors on the cell surface [70].

1.5.2 *O*-GlcNAcylation in cancer

In cancer cells, the metabolism, that is initially oxidative phosphorylation, shifts to aerobic glycolysis (Warburg effect) with a high glucose rate that will increase the metabolic flux through the hexosamine pathway. This alteration in metabolism promotes the increase of the UDP-GlcNAc substrate [38, 48, 62] and occurs because these cells are under stress [48]. This increase in substrate leads to increased glycosylation, particularly of *O*-GlcNAcylation. This modification is often increased in several types of cancer [38, 39, 62, 63, 73], leading to an anti-apoptotic effect [48], compared with controls, in which these levels are decreased [60, 74]. The *O*-GlcNAcylation has different functions in tumor progression, modulating protein interactions, localization in subcellular compartments, enzyme activity, affinity for the DNA, and transcription like cyclins, p53 and cMYC [39, 48, 63].

An important regulator of the glycolysis flux is the phosphofructokinase 1 (PFK1) that is negatively regulated by *O*-GlcNAc [75], being involved in a decrease of tumor cell

proliferation [48]. The *O*-GlcNAc modification of the other factors can influence the Warburg effect. An increase of the *O*-GlcNAc in hypoxia-inductor factor-1α (HIF-1α) helps its stabilization [48] and, in turn, this factor will regulate OGT. The OGT overexpression, associated with an increase of the *O*-GlcNAc levels, promotes the GLUT1 transcription. In turn, this leads to an increase in glucose uptake [75, 76], making HIF play a role in energy reprogramming in malignant cells, promoting survival and tumor growth [48].

Uncontrolled proliferation is a common characteristic of all tumors, leading to loss of the homeostasis in the organism [75]. This capacity can imply an autocrine regulation of the proliferative signaling [75]. The *O*-GlcNAcylation and phosphorylation compete by the same Ser/Thr sites [75] in cMYC influencing the cell signaling [48, 73]. Phosphorylation of the cMYC promotes protein degradation [75], while glycosylation promotes protein stabilization, being involved in oncogenesis [38, 48, 62, 75, 76] and cancer metabolism [76], promoting cancer progression [62, 74]. This competition is also present in the regulation of DNA damage being possible that *O*-GlcNAc is related to genomic instability in cancer. In fact, the DNA repair machinery is glycosylated [62, 74]. The *O*-GlcNAcylation has a dual role in condensation, promoting the protection of DNA from damage, and decondensation, for example when cells enter in G1 phase, of the nucleosome [77].

The regulation of tumor growth is controlled by p53 and retinoblastoma (RB), that have sites that can be glycosylated or phosphorylated [48, 62]. The decrease of the *O*-GlcNAc levels in RB leads to cell cycle progression [48] and an increase of p53 *O*-GlcNAcylation promotes tumor suppression [62]. The stabilization of the p53 also happens in mutant forms, influencing tumor progression and having a pro-oncogenic function [62].

In intratumor environment, hypoxia increase mutation levels and change regulation of the DNA damage repair, this associated with interaction between HIF-1α and cMYC leads to malignant progression [48]. The cMYC can be glycosylated or phosphorylated and its stability changes according to the post-translational modification, regulating the signaling for DNA damage repair [62].

The cancer cell survival is promoted by hyper-*O*-GlcNAc, contributing to a worse prognosis [48].

1.5.3 Lectins

Lectins are glycan-binding proteins [64, 78] being described for the first time in 1888 in plants and, some years later, 1960, were discovered in animals by Anatol Morell and Gilbert Ashwell [78]. There are many types of animal lectins, such as galectins, C-Type lectins, I-Type lectins, and P-Type lectins **(Figure 8)**. They are divided into two different groups, endogenous and exogenous lectins, taking into account the location of the ligands [78, 79]. Endogenous lectins are involved in fertilization, development, and cell-cell and cell-matrix interaction, and on the other side, the exogenous lectins play a role in recognition and organism defense [79]. Lectins are molecules that can be associated with the membrane, for example, siglecs and selectins, or soluble molecules such as galectins (Gal) [64].

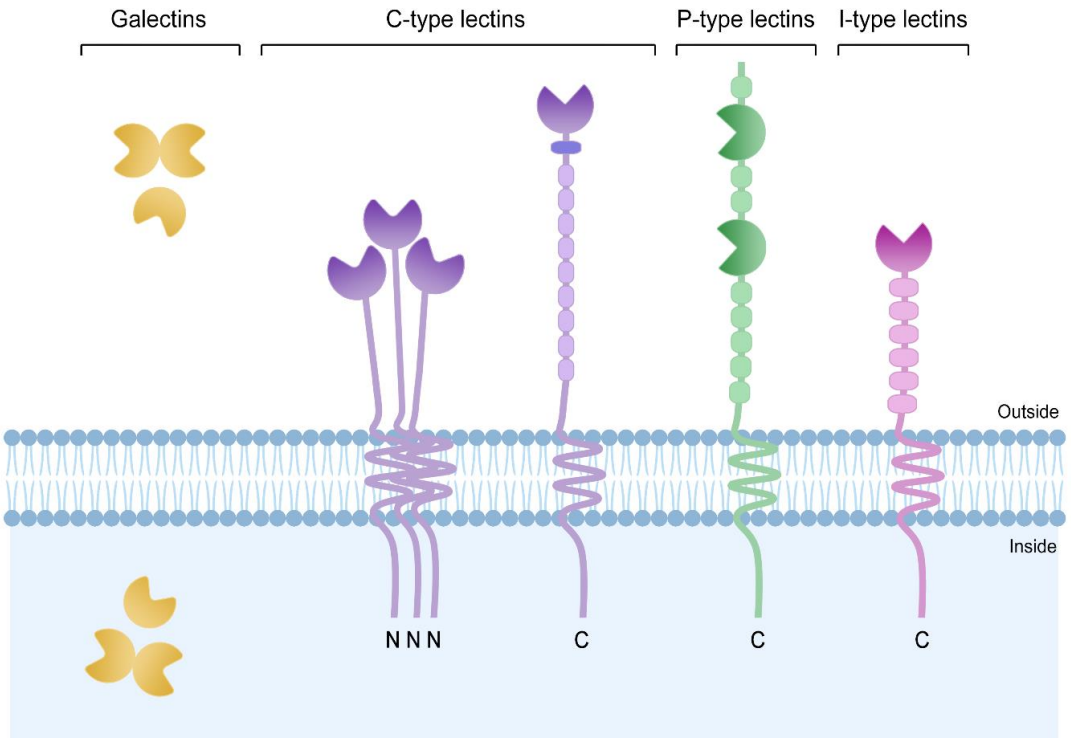

Figure 8 *- Types of glycans-binding proteins. Adapted from [78].*

Galectins, that were initially called S- Type lectins [68, 80], acquired this name in 1994. This type of lectin is expressed in all organisms [80] with highly evolution conservation [79], recognizing β-galactosidases [68, 79] in animals [79]. Galectins are also important for the development and regulation of immune system activity and play a role in the immune system's recognition function [76]. There are 15 different galectins that can be divided into three distinct groups [68, 80] according to their structure [80]. The prototype group is composed by Gal-1, -2. -5, -7, -10, -11, -13, -14 and -15; the second group, chimeric, is

constituted by Gal-3; and the last one, the tandem repeat group, comprises Gal-4, -6, -8, -9 and -12 [64, 80]. Each galectin has specificity for different glycans [68, 79, 80] **(Figure 9).**

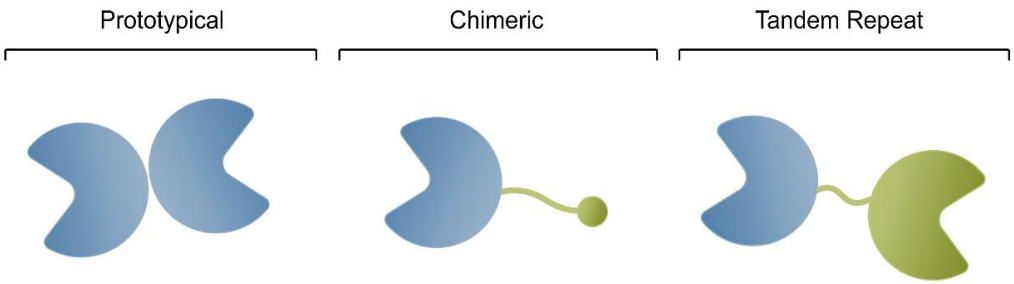

Figure 9 *- Types of Galectins. Adapted from [80].*

Different organisms express a wide variety of lectins, recognizing a vast repertoire of glycans. Plant lectins can bind to mammalian cell glycans, allowing their use for the study and evaluation of the glycoprofile of different cell types [81], being an important tool for biomedical research, diagnosis and therapies in different pathologies, for example cancer [82].

Lectins and anti-glycans antibodies are important sensors of cellular glycans, as they can discriminate the expression of biologically and chemically distinct carbohydrates **(Figure 10)** [83, 84]. In fact, there are several methods used to study glycosylation using lectins, such as Western Blotting and Enzyme-like lectin assay or Immunohistochemestry, where the latter displayed successful application in diagnosis and prognosis in cancer [82] and Inflammatory Bowel Diseases [85] .

The *Phaseolus vulgaris* leukoagglutinin (L-PHA) lectin detecting β1,6-GlcNAc branched *N*-glycans, product of *MGAT5* enzyme [9, 33, 50, 86], the polylactosamine structures in *N*-glycans is detectable by *Lycopersicon esculentum* agglutinin (LEL) [50] and bisecting *N*-glycans connect to *Phaseolus vulgaris* erthroagglutinin (E-PHA) [86]. For sialic acid termination exist *Sambucus nigra* agglutinin (SNA) for α2,6-linked sialic acid and *Maackia amurensis* agglutinin (MAL-II) that detect α2,3-linked sialic acid [50]. Another important lectin is the Concavalin A (ConA) used for high-mannose *N*-glycans detection [83, 86], and for α1-3 mannose detection is used *Galantus nivalis* agglutinin (GNA) [87].

Lectins enable the study of global alterations of the cellular glycosylation profile [83]. As it was discussed above, glycans expression can be altered due to several factors, related to cell-intrinsic features (genetic alterations) or cell-extrinsic factors (such as extracellular monosaccharide availability) In fact, when supplemented with GlcNAc, the increased expression of β1,6-GlcNAc branched *N*-glycans, can be detected by L-PHA reactivity [50].

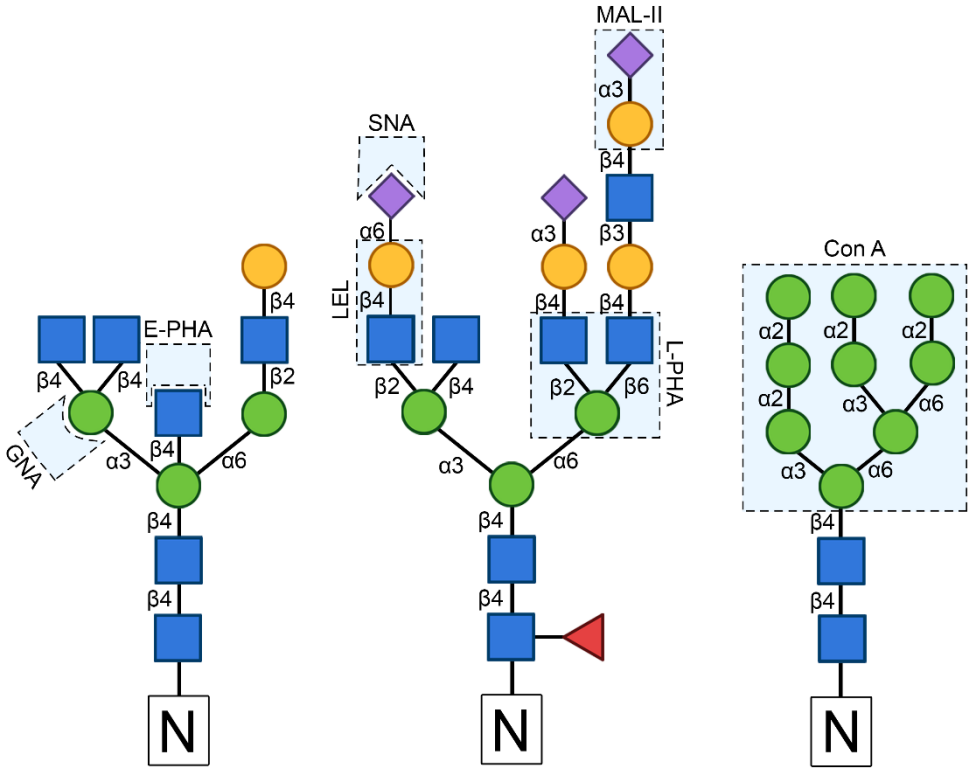

Figure 10 *- Binding site of lectins to glycans. Adapted from [50, 86,87].*

Alterations in cellular glycosylation are a hallmark of cancer progression [38, 41, 64]. Increase in complex *N*-glycans, sialylated structures and *O*-glycans levels, that can be observed by lectin assays [83, 84], are associated with pro-tumoral activity [38, 39, 48, 63, 66]. This alterations in glycosylation can be observed in different cancer types, for example hematological malignancies [88].

1.6 Leukemia

1.6.1 Carcinogenesis

Cancer is characterized by mutations in DNA that change tissue homeostasis [4]. These mutations can occur in oncogenes, that are involved in cell growth, or in tumor suppression genes, that decreases cell growth [1], and are related to cancer survival, proliferation, and growth [4].

[1]. The tumor microenvironment includes immune cells, innate and adaptive, cancer cells, and stroma [6]. The interaction of cancer cells with the immune system is an important step for tumor progression, [1]. During immunosurveillance, the immune system is often efficient in eliminating the cancer cells [89, 90]. The immunoediting is divided into three phases. The **elimination** phase corresponds to the effectiveness of the immune system to eliminate cancer cells, although some cells can resist to the immune response while other are eliminated. The **equilibrium** is a stage where the tumor is clinically dormant [89, 90]. The last phase is known as **escape**, where tumor cells are able to evade immunologic recognition and attack, which enable tumor growth and metastasis [89, 90].

Like the carcinogenesis, the hematological cancers have a cellular growth unregulated. Hematological cancer can be divided in leukemias (malignant transformation in hematopoietic cells in bone marrow), myelomas (malignant transformation in plasma cells) and lymphomas (solid tumors characterized of masses of lymphocytes). Leukemia can be originated by genetic mutations, environment factors, among others. Being important genetic mutations in cancer progression, the affected genes control cellular growth and apoptosis conferring the malignant cells phenotype.

The leukemia can affect lymphoblastic or myeloid, according with lineage cell [93, 94], being that inside of lymphoblastic leukemias affects B or T-cells [95].

1.6.2 T-cell acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is an aggressive hematological cancer that affects B or T–cells (B-ALL and T-ALL, respectively) [95]. Being a common cancer in pediatric ages [29, 95, 96], the B-ALL represents 85% [95, 97] and T-ALL corresponds to 15% of all ALL cases [27, 95, 97]. In adults it corresponds to 25% [27]. With a rate of relapse of about 50% of adult cases and 30% of pediatric cases [27], this pathology's biggest challenge is relapse and treatment [34, 95], as current treatments are very aggressive [34], being considered a disease with poor prognostic characteristics [97]. There are different mechanisms that lead to T-ALL, namely mechanisms that alter the IL7R / JAK / STAT signaling [27, 32, 95], TEL / JAK [98], and cell competition in the thymus [96]. However,

there are, also, some mutations very common in T-ALL, such as NOTCH 1, that have influence on IL-7Rα [29]

The cell competition model defends that malignant transformation in T–cells occurs in the DN2 and DN3 stages, when self-renewal happens, and the progression of the T-ALL is based on IL-7 competition [96]. This competition for cytokines and growth factors is important for cell homeostasis and can promote thymus autonomy and T–ALL development [99], conferring an advantage to tumor cells compared with normal ones [12, 96, 99]. The selection occurs in the thymic cortex, at the DN3 stage, and can be divided into two steps: thymus autonomy and cell proliferation control [99]. The hyperactivation of IL-7 signaling pathway can be founded in 70% of T–ALL patients [21, 27]. This oncogenic phenotype is due mutations/alterations in the signaling pathway, that render the signaling cascade to be constitutively active [12, 29]. IL-7R mutations that generate IL-7-independent signaling can be founded in 10% pediatric patients [27] with poor prognosis in relapse [12, 27]. These mutations activate constitutively the IL–7 signaling [21] promoting cell transformation, and leukemogenesis [21, 27]. With IL–7 signaling activated, the PI3K [21, 22] and STAT5 are activated and promote T–ALL [21]. Activation of the PI3K is important for cell proliferation and survival [12, 21, 32], as well as cell cycle progression [12]. On the other hand, STAT5 phosphorylation favors cell growth and progression of the cell cycle [21]. Adding to these factors, the up-regulation of the Bcl-2 promoted by IL–7 in the thymus [12, 22] is dependent of the STAT5 phosphorylation [12], forming the perfect environment for the T–ALL development, leading to cell proliferation and an antiapoptotic effect in cells of this neoplasia [22, 29]. In T–ALL cells, the IL–7R expression is maintained functional [34]. In fact, in a T-ALL-IL-7-dependent cell Line (TAIL7), IL–7 signaling promotes RB phosphorylation and JAK / STAT activation [34].

The TEL / ETV6 – JAK2 is a gene fusion that is involved in different types of leukemia, such as T-ALL [98]. This mutation is neoplastic because it causes the DN thymocytes to become independent of the IL-7 signaling, promoting their survival and proliferation, and associated with pre-TCR signaling leads to leukemogenesis [98]. In this leukemic model, the TEL-JAK2 leukemic cells active constitutively the STAT5 signaling that contributes for CD8 T-cells differentiation independent of TCR or pre-TCR presence [98]. The low levels of TCRβ and high levels of CD24 indicate an arrest of the cell cycle in an immature stage ($CD8^+$ ISP), transitioning to DP and CD8 SP stages [98].

1.6.3 The impact of Glycosylation alterations in Leukemia

Result of maladjusted proliferation of neoplastic cells [97], leukemia is a hematological malignancy that can be acute or chronic, can affect the myeloid or

lymphoblastic lineage [93, 94], being a common cancer in childhood [97]. Regarding infant leukemias, the ALL is more common than acute myeloid leukemia (AML)

The impact of carbohydrates has been studied for some leukemic subtypes. Leukemia can be associated with loss of the Ikaros factor [100], which represses the alpha-1,3-fucosyltransferase IV (FUT4), an enzyme that promotes FAK signaling which is important in cancer progression [100].

MUC1, a mucin widely expressed in epithelial tissues, has also been observed in several hematological cancers such as adult T-cell leukemia, chronic lymphocytic leukemia (CLL), ALL, and AML [101]. Glycosylation of the MUC1 is different between normal tissues and neoplastic cells, leading to differentiation, proliferation, invasion, and metastasis of the cancer cells [102]. This protein is composed of domains, MUC1-N and MUC1-C, being the latter involved in the uptake of glucose, and promoting tumor progression, such as of chronic myeloid leukemia (CML) and AML [101].

The enzyme codified by the *MGAT3* glycogene (GnT-III), in blasts of CML cells can be considered a cancer biomarker and GlcNAc bisecting *N*-glycans can be found in the CD45 receptor [103]. However, the GnT-III activity is different between patients with CML. In fact, some patients that have Philadelphia chromosome (typical of this pathology) show a decrease in the activity of this enzyme, while other patients present an increase [103].

Beyond CD45 receptor, the CD43 also is important in leukemic cells. The leukemic cells of the MLL/AF9 cell line express in their surface the CD43 receptor *O*-glycosylated, however the terminal sialic is different between these cells [104]. This difference indicate that sialylation levels can be responsible for different responses of these leukemic cells to the cytotoxic T cells [104]. Another important receptor in malignant cells is the CD147, that is overexpressed in these cells and, when associated with low levels of N-glycans, promote drug resistance [105].

In the human acute T-cell leukemia cell line, Jurkat cells, CD43 and CD45 express high levels of Tn antigens, which is characteristic of different cancer types, including leukemia [106]. This termination can be found in O-glycans branching and interacts with macrophage galactose-type lectin (MGL), a C-type lectin present in antigen-presenting cells (APC) surface [67, 106]. This leads to the repression of the T-cell activation mediated by TCR, promoting an anti-inflammatory response, fewer T-cells proliferation, and death of the Jurkat cells [106]. Not only the Tn antigen but also the T antigen can be found in precancerous states and several tumor types. In fact, in epithelial cancers it can be present in about 50% of the T-cell acute lymphoblastic leukemia (T-ALL) cases. Moreover, this antigen shows an important interaction with Gal – 3, promoting the metastasis and adaptative immune response modulation and can be founded in a human AML cell line, KG-1 [67]. With a similar biosynthesis pathway, the sTn antigens can also be over-expressed

in tumors cells [67]. This phenotype can be associated with ST6GalNAc-I overexpression, being involved in cell-cell and cell-matrix interactions and lectin modulation, leading to cancer progression through PI3k/Akt signaling activation [67].

The neoantigen biosynthesis in cancer cells [41] and the alteration in glycosylation patterns [107] can be a good cancer hallmark [41, 107], because are exclusive of the neoplastic cells, which make these neoantigens an excellent target for immunotherapies [108]. One example of neoantigen biosynthesis where glycan play a role is on the O-GlcNAcylation of MHC-linked peptides which promote a specific T-cell response, in mice [108].

Glycan-binding proteins, such as galectins, have an important role in hematological malignancies and can be found in lymphomas of B and T cells [109]. Leukemic cells may take advantage of anti-apoptotic activity of galectins, Gal-3 and Gal-7, which can interact with Bcl-2 and promote its activity [109]. These galectins have been found in patients with CLL, which may be a factor to evaluate the prognosis of this pathology [109].

Leukemia, namely **T-ALL**, is a product of different oncogenic mechanisms such as mutations and extracellular stimuli misregulation. As in solid tumors, alterations in glycosylation may as well be a transformation promoting factor. Alteration in enzyme activity and differences in glycosylation patterns may lead to novel cellular functions, related to the disease, posing as promising therapeutic targets. As it has been done in other disease contexts (such as IBD [50] and MS [35]), the knowledge of T-cell glycosylation has given rise to novel therapeutic strategies, based on the glyco-reprogramming of those cells. Inspired by such work, the present thesis addressed the questions regarding T-ALL glycoprofile existance and function, and the therapeutic potential of glyco-reprogramming of these cells.

Chapter Two

Aims

In this chapter, the general goal of this work will be revealed. Therefore, specific milestones were set, so that all which was envisioned for this thesis. Would be successfully achieved.

Glycosylation is a major post-translational modification essential for both normal cellular function and for malignant transformation. Its role in T-ALL leukemogenesis is far from being understood, although representing a major layer of regulation of T-cell function. The hypothesis of the existence of specific glycan signatures in leukemia and that these cells react to metabolic supplementation with glycans generated a thesis project that aimed to fill missing gaps in knowledge of T-ALL leukemogenesis.

General aim

To investigate (novel) glycofeatures of T-ALL and their therapeutic potential

Specific aims

1. To characterize the glycosylation profile of different leukemia cell lines/models

2. To determine the glycosylation dynamics associated with leukemic cellular activation in vitro

3. To modulate the glycosylation pathway in leukemic cell lines, evaluating its impact in cell death

4. To evaluate the impact of the presence of complex N-glycans in leukemic glycoprofile and mouse survival in a leukemic in vivo model

Chapter Three

Material and Methods

Throughout this chapter, all the experimental procedures that made it possible to achieve our goals will be covered. As well as all the materials needed for experimental tests as well as all protocols.

3.1 Cell Lines

The Jurkat cell line is derived from a human T-cell acute lymphoblastic leukemia, isolated in 1977, that show a deficiency in TCR signaling, making it a good cell line for studying T cell biology [110].

These cells grow in suspension in cell culture flasks. Cells were maintained at 37 °C in an atmosphere of 5% CO_2, in a complete medium composed by RPMI 1640 Medium (Gibco, ThermoFisher) with 10% of Fetal Bovine Serum (FBS) and 1% of Pen-Strep. The cells had their means changed every three days.

The second cell line that was used was the T-cell Acute-Leukemia IL-7- dependent cell line (TAIL-7) that was obtained from peripheral blood of the 7-years-old patient, being characterized by an immature phenotype. These cells was maintained in RPMI 1640 with 10% of FBS and recombinant IL-7 [34]. This cellular line was maintained at Instituto of Molecular Medicine, University of Lisbon Medical School, in Doctor João Barata´s group, that kindly gave us RNA samples and protein extracts.

3.2 Flow Cytometry

Fluorescence-activated cell sorting (FACS) can separate the cells into subgroups. The cells are labeled with fluorochromes conjugated to antibodies that subsequently allow the separation of cells.

The suspended cells were collected and centrifuged at 300g for 5 minutes at 4ºC subsequently washed in FACS buffer (Phosphate-Buffered Saline (PBS)1X with 1%FBS) and centrifuged. Subsequently, the cells subject to marking for cell death with Fixable Viability Dye APC-Cy7 (FVD 1:2000, ThermoFisher, Walthan, MA) for 20 minutes at 4ºC. After incubation, the samples were washed and centrifuged and incubated with primary antibody (L-PHA, GNA, SNA, LEL, E-PHA, ConA, MAL-II (1:1000, Vector)) for 30 minutes at 4ºC. Subsequently, the cells were washed and incubated with the secondary antibody (Streptavidin 1:200, ThermoFisher) diluted in FACS Buffer for 20 minutes on ice. The protocol continues with cells washed and the fixation of cells in paraformaldehyde (PFA) 2%, for 30 minutes at room temperature (RT) and in the end, cells were washed in Saponin 0,5% (Sigma-Aldrich), diluted in PBS 1X. The next step was the permeabilization with Saponin 0,5% for 10 minutes at RT, then there was incubation with *O*-GlcNAc (1:200, clone RL2 (APC)), diluted in Saponin 0,5%, for 30 minutes on ice, and washed in FACS buffer and resuspended in the same solution. When staining is realized with lectins and antibodies, after lectin incubation, cells were washed and incubated with antibodies (CD24 (1:800, eBioscience), CD25 (1:300, eBioscience), CD8 (1:500, eBioscience), CD4 (1:300,

eBioscience), CD3 (1:200, eBioscience)) for 30 minutes in ice and after this were washed and incubated with secondary antibody. In the end cells were washed, resuspended in FACS buffer and analyzed.

For the apoptosis analyzes, after the cells were washed one time in PBS1X, cells were washed a second time in Binding Buffer 1X, after centrifugation the cells were resuspended in Binding Buffer 1X. Later were added Annexin (Biolenged (APC)) for 10 minutes and added propidium iodide (PI, eBioscience (PE)). In the end, cells were analyzed.

The analysis of the staining was realized in FACS Canto II (BD Bioscience), using the FACSDiva software (BD Bioscience). This analysis allows separated cells in subgroups according to the fluorescence emission. Cells marked for the APC-Cy7 were detected using a 633nm laser and the 780/60 filter, for APC using the same laser but 660/20 filters, for PE and FITC using 488nm laser but different filters being them 585/42 and 530/30, respectively. The data were analyzed in FlowJo_V10 software. The statistical analyses were realized in GraphPad Prism 7 software (GraphPad Software), where results were considered statistically significant with $p<0.05$.

3.3 Western Blot

The western blot is a technique for protein detection through gel electrophoresis, using the protein extracts. The protein extracts were obtained smoothing the cells with RIPA Buffer, previously washed in PBS 1X, and were quantified by the method DC protein assay. The samples used in this protocol had a final volume of 30µL and were composed of 15µg of protein extract, and load buffer (Bio-Rad) which constitutes 1/3 of the final sample volume. The load cap was composed of 3x LB and 1/10 DTT. The samples were heated for 5 minutes at 95ºC, to denature the proteins.

For this technique are used two gels of acrylamide, the running gel (7.5% for lectins or 12% for other antibodies) and the stacking gel (4%). The samples were loaded and ran in Tris/Glycine/SDS Buffer (Bio-Rad), for 1 hour at 130 volts. Proteins were transferred from the gel to a nylon membrane (Amersham, GE Healthcare), in Tris/Glycine Buffer (Bio-Rad), for 1 hour at 330mA, using Mini-PROTEAN®Tetra Vertical Electrophoresis Cell (Bio-Rad). After this, the membrane was colored with Pônceau, cut, and washed in distilled water very well.

The membrane was blocked in Tris-Buffered Saline (TBS) with 0.1% TWEEN (TBS-T) containing 5% milk with overnight incubation, for antibodies or in PBS with 0.05% TWEEN (PBS-T) containing 5% Bovine Serum Albumin (BSA, Sigma) with 1 hour of the incubation, for lectins, under agitation. For *O*-GlcNAC detection, an antibody, the block

solution was 3% BSA in TBS-T. The next step was the membrane incubation with the first antibody, that can be antibodies (β-actin (Santa Cruz); *O*-GlcNAc (SIGMA), both to 1:1000) or lectins (L-PHA (Vector) at 1:1000), that were diluted in block solution and incubated for 1 hour and overnight, respectively, under agitation. After de incubation for the primary antibody, the membrane was washed in TBS-T (antibodies) or PBS-T (lectins), for 30 minutes, changing the solution from 10 to 10 minutes, and incubated with the second antibody diluted in TBS-T (antibodies) or PBS-T (lectins) for 1 hour, under agitation and washed the membranes. In case of *O*-GlcNAc is used Anti-Mouse Igs HRP at 1:2000 (DAKO), for Actin is incubated with Anti-Mouse IgG HRP at 1:2000 (GE Healthcare) and for lectins is used Streptavidina HRP at 1:5000.

The signaling was revealed with ECL Western Blotting detection reagents and ECL Prime Western Blotting detection reagents (Amersham, GE Healthcare) and chemiluminescence films (Amersham ECL, GE Healthcare), exposed in a Hypercassette (Amersham Bioscience) and revealed.

3.4 cDNA synthesis and RT-PCR

The Real-Time Polimerase chain Reaction (RT-PCR) allow evaluate any gene expression. For this procedure is necessary DNA, that can be synthetized from quantificated RNA samples, obtained complementary DNA (cDNA) using SuperScript™ IV Reverse Transcriptase (Invitrogen, CA).

The previous RNA extraction it is performed by TRI Reagent (Sigma-Aldrich) and with manufacturer's instructions.

The RT-PCR using cDNA for amplified gene by TaqMan assays: *MGAT5* (Hs00159136), *OGT* (Hs00269228_m1) and *18s*(Hs.PT.39a.22214856). The amplification was detected by 7500 Fast - Real time PCR system (Applied BiosystemsTM).

Chapter Four

Results

In this chapter, all the experiments will be detailed and the results obtained will be presented.

4.1 Leukemia cells Display an Altered Glycoprofile When Compared To Healthy Counterparts

The phenotyping of leukemic cells provides useful information for the design of novel therapies and to the unraveling of malignant pathways [111]. As it was mentioned in the Introduction section, the study of the glycoprofile of leukemic research models or patient samples is still missing in the literature. In this first section of the Results chapter the main focus was to characterize the glycoprofile of leukemic cells, from different origins, by lectin-based flow cytometry and glycogene expression by RT-PCR. Cell surface glycoprofile was done using the following lectins for the respective glycans: L-PHA for β1,6-GlcNAc branched, LEL for polylactosamine (poly-LacNAc), E-PHA for bisecting-GlcNAc, SNA for α2,6-Neu5Ac, GNA for α1,3-Man, MAL-II for α2,3-Neu5Ac and ConA for glycans (Figure 10 of the Introduction). In order to study the presence of the intracellular glycan *O*-GlcNAc, a monoclonal antibody against this post-translational modification was used [112]. The glycoprofiling of a human T-ALL cell line, Jurkat, and also healthy T cells, isolated from healthy Peripheral blood mononuclear cells (PBMCs), was done.

The results showed that Jurkat cells display a distinct glycoprofile when compared to their healthy counterparts (**Figure 11**). Regarding specific glycan expression in Jurkat cells, given by lectin binding, it was observed a statistically significant increase in α1,3-Man (by 3-fold), GlcNAc-bisecting *N*-glycans (by 10-fold), β1,6-GlcNAc branched *N*-glycans (by 2-fold), poly-LacNAc structures (by 3-fold) and *O*-GlcNAc (by 15-fold). Regarding carbohydrate terminal sialylation, Jurkat cells display a statistically significant decrease in

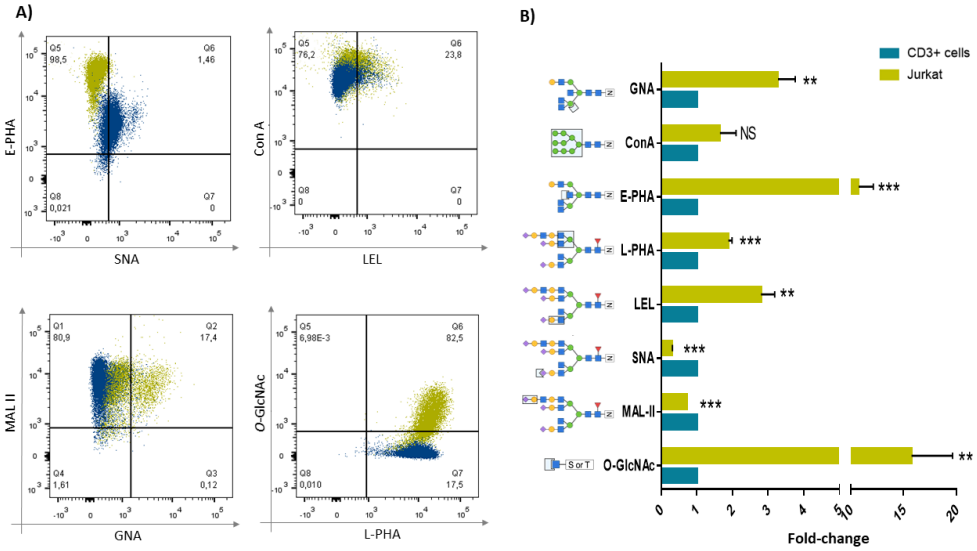

Figure 11 *- Jurkat cells display a specific glycoprofile. (A) Plots of different glycan expression, given lectin/antibody binding. (B) Mean Fluorescence Intensity (MFI) values of different glycan expression, normalized to the healthy CD3+ cells (n=3). * p-value < 0.05, ** < 0.005 and *** < 0.0005.*

levels of about 0.69-fold and 0.28-fold of α2,6-Neu5Ac and α2,3-Neu5Ac structures, respectively (**Figure 11B**).

The expression profile of glycans can be related to several factors that influence the glycosylation pathway, including the levels of glycogene expression; the substrate availability, ER and Golgi pH, among others [36]. The *MGAT5* and *OGT* glycogenes have been demonstrated to be involved in T cell function and cancer [38] and their expression was evaluated by RT-PCR using different patient-derived leukemic cells: Cutaneous T-cell lymphoma / Sézary Syndrome (samples 110/60 and 106/51), Large granular T lymphocyte leukemia (samples 109/22 and 110/8), Prolymphocytic T leukemia (samples 39 and 45) – and a T-ALL cell line (Jurkat cells). It was also included in this analysis a healthy patient (Ly) **(Figure 12)**. *MGAT5* expression was found to be significantly increased in Sézary Syndrome and decreased in one patient with Large granular T lymphocyte leukemia. In the healthy donor sample *OGT* was not detected (nd) as well as in one patient with Large granular T lymphocyte leukemia and one patient with Prolymphocytic T leukemia. In all other leukemic samples *OGT* expression has found to be significantly increased.

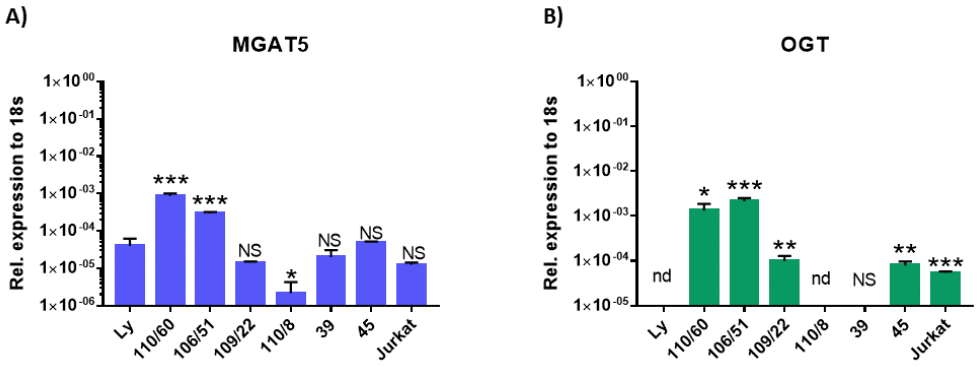

Figure 12 *- Relative exprexion to 18s of different types of Human T-cell leukemia show disease-specific glycogene expression. (A)* MGAT5 *gene expression. (B)* OGT *gene expression. Ly- healthy donor; 110/60 and 106/51- Cutaneous T-cell lymphoma / Sézary Syndrome; 109/22 and 110/8- Large granular T lymphocyte leukemia; 39 and 45- Prolymphocytic T leukemia; and Jurkat cells- T-ALL. For each sample a total of 3 technical replicates were done. * p-value < 0.05, ** < 0.005 and *** < 0.0005.*

To gain further insights on T-ALL distinct glycosignature, given by the glycan expression of Jurkat cells, the glycoprofiling of an *in vivo* model of this type of leukemia was done. The spontaneous T-ALL model that was studied here derives from the TEL-JAK2 chromossomal fusion expression in murine DN cells [98, 113]. The transformed thymocytes express both CD8 and CD25, and may express CD4, as they sit at the ISP-DP-CD8SP transition [98]. Our results showed that TEL-JAK2 cells display a significant 0.25-fold increase in β1,6-GlcNAc branching (given by higher L-PHA binding) and 0.82-fold decrease in poly-LacNAc strutures (LEL binding) when compared to their healthy counter-parts (immature single positive CD8 cells), (**Figure 13**).

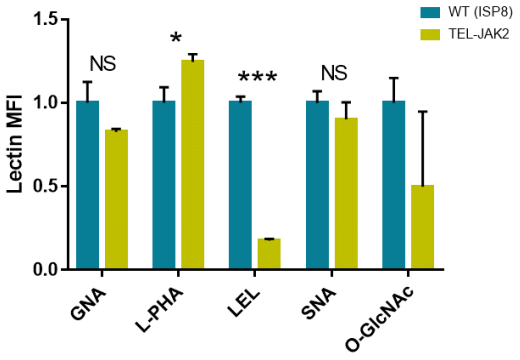

Figure 13 *- TEL-JAK2 glycoprofiling reveals acquisition of distinct profile upon malignant transformation. Used for analysis a n=3 for GNA, L-PHA, LEL and SNA and n=2 for O-GlcNAc). * p-value < 0.05, ** < 0.005 and *** < 0.0005.*

Collectively, these results reveal another layer of complexity in the study of T-ALL, which is the cellular glycosylation profile. Moreover, it is clear that stable leukemic cells reveal a malignant specific glycosignature, characterized by glycogene expression and glycan presence.

4.2 Glycoprofile Acompanies Leukemic Progression

Glycans play a key role in essentially all T cells functions [11] namely during development, migration and activation. As it was observed in the previous section, malignant T-cells express a distinct glycosignature, that may have been established upon malignant transformation and cell proliferation. Therefore, the study of the glycan expression profile upon the oncogenic hit should provide important information in the research on T-ALL.

As it was mentioned in the Introduction section, TAIL7 cells are dependent on IL-7 signaling for leukemic progression. Upon exposure of IL-7, these cells begin to proliferate and display oncogenic features *in vitro* and *in vivo* [34]. Using these leukemic cells as a model of malignant transformation/activation, the expression of two key glycogenes were evaluated, *MGAT5* and *OGT*, as well as their associated glycans, β1,6-GlcNAc branching and O-GlcNAcylation (**Figure 14**). The main conditions set were TAIL7 with or without IL-7 in the culture medium, for 2, 8, 24 and 48 hours. Our results showed that IL-7 significantly promotes *MGAT5* gene expression upon 2 hours of stimulation, stabilizing at 24 hours of incubation (**Figure 14**). This increase in *MGAT5* expression is translated in an increase of L-PHA binding levels of protein extracts, when cells are treated with IL-7 at 24 hours, being less evident upon 48 hours of incubation (**Figure 14A top and 14B right**). The expression of *OGT* was also increased, but to a lesser extent than *MGAT5*, following a 24 hours culture

in the presence of IL-7, which was also confirmed by O-GlcNAc levels, and again fading by 48 hours of culture (**Figure 14A bottom and 14B left**).

Altogether, the results suggest that a shift for leukemic behavior, enabled by the presence of IL-7 (in this model), appears to drive the β1,6-GlcNAc branching and *O*-GlcNAc glycans modulation.

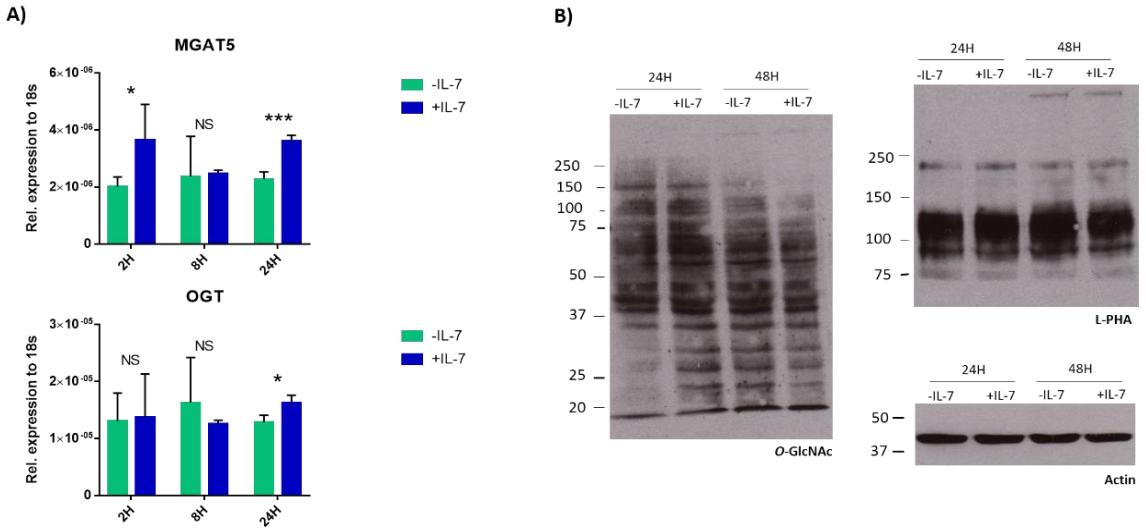

Figure 14 *– Glycan expression changes in IL-7-stimulated TAIL-7 cells. (A) Relative expression to 18s of* MGAT5 *(top) and* OGT *(bottom). (B) Westerns Blots analysis of glycan expression:* O-GlcNAc *(left) and ß1,6-GlcNAc branching (right) and Actin, as a loading control (right, bottom). * p-value < 0.05, ** < 0.005 and *** < 0.0005.*

4.3 Glycoprogramming Of Leukemic Cells: impact in cell viability

Taking into consideration that both *MGAT5* and *OGT* expression were increased in leukemic cells during proliferation, it may indicate an important role of both *N*-glycosylation and *O*-GlcNAcylation pathways in disease progression. As both share the same substrate, GlcNAc, which intracellular presence is highly regulated, it was hypothesized that metabolic supplementation of this monosaccharide could drive a misregulation between its availability to GnT-V (protein coded by *MGAT5*) and OGT, influencing leukemic progression.

In order to glycoprogramme T-ALL cells, different GlcNAc concentrations were added to the Jurkat cell line and the levels of expression of both β1,6-GlcNAc branching *N*-

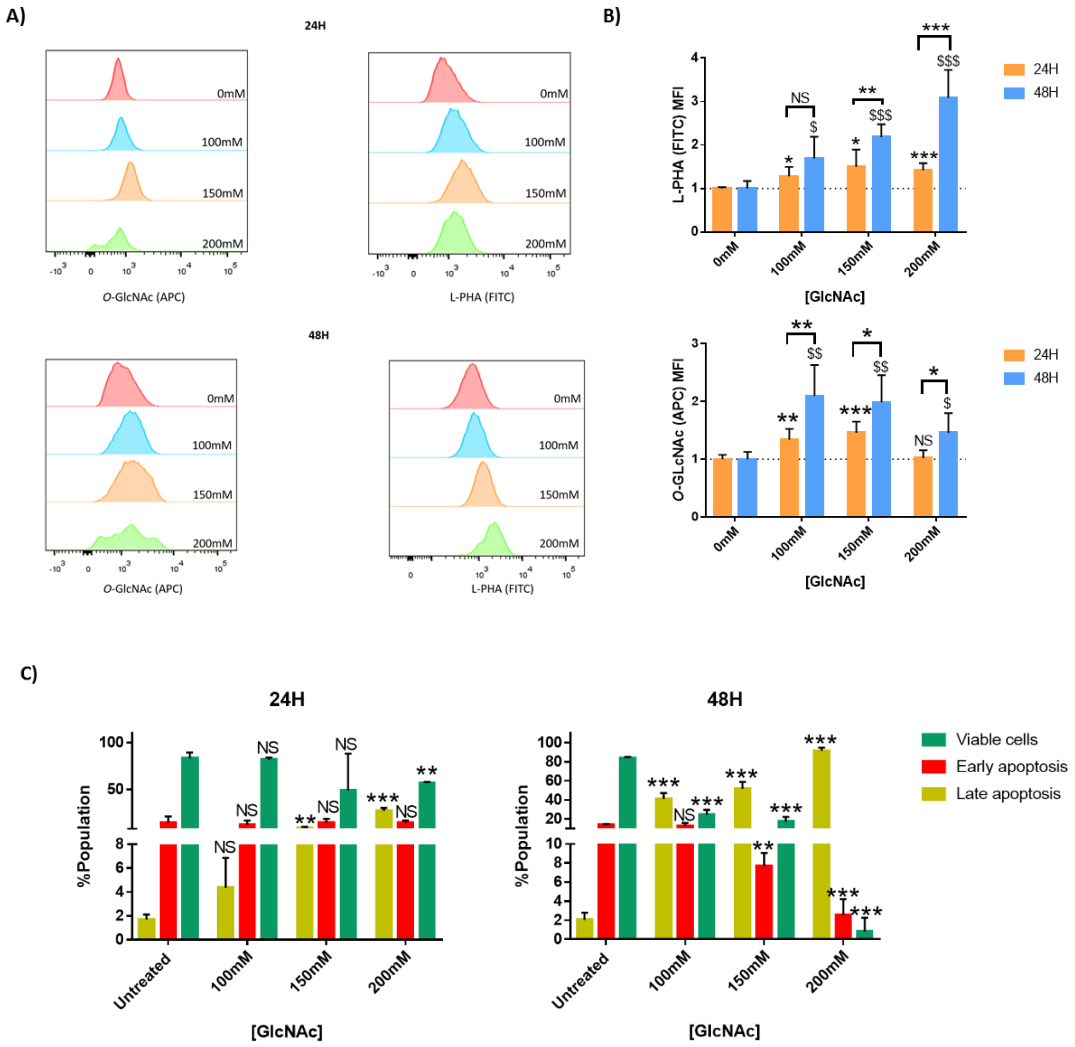

Figure 15 *- Influence of different GlcNAc concentrations in glycan expression and cellular viability. (A) Histograms of lectins expression (on) 24H and (below)48H. (B) MFI of Lectins expression (on) L-PHA and (below) O-GlcNAc. (C) Apoptosis analysis (left) 24H and (right) 48H. * p-value < 0.05, ** < 0.005 and *** < 0.0005.*

glycans and *O*-GlcNAc were evaluated in two different timepoints (24 and 48 hours) (**Figure 15**). The results showed that branched *N*-glycans significantly increase with GlcNAc concentration and exposure time, suggesting a dose dependent response in terms of GnT-V activity, according to previous studies done by the group [50] (**Figure 15B**). On the other hand, *O*-GlcNAc levels showed a peak of increased expression with 100mM of GlcNAc supplementation, being also dependent on exposure time (**Figure 15B**). Interestingly, when apoptosis rates were evaluated in the same conditions, it was observed that Jurkat cells are susceptible to GlcNAc presence in a dose dependent manner with a significant increase in apoptosis rate when cells were supplemented with GlcNAc, in a dose dependent manner (**Figure 15C**). GlcNAc supplementation reveals more toxicity when cells are exposed for longer times, which can be observed by the increase in apoptosis levels from 24 to 48 hours.

To gain more insights on the mechanism driving increased apoptosis of Jurkat cells in the presence of GlcNAc, the pathways of *N*-glycosylation and *O*-GlcNAcylation were chemically inhibited.

Jurkat cells were treated with 10 μM Kifunensine (KF, Sigma-Aldrich), which inhibits α-Mannosidase I, blocking *N*-glycosylation, and 100 mM of GlcNAc, to understand how the increase of β1,6-GlcNAc branching influenced cell viability. The KF alone and KF + GlcNAc treated cells show a drastic decrease in β1,6-GlcNAc branched *N*-glycans levels, accompanied by an increase of *O*-GlcNAc levels. When cells are only treated with GlcNAc, β1,6-GlcNAc branched *N*-Glycans (L-PHA levels) and *O*-GlcNAc levels are increased, when compared to untreated cells (**Figure 16A**). When apoptosis was evaluated, an increase in apoptotic cells, namely in early apoptosis, was observed in both GlcNAc and GlcNAc + KF conditions in the 24 hours timepoint, being only present for the GlcNAc supplementation at 48 hours (**Figure 16B**).

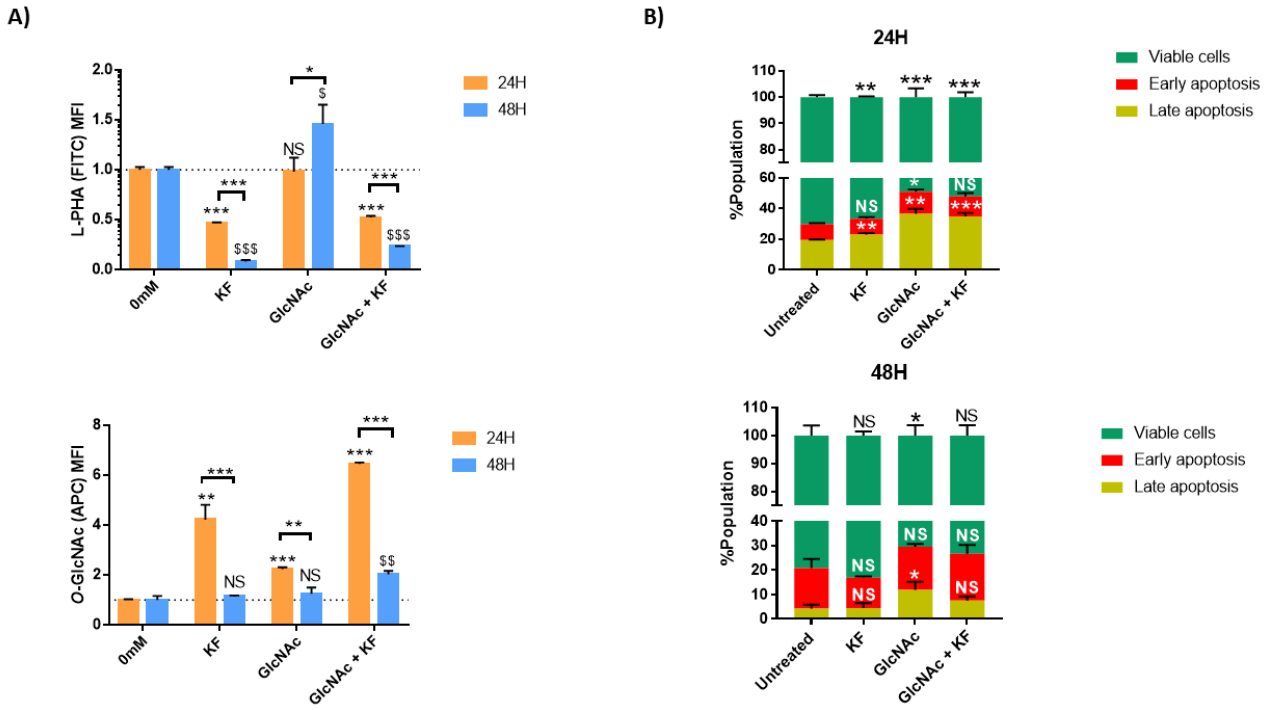

Figure 16 *– Modulation of N-glycosylation pathway in Jurkat cells. (A) MFI of glycan expression at 24 and 48 hours after treatment with KF (top) L-PHA and (bottom) O-GlcNAc. (B) Apoptosis levels, in cells treated with KF, at (top) 24 hours and (bottom) 48 hours. * p-value < 0.05, ** < 0.005 and *** < 0.0005.*

As cellular apoptosis was similar in cells treated with GlcNAc alone or with GlcNAc + KF, it was hypothesized that *O*-GlcNAcylation may be the driving factor of cellular death, as the only consumption of supplemented GlcNAc is done by OGT-mediating O-GlcNAcylation. The next step of this study was the incubation of the Jurkat cells with 50μM Osmi-1 (an OGT inhibitor, [114], Sigma-Aldrich), for 6 hours (as increased times of incubations drove cellular death due to solvent toxicity). In this experiment it was possible to observe that *O*-GlcNAc levels decrease when cells are incubated with Osmi-1 alone and

Osmi-1 + GlcNAc, although no major differences were observed in β1,6-GlcNAc branched *N*-glycan levels (**Figure 17**).

Regarding cellular apoptosis, when cells were incubated with Osmi-1 + GlcNAc (showing reduced O-GlcNAcylation), they showed a decreased mortality when compared with cells supplemented with GlcNAc alone (with increased branching glycosylation and O-GlcNAcykation). Moreover, when compared with untreated cells the viability remains unaltered.

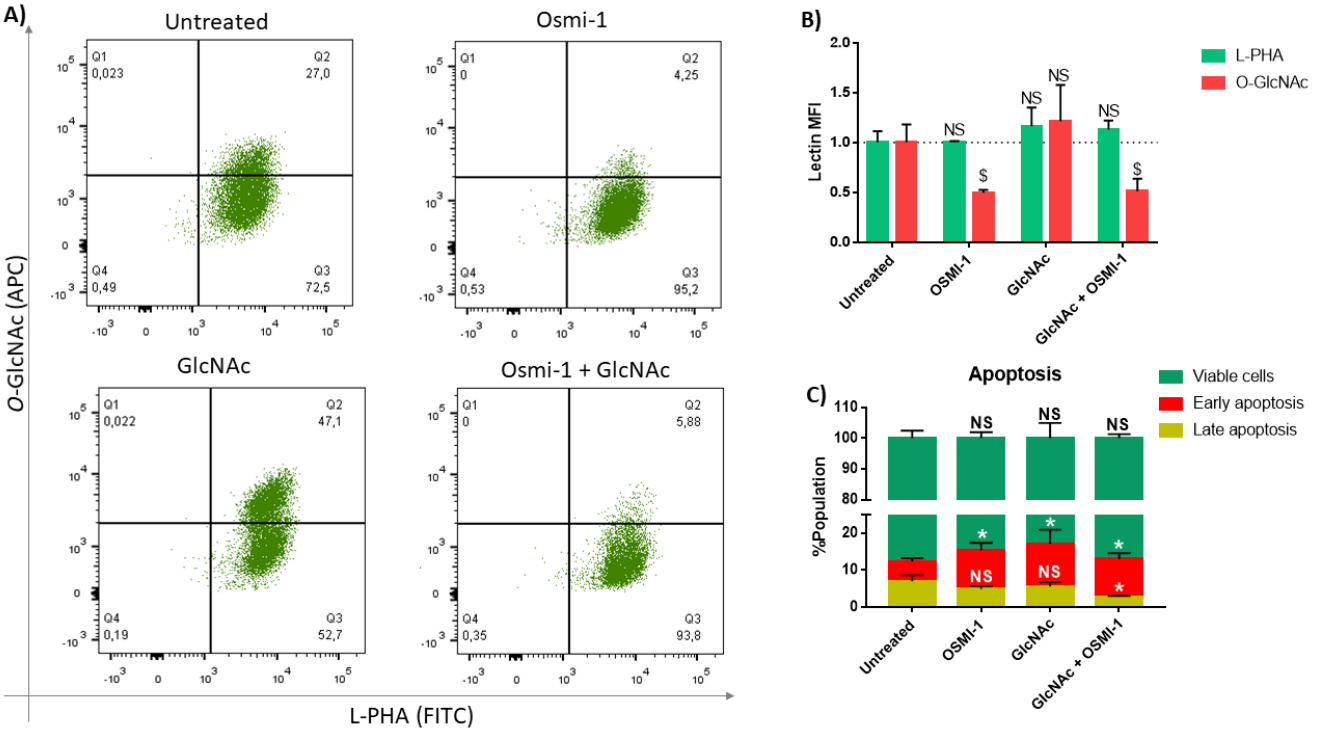

Figure 17*-Modulation of O-GlcNAcylation pathway in Jurkat cells. (A) Plots with lectins expression in Jurkat cells treated with Osmi-1, for 6 hours. (B) Lectin MFI. (C) Apoptosis levels of Jurkat cells with Osmi-1. * p-value < 0.05, ** < 0.005 and *** < 0.0005*

The previous findings showed that an increase O-GlcNAcylation is related to apoptosis induction. To further validate this relationship, GlcNAc supplementation and KF treatment of Jurkat cells was done for 24 hours, following Osmi-1 incubation for 6 hours. Glycans expression was evaluated and the major effects seen were regarding the initial 24 hours culture period (KF or GlcNAc) . As expected, a decrease in β1,6-GlcNAc branched *N*-glycans was observed in cells treated with KF and in *O*-GlcNAcylation in cells treated with Osmi-1. This latter observation was not observed in cells treated with Osmi-1 that were beforehand supplemented with GlcNAc. As it was seen before, GlcNAc + KF treatment resulted in the highest O-GlcNAc presence, with or without Osmi-1 **(Figure 18A and 18B)**. When apoptosis induction was measured, the previous results were validated, GlcNAc treatment only increases apoptotic cells when compared to its control condition when it is supplemented alone, or together with KF, and not with OSMI. Interestingly, the combination

of all treatments resulted in increased presence of late apoptotic cells, when compared to the KF + OSMI condition **(Figure18C)**.

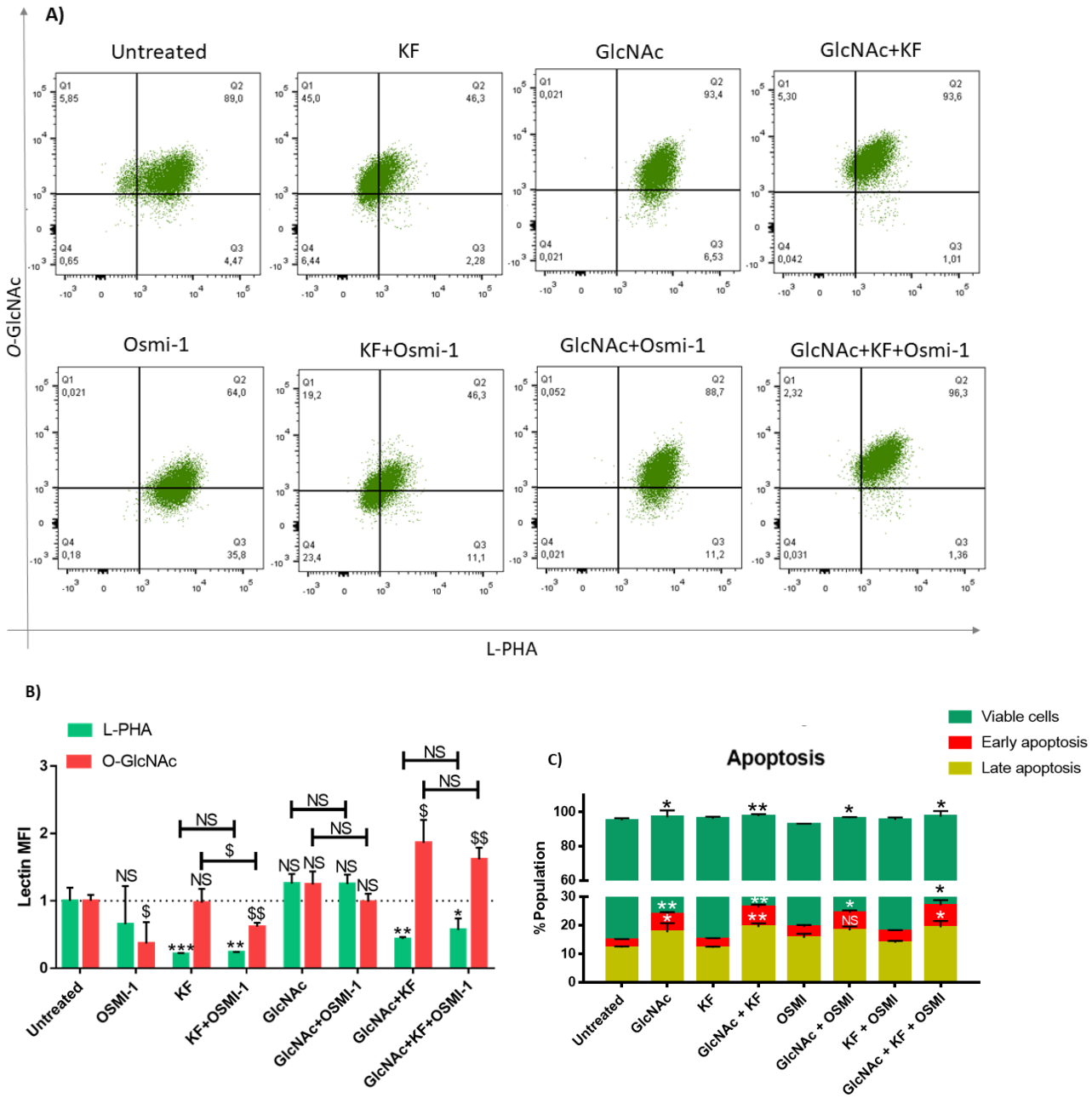

Figure 18 *- Glycomodulation of Jurkat cells with KF and Osmi-1. (A) Plots of the lectins expression in different conditions. (B) MFI of lectins expression. (C) Apoptosis levels of Jurkat cells with Osmi-1, GlcNAc and KF. * p-value < 0.05, ** < 0.005 and *** < 0.0005*

The results point towards the fact that a glycoprogramming of Jurkat cells have a role in the modulation of apoptosis of leukemic cells, revealing potential new avenues in leukemia treatment. To detect the influence of glycans, more specifically of β1,6-GlcNAc branched N-glycans, TEL-JAK2 (TJ2) mice were crossed with *Mgat5*$^{-/-}$ ones, generating

three different genotypes: TJ2*Mgat5*$^{+/+}$, TJ2*Mgat5*$^{+/-}$ and TJ2*Mgat5*$^{-/-}$. As it was already mentioned, this murine model enables the spontaneous T-ALL generation. Upon symptoms associated with leukemia detection, related with humane experimental endpoints, such as weight loss, breathing struggle or lack of movement or response, mice were euthanized and leukemias were phenotyped, according to T-cell markers and glycoprofile. As it was described, TJ2*Mgat5*$^{+/+}$-derived leukemic cells express high levels of CD8, CD25 and CD24 [98] **(Figure 19)**. Interestingly, the initial analysis of leukemias revealed aberrant expression of these markers **(Figure 19)**.

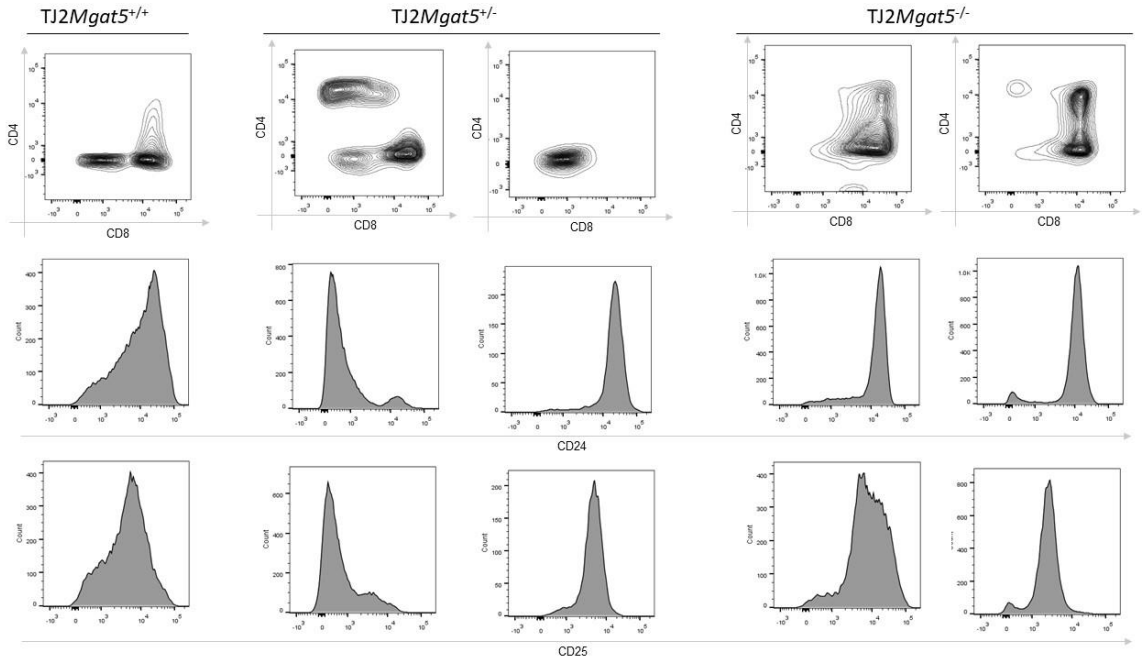

Figure 19 *- Immunophenotyping of TJ2 leukemias upon Mgat5 deletion. Representative plots are shown for each genotype.*

The different genotypes of TJ2*Mgat5* crossings show distinct glycoprofiles and overall survival **(Figure 20)**. As expected, TJ2*Mgat5*$^{-/-}$ display a significant loss of L-PHA and increased GNA binding, related to loss of β1,6-GlcNAc branching (**Figure 20A**). The other lectins reveal slight changes in glycan expression, which will be more evident upon increased samples. Nevertheless, α2,6-Neu5Ac is increased and *O*-GlcNAc decreases slightly upon Mgat5 mono- or bi-allelic knockout **(Figure 20A)**, however. Regarding mice survival, even though the sample number is still low, full deletion of *Mgat5* leads to higher mouse survival rate **(Figure 20B)**.

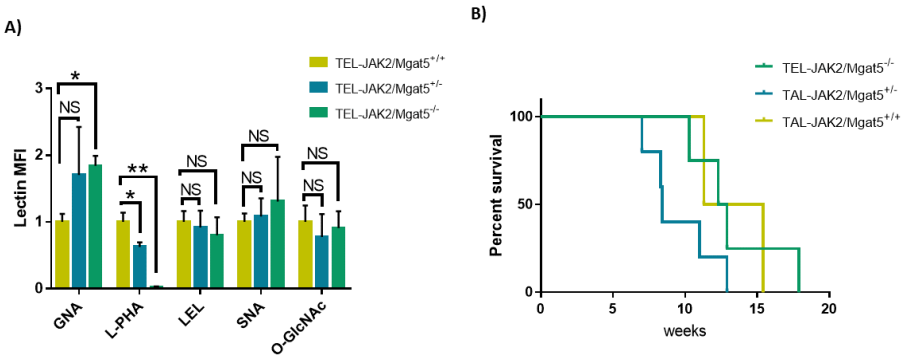

Figure 20 *- Evaluation of glycomodulation in an in vivo leukemic model. (A) Glycoprofile in different genotypes (n=2 for Tel-Jak2/Mgat5+/+ and n=3 for Tel-Jak2/Mgat5+/- and Tel-Jak2/Mgat5-/-). (B) Survival curve.*

Altogether, the results presented in this thesis demonstrate novel information regarding leukemic cells, glycan and glycogene expression. Moreover, glycan modulation is shown here of leukemic cells under expansion, both in vitro and in vivo. Finally, the glycoprogramming of a T-ALL cell line was revealed to be toxic and to induce cellular apoptosis.

Chapter Five

Discussion and Conclusion

In this chapter we will address the discussion of the dissertation where the results obtained in the experimental part will be crossed and compared with those described in the literature. Finally, the conclusion of the work will be explained, where future perspectives will be presented.

5.1 Discussion

Glycosylation is an important post-translational modification, which is considered a cancer hallmark [38, 40]. Having great influence in several cellular mechanisms, the glycosylation pathways are involved in cell growth, proliferation and survival, and when altered can promote malignant transformation and cancer progression [38]. It is known that these changes in glycosylation patterns contribute for different features of tumor cells, such as the increase in complex *N*-glycans in colorectal cancer cells associated with tumor progression [71].

The pattern of glycans expression in T-cells is important in many biological processes associated with T cell development, immune activation and response [11]. Complex N-glycans, which contain four glycan antennas, are responsible for the regulation of TCR clustering, being able to control thresholds of T cell activation [115]. Lack of β1,6-GlcNAc branching N-glycans in T-cells from IBD patients was shown to increase T-cell activation associated with disease severity. T-cell pro-inflammatory profile was rescued upon glycan supplementation, which led to increased complex N-glycan presence [50] Terminal sialylation was shown to mediate galectin binding of T-cells, enabling resistance to galectin-1-mediated apoptosis-induction [116].

The study of glycans in malignant T-cells, namely in T-ALL, remains poorly understood. T-ALL is an hematological cancer common in pediatric ages [29, 95, 96]. Research endeavors have been called for T-ALL, as it poses a more challenging healthcare issue [34, 95], due to high rates of clinical relapse (above 30%) [27].

Our results showed that the glycosylation profile of Jurkat cells revealed an unique pattern of glycans expression, with major differences in complex *N*-glycans and *O*-GlcNAcylation. O-glycans, were not possible to be studied in this cell line, as it harbors a commonly found mutation in cancer, in the *COSMC* gene, a chaperone responsible for the initial synthesis of these O-linked glycans [110, 117, 118]. In fact, the extra availability of monosaccharide substrates due to this mutation may contribute to glycoprofile changes in Jurkat cells, in combination with altered glycogene expression. Interestingly, when compared to healthy T cells, Jurkat cells display a significant increase in β1,6-GlcNAc branching and *O*-GlcNAcylation, which is only accompanied by *OGT* increased expression, as the one of *MGAT5* is not altered.

Terminal sialylation was shown in Jurkat cells to be decrease, as it was in the present thesis [119, 120]. In fact, it was described that terminal sialic acid reduction in these cells is compensated by an increase in polylactosamine structures, that corroborates the results observe in this dissertation [120]. The same study revealed a decrease in *MGAT3* expression, conflicting with the results presented here, showing yet again that both

glycogene and glycan expression should be taken into consideration when performing cellular glycophenotyping.

The presence of complex *N*-Glycans and *O*-GlcNAc was found to be increased in Jurkat cells, when compared to healthy counterparts. Several factors can regulate and influence the glycogene expression and activity, such as IL-7, an important interleukin for T-cell activation and proliferation [30], contributing for malignant transformation [34]. Signaling through the IL-7 pathway has been shown to be related with *N*-glycosylation modulation [35]. Resting healthy T-cells incubated with IL-7 have been shown to increase *MGAT1* and decrease *MGAT5* expression, resulting in reduced levels of β1,6-GlcNAc branching, contributing for their activation. In contrast, when activated T-cells are cultured with IL-7, the presence of β1,6-GlcNAc branched *N*-glycan increases, related to TCR signaling regulation [35]. TAIL7 cells behave as activated T-cells, as they increase *MGAT5* expression and L-PHA binding upon a 24 hour incubation with IL-7. The implications of this glycan upregulation can be seen in a vast horizon of cellular functions, such as in increased receptor surface expression. In fact, it may as well be observed in the receptor responsible for leukemic activity, the IL-7R. The CD127 chain, responsible of IL-7 recognition, was shown to increase its substrate affinity by 300-fold, when glycosylated, compared to its unglycosylated counterpart [23]. The upregulation of *MGAT5* should take part in a positive feed-back loop, that drives IL-7 signaling, and leukemia, in TAIL7 cells.

The TEL-JAK2 murine model display a mutation that lead the independence of the cells for IL-7 stimulus, activating constitutively signaling pathways promoting cell survival [98]. Interestingly, these cells display an increase in L-PHA binding, as they should also be behaving as activated T-cells. Their lineage choice appears to be altered upon *Mgat5* deletion, which can be related to the required *N*-glycans in the CD4 and CD8 co-receptors in thymocytes [9]. The genetic deletion of *Mgat5* may also disrupt leukemic activity, resulting in increased mouse survival, as it can promote thymocyte negative selection, due to its influence on TCR signaling [9]. In fact, despite TJ2 leukemias are observed in TCR-deficient mice, TCR signaling may be detrimental to leukemic cells, inducing a process similar to thymic selection [121].

The increase of *O*-GlcNAc in Jurkat cells was already described in a previous study, that shows the importance of *O*-GlcNAcylation of the cRel (a monomer of the NK-κB complex, important for cell survival and apoptosis) [122]. Curiously, OGT expression was increased in all leukemic samples. In fact, *OGT* was shown to be necessary for malignant transformation in a mouse model [47], which agrees with the increased *O*-GlcNAcylation found in Jurkat (both at *OGT* expression and glycan presence) and TAIL7 (found in *OGT* expression, when compared to healthy T-cells) cells.

O-GlcNAc biosynthesis is important for T-cell activation [61], the modulation of the cell cycle and apoptosis [123]. When supplemented with GlcNAc, Jurkat cells display increased apoptosis rates, being related to higher *O*-GlcNAcylation levels. However, conflicting results are seen upon the comparison between KF treatment and GlcNAc supplementation alone or combined. The combination of both results in the highest levels of *O*-GlcNAc presence, but only GlcNAc alone increases apoptosis. It has been described that KF-treated Jurkat cells have elevated UDP-GlcNAc presence in the Golgi apparatus [124]. Upon cellular entry, the GlcNAc monosaccharide is converted into UDP-GlcNAc, which is the substrate of OGT and GnT's (GnT-V, for instance). As with KF treatment there is an excess in UDP-GlcNAc availability, which can transported out of the Golgi [124], the result should be an increase of *O*-GlcNAcylation, as observed whenever KF is used. The reason why this increase is not driving apoptosis as well as GlcNAc supplementation is puzzling, and should be related to GlcNAc cytoplasmic presence, to the rate limiting enzyme that converts GlcNAc into UDP-GlcNAc, glutamine-fructose-6-phosphate transaminase (GFPT) [86] and, finally to the remaining levels of β1,6-GlcNAc branched *N*-glycans. The flux of substrate to the hexosamine biosynthetic pathway is regulated by GFPT activity, and it has been shown that it controls not only levels of overall *O*-GlcNAcylation, but also its targets [125, 126]. The existence of β1,6-GlcNAc branching may also pose as a protective feature of high levels of *O*-GlcNAcylation, as once they drop, O-GlcNAcylation has also decreased to untreated cells' levels. In fact, when both inhibitors, KF ans OSMI, were used alone or combined with GlcNAc, cellular apoptosis was promoted in the latter condition, indicating that increased cytoplasmic GlcNAc, substrate for GFPT, should be misregulating cellular viability.

Consensus has not yet been reached about the *O*-GlcNAc role in apoptosis in T-cell biology. Several studies show that *O*-GlcNAc levels reduction, through *OGT* gene knockdown, increases Jurkat cellular apoptosis [61, 123]. However, the results of this thesis support the that increased *O*-GlcNAcylation is related to cellular death. It is clear that a balance of *OGA* and *OGT* action, which render an interval of levels of *O*-GlcNAcylation, is necessary for leukemic cell viability. If that balance is somehow disrupted, with metabolic glycoprogramming for instance, as it is described in this dissertation, cellular viability decreases. In fact, high levels of *O*-GlcNAcylation promote cell cycle progression arrest in interphase, namely at the G2/M transition, and low levels disrupt cytokinesis [127].

The results presented in this master thesis highlight the relevance of glycosylation in T cell leukemia development and progression. The balance and relationship between *O*-GlcNAcylation and branched *N*-glycosylation in defining resistance to cell death *versus* promotion of apoptosis need to be further explored and validated both in vitro and *in vivo*. In particularly, the impact of the treatment with inhibitors of *O*-GlcNAcylation (Osmi) or

branched *N*-glycosylation (KF) in the regulation of leukemic cells apoptosis (single or in combination) needs to be further clarified.

Altogether, the work presented in this thesis shows the importance of glycans evaluation in leukemia and presents cellular glycoprogramming as a good therapeutic target in hematological malignancies.

5.2 Conclusion

In this dissertation, results show an importance of glycans in hematological malignancies progression, namely in T-ALL. During for this work was possible take some important conclusions, such as:

- ✓ The glycoprofile between leukemic and normal T-cells is distinct, showing that glycophenotyping can be a target for leukemic hallmarks;
- ✓ The complex *N*- and *O*-GlcNAc glycans show a crucial role in hematological malignancy progression;
- ✓ The glycoprogramming of T-ALL cells shows significant evidence in the induction of malignant cell death;
- ✓ The absence of complex *N*-glycans in the TJ2 model leads to defects on cellular lineage choice and overall increased survival.

These results show an importance of glycosylation in Leukemic progression, and the evidence suggest that this modification can be a good therapeutic target. However, this dissertation is the begin of the study of the glycosylation role in leukemia. In the future, should be realized more studies of malignant cell viability, in different cell lines and *in vivo* models, also should be included cellular cycle evaluation studies.

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