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Insights into O-GlcNAc-mediated regulation of galectin expression and secretion in promyelocytic HL-60 cells

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Abstract

Galectins are a family of β-galactoside-binding proteins involved in cell stress responses and differentiation. Galectins are multifunctional proteins widely studied in many cell models including acute myeloid leukemia HL-60 cells where they mediate numerous intra- and extracellular functions in response to many stress-inducing stimuli. *O*-GlcNAcylation is a dynamic post-translational modification implicated in the regulation of many cellular diseases including cancers. The *O*-GlcNAc mediated expression and secretion of galectins during neutrophilic differentiation was examined in HL-60 cells. Galectin gene (*LGALS*), galectin protein expression, and galectin secretion were determined by RT-qPCR, immunoblotting, and ELISA, respectively. Inhibition of *O*-GlcNAcylation induced markers of differentiation namely growth arrest, segmented nuclear morphology, and H_2O_2 production by NADPH oxidase comparable to ATRA-induced differentiation. The reduction of *O*-GlcNAcylation modified galectin expression, and increased galectin secretion in a manner similar to ATRA. This study confirms that inhibition of *O*-GlcNAcylation is one of the important mechanisms regulating expression and secretion of galectins and cellular differentiation.

Keywords

Galectins, *O*-GlcNAcylation, cellular differentiation, secretion, all-*trans* retinoic acid, 6-diazo-5-oxo-L-norleucine, neutrophil cytosolic factor 1, HL-60 cells

Summary for Lay Audience

Galectins are a family of β-galactoside-binding proteins widely studied in many cell models including HL-60 acute myeloid leukemia cells where they mediate many intra- and extracellular functions in response to many stress inducing stimuli. Acute myeloid leukemia (AML) is cancer occurring in immune cells of the myeloid lineage. Cancer is characterized as uncontrollable cell growth and division that results in the formation of tumors which negatively affect the normal cells and tissues nearby. Uncontrolled growth is often caused by gene mutations that can both upregulate genes that force cells through signaling pathways that promote growth and downregulate genes that keep cell division in check and suppress tumor formation. In AML, the cancer occurs in cells with more stem-like features that promote high growth and proliferation. These cells ordinarily will continue to develop and differentiate throughout their life cycle until they terminally differentiate into a cell type that can no longer divide. AML can be treated clinically using therapies that induce this terminal differentiation thus limiting tumor expansion. However, some differentiation therapies are not universally applicable therefore in this study, we sought to investigate the potential of *O*-GlcNAcylation modulation as a method of terminal differentiation. Many cancers display aberrant *O*-GlcNAcylation, a post-translational modification that regulates protein function, with many more proteins modified by this sugar in cancer cells than in healthy cells. In this study, I used ATRA to induce granulocytic differentiation of HL-60 cells and compares the results to those obtained by treatment biochemical inhibitors of enzymes that regulate *O*-GlcNAcylation to disrupt *O*-GlcNAc homeostasis. Reduction of protein *O*-GlcNAcylation led to slowed growth of AML cells, increased expression of galectins, and phagocytic activity similar to what is seen when cells are treated with established differentiation chemicals. Neutrophils induced by either ATRA, or inhibition of *O*-GlcNAc showed high expression levels of neutrophil-specific genetic markers, segmented nuclear morphology, and increased reactive oxygen species production compared to promyeloid cells, confirming that targeting *O*-GlcNAcylation can induce terminal differentiation. This provides novel insight into AML regulatory mechanisms and could be used to develop more utilitarian chemotherapies.

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List of Abbreviations

AC	Ac-5SGlcNAc
AML	Acute myeloid leukemia
ANOVA	Analysis of variance
APL	Acute promyeloid leukemia
ATRA	All-*trans* retinoic acid
BSA	Bovine serum albumin
C/EBP	CCAAT enhancer binding protein
CD	Cluster of differentiation
CRD	Carbohydrate recognition domain
DAPI	4’,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DON	6-diazo-5-oxo-L-norleucine
DPBS	Dulbecco’s phosphate buffered saline
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
G6PD	Glucose-6-phosphate dehydrogenase
GFAT	Glutamine fructose-6-phosphate aminotransferase
HBP	Hexosamine biosynthesis pathway
HL-60	Human promyelocytic leukemia cells
IMDM	Iscove’s modification of Dulbecco’s modified eagle medium
IR	Insulin receptor
ITS	Insulin, transferrin, selenous acid
mTOR	Mechanistic target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NCF1	Neutrophil cytosolic factor 1
NCF2	Neutrophil cytosolic factor 2
O-GlcNAc	*O*-Linked β-N-Acetyl-D-glucosamine
OGA	*O*-GlcNAcase
OGT	*O*-GlcNAc transferase
PKC	Protein kinase C

PMA	Phorbol 12-myristate 13-acetate
PML	Promyeloid leukemia gene
PPP	Pentose phosphate pathway
RARα	Retinoic acid receptor alpha
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Quantitative Reverse transcription polymerase chain reaction
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBST	Tris-buffered saline with Tween
TG	Thiamet G
Tim-3	T-cell immunoglobin domain and mucin domain 3
UDP	Uridine diphosphate

Chapter 1

1 Introduction

1.1 Galectins

Galectins are a family of multifunctional, soluble, glycan-binding proteins found in animal cells characterized by carbohydrate recognition domains that preferentially recognize β-galactosides (Robinson et al., 2019). First discovered in 1975 as a family of S-type lectins, galectins differ from other lectins in their recognition of β -galactosides (Robinson et al., 2019). Galectins were first distinguished by the name galectin in 1994 (Barondes et al., 1994) and are distinguished from previously discovered carbohydrate binding proteins by the lack of calcium-dependence in the glycan binding that is observed in C-type lectins. Galectins are found in many cellular compartments, in the extracellular matrix, and in circulation. The secretion of galectins is poorly understood, and the mechanisms are not well documented in the literature. Galectins lack an N-terminal sequence necessary for secretion through the canonical secretion pathway, and as such galectins must be secreted through non-canonical mechanisms (Popa et al., 2018).

Galectins can be biomarkers of differentiation status of cells. Some galectins increase in expression in more differentiated cells whereas others are more highly expressed in less-differentiated cells (Abedin et al., 2003; Vinnai et al., 2017).

The galectin proteins fall into three main categories based on their structural properties: prototype, tandem-type, and chimeric galectins (Figure 1) (Timoshenko, 2015). Prototype galectins contain only one carbohydrate-recognition-domain (CRD) and can exist as monomers or in some cases as homodimers as reported by Robinson and co-workers (Robinson et al., 2019). Prototype galectins include galectins – 1, -2, 5, -7, -10, -11, -13, -14, -16. Tandem type galectins contain two CRDs which are joined by a non-CRD linker region and are larger than prototype galectins. Tandem-type galectins may homo- or heterodimerize (Robinson et al., 2019). Tandem-type galectins include galectins -4, -8, -9, and -12. The only known chimera type galectin is galectin-3. Galectin-3 has the ability to pentamerize via the N- terminal domain. Galectin-3 is a widely studied 26 kDa protein

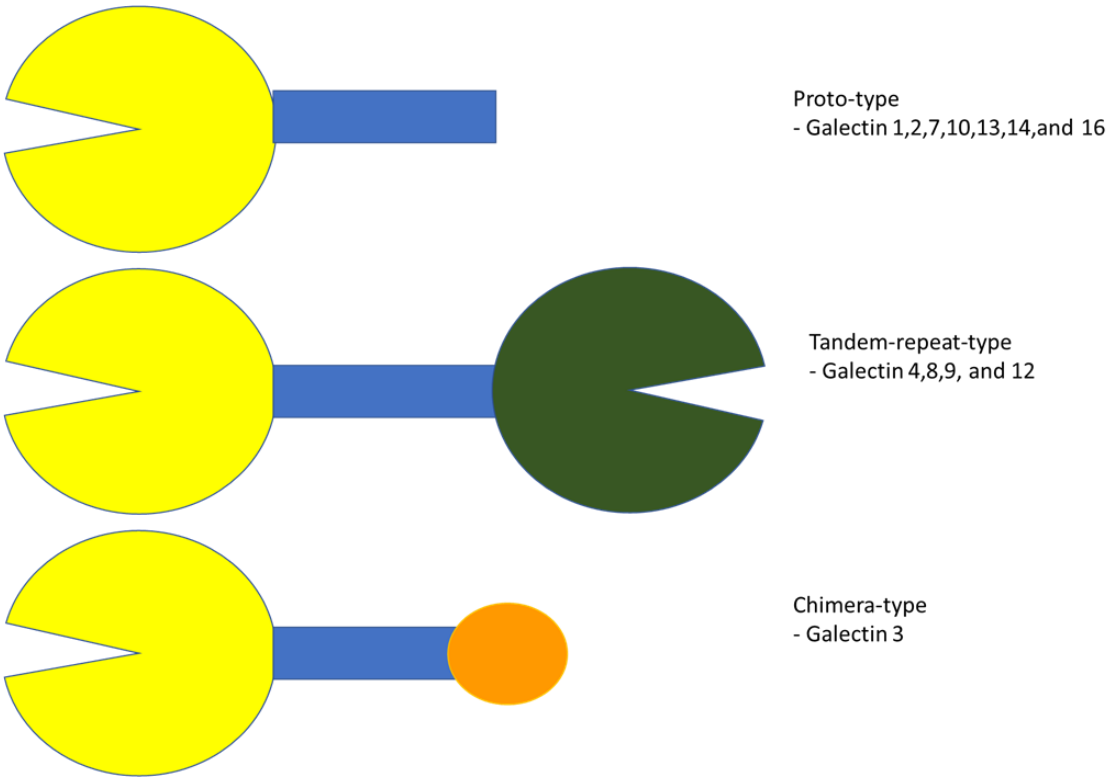

Proto-type
- Galectin 1,2,7,10,13,14,and 16
Tandem-repeat-type
- Galectin 4,8,9, and 12
Chimera-type
- Galectin 3

Figure 1. The molecular structure of galectins.

Galectins are classified based on the carbohydrate recognition domain (yellow and green), non-lectin linker domain (blue) and N-terminal end domain (orange). Prototype galectins contain one CRD and can form homodimers. Tandem-repeat galectins contain two non-identical CRDs connected by a non-lectin linkage peptide region. Chimera-type galectin-3 is monomeric and may pentamerize via the N-terminal end domain (orange circle). The figure is adapted from (Timoshenko, 2015).

which functions ranging from participation in antimicrobial activity of leukocytes, to mitotic cleavages with galectin-3 being a binding partner of the nuclear mitotic apparatus protein (Magescas et al., 2017).

1.1.1 The roles of galectins expressed in HL-60 cells

Previously, the expression of six galectins was confirmed in HL-60 cells by RT-qPCR (Vinnai et al., 2017) including galectins -1, -3, -8, -9, -10, and -12. Thus, there are two prototype galectins -1, and -10, three tandem type galectins -8, -9, and -12, and the chimera type galectin-3. The HL-60 cell line originated from the blood of a 36-year-old Caucasian female with acute myeloid leukemia and has been used to study many cellular pathways in the context of cell differentiation. Previous reports indicate that some galectins are inducible by differentiation stimuli (Abedin et al., 2003; Vinnai et al., 2017). It has also been reported that galectins play many roles in neutrophil function *in vivo* (Robinson et al., 2019) thus making HL-60 cells a suitable functional model to study differential regulation of galectins.

1.1.1.1 Galectin-1 (*LGALS1*)

Galectin-1 is a prototype galectin expressed in HL-60 cells whose role in neutrophilic differentiation and relation to the fate of the cell is reported in different ways. Expression of this protein in the undifferentiated promyeloid cell has been documented by many (Abedin et al., 2003; Vinnai et al., 2017), yet the expression profile among the differentiated neutrophil like cell has been differentially reported (Vinnai et al., 2017; Vakrushev et al., 2018). While neutrophilic differentiation can be induced by at least two chemicals, dimethyl sulfoxide (DMSO) (Breitman et al., 1980b) and all-*trans*-retinoic-acid (ATRA) (Breitman et al., 1980a), previous work shows that galectin-1 is upregulated in HL-60 cells induced to differentiate with DMSO (Vinnai et al., 2017), yet is downregulated when induced by ATRA (Vakrushev et al., 2018). Chiarotti et al. (1994) also report differential induction of the *LGALS1* gene where they observed upregulation by thyroid stimulating hormone and downregulation by retinoic acid. Galectin-1 participates in differentiation of different types of cells, as it reported to be important in the skeletal muscle differentiation in human mesenchymal stem cells (Chan et al., 2006) and in granulocytic differentiation. In mature neutrophils, galectin-1 promotes human

neutrophil migration by inducing actin cytoskeleton rearrangements during non-inflammatory conditions (Auvynet et al., 2012)**.** Galectin-1 is multi-functional and has widely reported roles, some of which appear contradictory. Galectin-1 is reported to be a pro-survival molecule in AML, by activating Ras GTPase and stimulating phosphatidylinositol-3-kinase and protein kinase B activity, and downstream mitogen activated protein kinase signaling which progresses the cell through the cell cycle and proliferation (Elad-Sfadia et al., 2002; Ruvolo et al., 2020).

Galectin-1 promotes immunosuppression and tumor progression by remodeling the tumor endothelium to suppress T-cell infiltration thus inhibiting T-cell mediated immune clearance (Nambiar et al., 2019). This is achieved by galectin-1-mediated T-cell apoptosis (Perillo et al., 1995; Kovács-Sólyom et al., 2010). Galectin-1 like other galectins has opposing roles reported in the literature as it has also been reported that galectin-1 expression correlates with tumor aggressiveness in other cancer types (Saussez et al., 2008).

1.1.1.2 Galectin-3 (*LGALS3*)

Galectin-3 is a 26 kDa lectin protein. Mature leukocytes express galectin-3 and secrete it to the circulation where it can interact with immunoglobin E and the immunoglobin E receptor, signaling an oxidative burst through the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex to produce reactive oxygen species (ROS) which are used as microbicides by neutrophils (Robinson et al., 2019), and this signaling can be inhibited using neutralizing antibodies. Galectin-3 is important in some types of cells for proper mitotic cleavage and cytokinesis of daughter cells after DNA replication. In previous studies, galectin-3 associated with the nuclear mitotic apparatus protein to regulate proper spindle pole formation and allowed both mitosis and cytokinesis to occur properly (Magescas et al., 2017). When galectin-3 was inhibited or knocked out, the nuclear mitotic apparatus protein did not function as expected and as such, spindle pole formation occurred improperly and at inappropriate cytosolic locations. This led to bizarre cleave patterns and atypical distribution of chromosomes among daughter cells. The interaction of galectin-3 with the nuclear mitotic apparatus protein is *O*-GlcNAc dependent (Magescas et al., 2017).

Galectin-3 signaling through CD66a and CD66b may result in ROS production and IL-8 secretion (Robinson et al., 2019). Galectin-3 deficient neutrophils have an impaired laminin binding to the endothelium, a necessary process in neutrophil mediated immunity (Gittens et al., 2017). Inhibition of galectin-3 results in reduced TNFα KC, TGFβ, and MCP-1 levels and neutrophil accumulation, suggesting a role for galectin-3 in extravasation and chemotaxis (Pan et al., 2018). Extravasated neutrophils may bind to laminin and fibronectin in galectin-3-mediated mechanisms suggesting a role for galectin-3 in chemotaxis (Zhou et al., 1993; Ozeki et al., 1995; Kuwabara and Liu, 1996; Kerszetes et al., 1997). Galectin-3 also facilitates neutrophil phagocytosis of *Streptococcus pneumoniae* (Farnworth et al., 2008). Not only bacteria can be cleared in this way, as there were fungal infections such as *Candida parapsilosis*, and *Candida albicans* can be phagocytosed by neutrophils through galectin-3 mediated mechanisms (Linden et al., 2013; Kohatsu et al., 2016).

1.1.1.3 Galectins-8, -9, -10, and -12 (*LGALS8, LGALS9, LGALS10, LGALS12)*

Galectin-8 is a tandem repeat galectin implicated in many cell functions including adhesion, proliferation, and apoptosis (Robinson et al., 2019). Galectin-8 has opposing roles, as it has been reported to induce apoptosis in thymocytes (Tribulatti et al., 2007), whilst in brain cells it is reported to prevent apoptosis (Metz et al., 2016). Galectin-8 has been reported in the ROS production aspect of neutrophils by signaling through the C-terminal domain (Nishi et al., 2003; Nishi et al., 2006). Galectin-8 can regulate neutrophil adhesion to the endothelium (Yamamoto et al., 2008).

Galectin-9 is also a tandem-repeat galectin and is widely studied for its association with T-cell immunoglobin domain and mucin domain 3 (Tim-3). Tim-3 mediates galectin-9 enhancement of neutrophil microbial killing (Robinson et al., 2019). Galectin-9 also causes neutrophil degranulation and ROS production in a Tim-3 dependent manner (Vega-Carrascal et al., 2014; Steichen et al., 2015). Galectin-9 can also regulate neutrophil chemotaxis (Hirao et al., 2015). Galectin-9 was previously known by the name eotaxin, as it was discovered in eosinophils where researchers noted that galectin-9 exhibited chemotactic activity (Matsumoto et al., 1998; Hirashima, 1999). Galectin-9 was

also shown to enhance phagocytosis of *P. aeruginosa* (Farnworth et al., 2008; Stowell et al., 2010; Arthur et al., 2015). Tim-3 appears to be a necessary partner in galectin-9 mediated phagocytosis. Galectin-9 and the interaction with Tim-3 is well documented. The binding of these two proteins is implicated in many pathways associated with cell growth and proliferation (Zheng et al., 2019). Galectin-9 and Tim-3 interaction activate phosphatidylinositol-3-kinase and mTOR resulting in increased expression of hypoxia inducible factor 1α and the secretion of vascular endothelial growth factor (Gonçalves Silva et al., 2015 Prokhorov et al., 2015). These proteins play a role in angiogenesis (Zhang D et al., 2018) and are reported as important for the progression of leukemias (Lin et al., 2018). There is additional evidence for galectin-9 in the promotion of cell growth. The galectin-9-Tim-3 interaction may also activate extracellular signal regulated kinase and protein kinase B resulting in β-catenin signaling and activation of nuclear factor κB (Kikushige et al., 2015). Nuclear factor κB activation promotes the expression and secretion of cytokines and chemokines and growth factors stimulating the growth and proliferation of leukemias (Jia et al., 2016; Gao B et al., 2018). Galectin-9 also induces T-cell death through its interaction with Tim-3 (Cao et al., 2018). The T-cell apoptosis mediated by galectin-9 is consistent with reported functions of galectin-1 in remodeling the tumor microenvironment conferring immunosuppression to the tumor microenvironment and promoting tumor progression (Saussez et al., 2008; Kovács-Sólyom et al., 2010; Nambiar et al., 2019). Galectin-1 and -9 can thusly be seen as oncogenic markers and as such implicates them as target molecules for potential future cancer therapies.

Genetic expression of *LGALS10* can be induced after neutrophilic differentiation of HL-60 cells (Abedin et al., 2003). Largely studied in eosinophil models, galectin-10 is also known as the Charcot-Leyden crystal protein. It was initially thought to belong to the lysophospholipase family due to weak lysophospholipase activity (Swaminathan et al., 1999) even though there was little to no structural or sequence homology to other proteins of this family. However, it was reclassified in 1997 as a member of the galectin superfamily when it was discovered to have sequence homology to other galectins (Leffler et al., 2002). Charcot-Leyden crystal proteins oligomerize in the extracellular

matrix to form crystals visible by brightfield microscopy (Dvorak et al., 1998). These crystals have a role in allergy mediation and responses.

Galectin-12 according to the literature can play opposite roles in cellular differentiation. While some (Yang et al., 2004) report that galectin-12 expression promotes adipocyte differentiation, others (Xue et al., 2016) report that knockdowns of galectin-12 enhance ATRA-induced neutrophilic differentiation of the human NB4 promyelocytic leukemia cells. However, this does not mean that galectin-12 inhibits differentiation, as this same group (Xue et al., 2016) reports that absent a knockdown, there is still a high degree of differentiation as assessed by ROS production using a NADPH-oxidase functional assay.

1.1.2 The secretion of galectins

Galectins as previously mentioned are multifunctional proteins that have roles in both the intracellular and extracellular environments (Robinson et al., 2019). Extracellularly, galectins have been reported to regulate differentiation of various cell types (Hikita et al., 2000; Hirashima et al., 2004; Chan et al., 2006). Secretion of proteins through the conventional vesicular secretion pathway is well documented and understood. Proteins possessing an N-terminal signal sequence or hydrophobic segment are directed to the endoplasmic reticulum and anchored into its membrane by the N-terminal sequence. Proteins then pass through the endoplasmic reticulum to the Golgi apparatus and finally trafficked to the plasma membrane in membrane bound vesicles (Popa et al., 2018). However, not all proteins are conventionally secreted, and galectins are included in this group of proteins that use non-conventional secretion mechanisms (Popa et al., 2018).

Galectin-1 may use the same route as FGF2 for its secretion, the most studied non-conventionally secreted protein that uses direct translocation (Popa et al., 2018). Both galectin-1 and -3 may accumulate under the plasma membrane prior to secretion (Cooper and Barondes, 1990; Mehul and Hughes, 1997) which may suggest a direct translocation mechanism for their secretion. Cooper and Barondes (1990) also showed that galectin-1 may be secreted by extracellular vesicle formation. Galectin-1 accumulates under the cell surface, where micro-vesicles form and load the galectin-1, which then bleb out and release galectin-1 into the extracellular matrix. Galectin-3 can be secreted by clathrin-

independent carriers (Lakshminarayan et al., 2014). The mechanisms for secretion of galectin-1 are not confirmed but the literature does suggest a few different mechanisms for secretion absent an N-terminal sequence. One study (Seelenmeyer et al., 2005) hypothesized the secretion of galectin-1 was accomplished by counter receptors. Secretion of galectin-3 may require the removal of *O*-GlcNAc beforehand (Mathew et al., 2021). Galectin-3 can be secreted from cells when glycosylated proteins are unavailable (Popa et al., 2018) however, others have reported that cells grown in a serum-free medium showed secrete less galectin-3 than cells grown in a serum-containing medium (Sato et al., 1993). Currently, while it is known that galectins can be secreted from cells and act on extracellular surface proteins (Barondes et al., 1994), the mechanisms for the secretion of galectins remain to be elucidated (Popa et al., 2018).

1.2 Acute myeloid leukemia

Acute myeloid leukemia (AML) is cancer occurring in the myeloid lineage and are subdivided into eight subtypes under the French-American-British classification. The different AML subtypes occur in different cell types within the myeloid linage, at varying stages of maturation and differentiation (Figure 2). As such, an AML patient will have their cancer classified as one of the eight French-American-British subtypes (M0-M7) based on morphological and genetic characteristics of the cancer (Dalton et al., 1988). Acute promyeloid leukemia (APL) is designated as an M3 AML under the French-American-British classification system. APL is a rare form of AML comprising approximately 5% of all leukemia. M3 leukemias are famously known for their susceptibility to all-*trans*-retinoic acid (ATRA). ATRA has long been used in the clinical setting as a chemotherapeutic for M3 AML with great success. Previous literature reported a remission rate over 90% for M3 AML patients administered ATRA. Unfortunately, some other forms of AML exhibit ATRA-resistance and as such are unable to be clinically treated in this manner (Johnson and Redner, 2015). Early research into this phenomenon reported that 98% of APL patients have the t(15:17) translocation which resulted in a fusion of the retinoic acid receptor alpha (RARα) with the

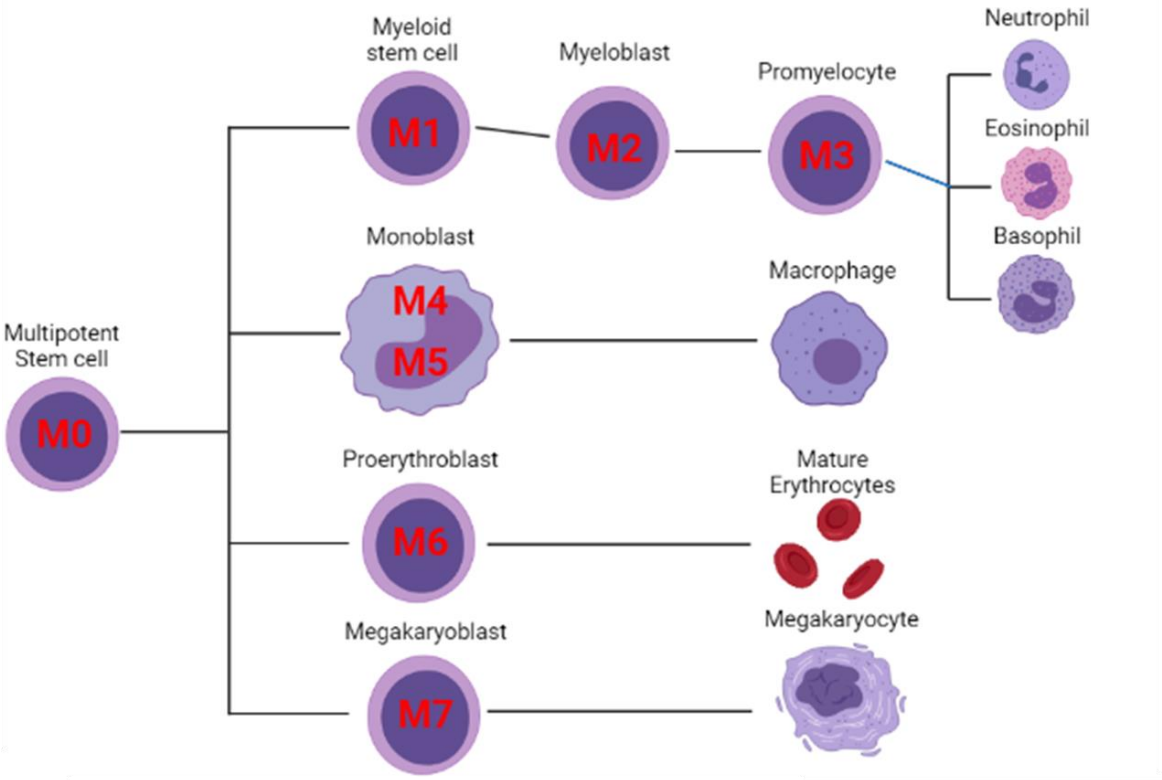

Myeloid stem cell
Myeloblast
Promyelocyte
Neutrophil
Eosinophil
Basophil
M1
M2
M3
Monoblast
Macrophage
M4
M5
Multipotent Stem cell
M0
Proerythroblast
Mature Erythrocytes
M6
Megakaryoblast
Megakaryocyte
M7

Figure 2. The French American British classification system of acute myeloid leukemia.

The above figure shows the designation of AML subtypes among various cells within myeloid linage (adapted from Szalontay and Shad, 2014). Myeloid leukemia is classified into eight different subtypes numbered M0-M7 based on the cell type carrying the leukemia within the myeloid linage. Leukemias are classed on maturity of the cell type within the myeloid lineage. This is determined by light microscopy to determine morphology and by the expression of genetic biomarkers.

promyelocytic leukemia gene (PML) called the PML-RARα (Gallagher, 2002; Tomita et al., 2013). ATRA-induced differentiation does not need to signal through the PML-RARα fusion protein however, as some non-M3 leukemias including the AML cell line HL-60, classified as M2 (Dalton et al., 1988), are also susceptible to ATRA-induced differentiation through RARα or retinoid X receptor signaling (Breitman et al., 1980a; Tassef et al., 2017). ATRA works as a differentiation agent by interacting with the RARα and downstream signaling leads to the activation of transcription factors C/EBPβ and PU.1 which activate the transcription of genes involved in neutrophilic differentiation of promyeloid cells (Bjerregaard et al., 2003; Akagi et al., 2010; Ai and Udalova, 2019). Past studies have shown that *in vivo* deficiencies of C/EBP family transcription factors specifically the β and ε proteins resulted in defective myeloid differentiation and mice lacking these proteins were highly susceptible to fatal infections (Akagi et al., 2010).

The RAR family has three proteins, α, β, and γ, which are all structurally similar yet differ in the tissue type in which they are expressed. RARγ is highly expressed in epithelial tissues whereas α is highly expressed in hematopoietic tissues (Johnson and Redner, 2015). The RAR is usually always DNA bound and the target genes remain inactive due to the additional binding of a co-repressor complex (Nagy et al., 1999). Binding of the ligand (ATRA) induces a conformational change and dissociation of the co-repressor complex leading to activation and transcription of the target genes (Nagy et al., 1999; Ablain and de The, 2011). Retinoic acid is also used as a differentiation agent in other cell models including SY5Y human neuroblastoma cells (Ammer and Schulz, 1994).

1.2.1 The HL-60 cell line as a model of neutrophilic differentiation

HL-60 cells are an M2 AML isolated from a 36-year-old female patient in 1976 and in the following year the immortalized cell culture which came to be known as the HL-60 line was established at the National Cancer institute (Collins et al., 1977). This cell line was later discovered to be susceptible to ATRA-induced terminal differentiation confirming the original clinical diagnosis of M3 APL (Breitman et al., 1980a). Although originally thought to belong to the M3 subtype because the patient had a high proportion

of progranulocytes, HL-60 cells were reclassified as M2 in 1988 due to the morphology of these cells better fitting the M2 classification (Dalton et al., 1988). Although HL-60 cells were reclassified as an M2 leukemia due to the morphological features, the HL-60 cell line is susceptible retinoic acid signaling and resulting in granulocytic differentiation (Breitman et al., 1980a). As such, HL-60 remains a suitable model for studying neutrophilic differentiation and ATRA signaling in APL. These cells can be cultured in a serum free growth medium as was first discovered in the 1980s by Breitman et al. (1980b). Commercial fetal bovine serum (FBS) contains many growth factors, proteins, and hormones that are used to enhance the growth of cell cultures *in vitro*. The Breitman group determined the minimal essential supplements needed to add to a culture media to allow HL-60 cells to grow, these being insulin, transferrin, and selenous acid (ITS) (Breitman et al., 1980b). Insulin is necessary to allow glucose uptake and metabolism and transferrin allows iron uptake which cells need to use as a cofactor for protein activity in many cellular processes. Selenous acid is a compound of the element selenium, a cofactor for enzymes in redox pathways such as peroxiredoxin. The absolute necessity of selenium compounds is however debatable as the Breitman group, even in their serum-free pioneering paper show that absent a selenium supplement, HL-60 cells were still culturable in RPMI. They noted previous literature which suggested the necessity of selenium (Guilbert and Iscove, 1976; McKeehan et al., 1976) yet did not show that growth and culture was impossible absent selenium. The Breitman group however kept selenium in their supplement and even today this remains a common supplement for cell culture, as there are several commercially available ITS cocktails. The insulin and transferrin requirements are absolute, as absent these growth factors, cell growth ceases immediately (Breitman et al.,1980a; Collins, 1987).

A serum-free method of culture is important for this work which seeks to learn about the secretion of galectins from HL-60 cells. As galectins are soluble proteins and previously secretion has been reported, they are found in serum as was confirmed by several groups independently (Barrow et al., 2011; He et al., 2017; Asiamah et al., 2019). The presence of galectins in serum will contribute to background signal in ELISA assays or other assays. To remove the background and measure the extracellular galectins, ensuring that only galectins which were secreted by the HL-60 cells of interest, we will employ the

serum-free culture method pioneered by Breitman et al. (1980b). Serum-free cell culture has also been used to assess galectin secretion by murine B cells (Yu et al., 2006). Since there are many components of serum aside from the minimal necessary for culture (ITS) and the effect of these with regards to growth, proliferation, signaling, differentiation, oncogenesis and more may not be fully understood, this study will use both a serum-containing and serum-free method of culture to compare the measurements and assess the role that serum may play.

HL-60 cells have a homozygous deletion of the p53 gene (Soddu et al., 1994). P53 induces G1 growth arrest in cells and the absence of this gene product in part contributes to the infinite proliferative capacity of this cell line (Ray et al., 2010). P53 is known as the guardian of the genome (Lane, 1992) that activates the expression of genes that control cell cycle progression and apoptosis (Levrero et al., 2000 Yang A et al., 2004). P53 degradation by Mdm2 signals cells to undergo apoptosis (Ray et al., 2010). However, HL-60 cells transfected with p53 show increases in apoptosis and differentiation (Soddu et al., 1994) showing that normal cell cycle regulation can be rescued by targeting those pathways dysregulated in cancers.

There are several inducers of neutrophilic differentiation of HL-60 cells (Stegmaier., et al 2004; Peck et al., 2006). Collins (1987) published a review paper detailing the different inducible lineages of HL-60 cells and reporting the markers for each type of differentiation. Specifically, for granulocytic differentiation many markers of differentiation are listed. These include upregulation of surface receptors confirmed using flow cytometry-based assays, such as CD11b and CD15, down-regulation of surface markers like the insulin receptor (IR) and transferrin receptor , increased expression of components of the NADPH oxidase complex such as neutrophil cytosolic factor 1 (*NCF1*) and neutrophil cytosolic factor 2 (*NCF2*), increased capacity for reduction of nitro blue tetrazolium dye, and increased ROS production by directly measuring hydrogen peroxide evolution using an activator of the NADPH oxidase complex. While there are many markers of granulocytic differentiation, HL-60 cells need not meet all the criteria in order to be classified as differentiated. Indeed, previous experiments by Sham et al, (1995) and Manda-Handzlik et al, (2018) sought to elucidate how HL-60 cells

induced to the granulocyte lineage by different drugs differentially express and regulate key components of the neutrophil.

HL-60 cells are an adequate model to study granulocytic differentiation and to study proteins and post-translational modifications in the context of differentiation. A promyelocyte which is pre-leukocytic can differentiate into all types of mature leukocytes and indeed there is much literature on the induction of differentiation of HL-60 cells to many cell types. Terminal differentiation can be induced by phorbol 12-myristate 13-acetate (PMA) which will yield monocyte cells or by sodium-butyrate which will yield eosinophil cells. Most interestingly, HL-60 cells can be induced to the granulocyte lineage with the use of several chemicals (Abedin et al., 2003). DMSO is a long used and widely used differentiation agent for HL-60 cells (Abedin et al., 2003). ATRA is another drug which has long been used in research settings to induce HL-60 cells to the granulocyte lineage (Breitman et al., 1980a). What's more is that ATRA has also been used to some success in the clinical treatment of acute myeloid leukemia. While DMSO and ATRA may both induce neutrophilic differentiation and this can be confirmed by the expression of neutrophil specific genes like *NCF1*, and also by the NADPH-oxidase complex production of hydrogen peroxide, the entirety of induction and suppression between these two drugs varies quite a lot. Some biomarkers do not have the same magnitude of change between DMSO and ATRA, such as the reduction of global *O*-GlcNAcylation. Sherazi et al, (2018) used DMSO and reported a 95% drop in *O*-GlcNAcylated proteins, whereas Asthana et al. (2018) used ATRA and show a less severe reduction, although this group did not quantify this reduction in their report. The method of action of both of these drugs is not the same and as such there should be some expected differences in the cellular response.

Galectins have previously been studied in relation to DMSO-induced granulocytic differentiation of HL-60 cells (Vinnai et al., 2017). Although galectin regulation during granulocytic differentiation has only been studied by few, more classical markers of differentiation have been studied in response to multiple known inducers of granulocyte differentiation (Manda-Handzlik et al., 2018). Retinoic acid induced galectin gene transcription utilizes specificity protein 1 consensus binding sites (Lu and Lotan, 1999;

Lu et al., 2000). Many galectins have this promoter, and this appears to be a common feature of gene regulation in this gene family (Chiarotti et al., 1999; Dyer and Rosenberg, 2001). The previous association of galectin upregulation and DMSO-induced neutrophilic differentiation (Vinnai et al., 2017) combined with the many reported roles of galectins in neutrophils reviewed by Robinson et al. (2019) and the differential regulation of neutrophilic markers reported by Manda-Handzlik et al. (2018) bring into question the utility of galectins as biomarkers using either DMSO or ATRA as an inducing agent.

Leukemia can be treated by the induction of terminal differentiation. Cancer cells upregulate Yamanaka factors which are oncogenes, and display a more stem cell-like morphology, genetic profile, and functional activity, lending to the nigh-infinite proliferative capacity. Induction of terminal differentiation forces stem-cell-like cancer cells through their respective developmental stages to a cell type which lacks expression of oncogenes and does not have the capacity to proliferate indefinitely (Breitman et al., 1981). Regarding acute myeloid leukemias, differentiated cells have lower expression levels of genes that signal cells through the proliferative pathways, specifically the IR, and the transferrin receptor. Chaplinski et al. (1986) showed that HL-60 cells fated to the granulocyte linage by DMSO or ATRA express significantly less IR than untreated HL-60 cells. The transferrin receptor also known as CD71 is similarly downregulated upon DMSO induced differentiation (Mollinedo et al., 1998; Santos-Beneit and Mollinedo, 2000). The downregulation and subsequent ablation of signaling through these receptors is not immediate however, as the expression decreases further and further over time (Mollinedo et al., 1998). Differentiation by DMSO or ATRA is achieved 3-7 days post stimulation with the differentiation agent (Mollinedo et al., 1998).

Neutrophils are leukocytes involved in the process of innate immunity. Neutrophils are highly abundant in circulation (Ai and Udalova, 2019), and are one of the earliest and most effective responders to microbial infection (Kobayashi et al., 2005). Neutrophils can use less specific response mechanisms to recognize and remove infection without antibody mediated opsonization of pathogens (Barton, 2006). Neutrophils can also interact with antibodies to focus effector function on more specifically targeted pathogenic invaders as well (Barton, 2006), as is evidenced by the increased expression

of immunoglobin E receptors and Fc receptors on the cell surface of mature neutrophils (Collins, 1987). Neutrophils are dedicated phagocytes that mediate innate immune function through many microbicidal activities (Belambri et al., 2018). One important and highly functional method of dealing with invading pathogens is by the production of ROS through the NADPH oxidase complex (Belambri et al., 2018). This complex is formed by several protein subunits which are upregulated in more maturated leukocytes relative to the promyeloid precursor cell (Vinnai et al., 2017; Dakik et al., 2021). Additionally, upregulation of subunit proteins of the NADPH oxidase complex during granulopoiesis is associated with upregulation of galectins-3 and -8 (Drewniak et al., 2008). The complex however is not constitutively active and does not produce ROS until it is assembled and activated through a series of phosphorylation steps by protein kinase C (PKC) (Bedard and Krause, 2007). Neutrophils can be activated by a variety of stimuli including recognition of pathogen associated molecular patterns through the abundant pathogen recognition receptors on the cell surface (Robinson et al., 2019). Signaling through pathogen recognition receptors leads to downstream activation of PKC which phosphorylate the many subunits of the NADPH oxidase complex, inducing the association of the subunits and initiating functional microbicidal activity. There are also agonists for this pathway as the ROS production can be stimulated by formyl-methionyl-leucyl-phenylalanine which is a bacterial cell wall component which is recognized by the formyl peptide receptor or can be activated by direct activation of the PKC enzyme using chemicals like PMA (Dakik et al., 2021). The NADPH oxidase complex oxidizes NADPH and transfers that electron to diatomic oxygen to create a superoxide ion and ultimately hydrogen peroxide as a result (Figure 3) (Bedard and Krause, 2007; Belambri et al., 2018). Hydrogen peroxide and other ROS exert phagocytic activity by inducing oxidative damage on the pathogens within phagolysosomes (Bedard and Krause, 2007).

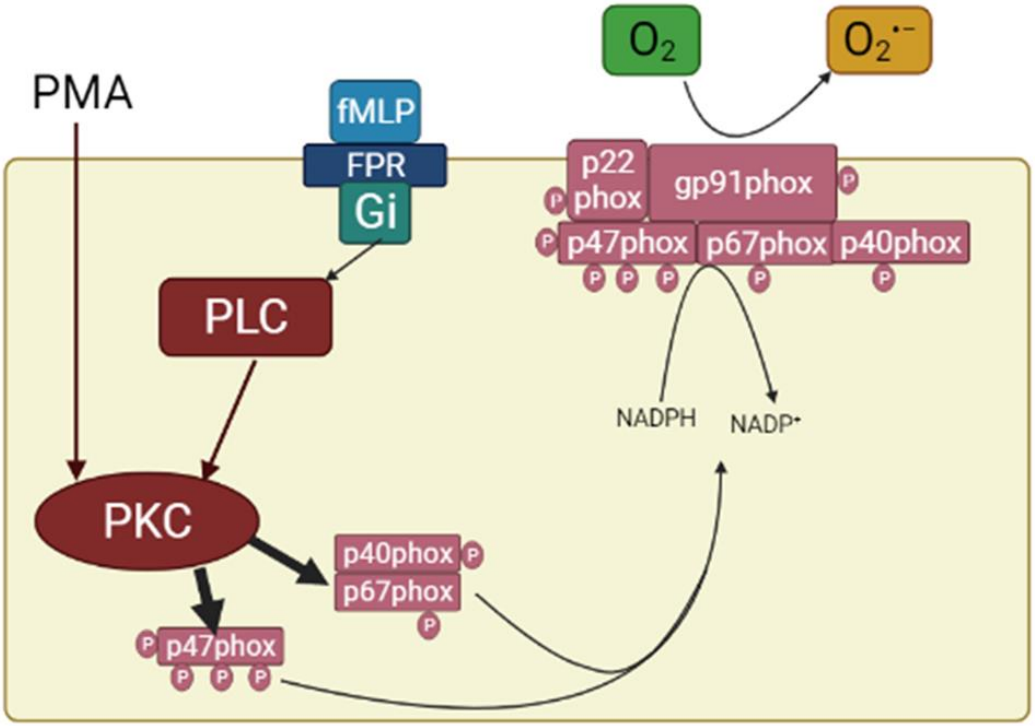

PMA
fMLP
FPR
Gi
PLC
PKC
p40phox
p67phox
p47phox
O2
O2•−
p22 phox
gp91phox
p47phox
p67phox
p40phox
NADPH
NADP•

Figure 3. ROS production via the NADPH oxidase complex requires neutrophil activation and PKC activity.

The above figure details the activation and relocation of the NADPH oxidase complex subunits (adapted from Belambri et al., 2018). The protein subunits of the NADPH oxidase complex are expressed in dedicated phagocytes. The NADPH oxidase activity is only exerted upon activation of the cell which can be achieved by formyl-methionyl-leucyl-phenylalanine (fMLP) signaling to activate PKC or directly activating PKC using PMA. PKC activation phosphorylates the subunits and induced the association of the subunits which oxidize NADPH to convert oxygen into superoxide. Superoxide is rapidly converted into hydrogen peroxide by superoxide dismutase.

1.3 *O*-GlcNAcylation

O-GlcNAcylation is a cellular post-translational modification where an *O*-linked, β-N-acetyl-D-glucosamine (*O*-GlcNAc) is added to the hydroxyl groups of serine and threonine residues of proteins (Figure 4). The precursor molecule, uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), is produced by the hexosamine biosynthesis pathway (HBP) which catalyzes intermediates from the glycolysis pathway into UDP-GlcNAc which participates in many cellular processes (Akella et al., 2019). The rate-limiting step of the HBP is the conversion of fructose-6-phosphate to glucosamine-6-phosphate, a reaction which is catalyzed by the enzyme glutamine fructose-6-phosphate aminotransferase (GFAT) (Martinez et al., 2017). UDP-GlcNAc is also used to produce proteoglycans, glycolipids, and glycoproteins (Grønning-Wang et al., 2013). *O*-GlcNAcylation occurs on serine and threonine residues and thus, competes with phosphorylation. The large size of the GlcNAc moiety provides steric hinderance to the residue, prevent other post-translational modifications from occurring on that site and on adjacent sites (Liu and Li, 2018). *O*-GlcNAc inhibitors used in cell culture also showed that for hundreds of proteins, when *O*-GlcNAcylation is inhibited that the phosphorylation of many proteins was enhanced (Wang et al., 2008) and vice versa, when *O*-GlcNAc is elevated, the phosphorylation of many proteins was decreased. *O*-GlcNAcylation appears to be controlled by only two enzymes: *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA). OGT catalyzes the addition of the sugar moiety by cleaving the UDP region and linking the GlcNAc to the hydroxyl group of serine or threonine residues, hence the nomenclature "*O*-linked." The reverse reaction is catalyzed by the enzyme OGA (Asthana et al., 2018). The two enzymes involved are very important biologically and evolutionarily playing important roles in cellular functions. As such, significant disturbances to this homeostasis are stressful to the cells, with some reporting that OGT knockouts were fatal in mice (Zhang et al., 2015).

O-GlcNAc is implicated in cytokinesis of cells (Li et al., 2017). These authors reported that OGT knockdown by siRNA led to decreased phosphorylation at Ser-71 which hampered vimentin filament severing and led to failure of cytokinesis. This agrees with other literature which reports decreases in cell growth and mitosis when treated with

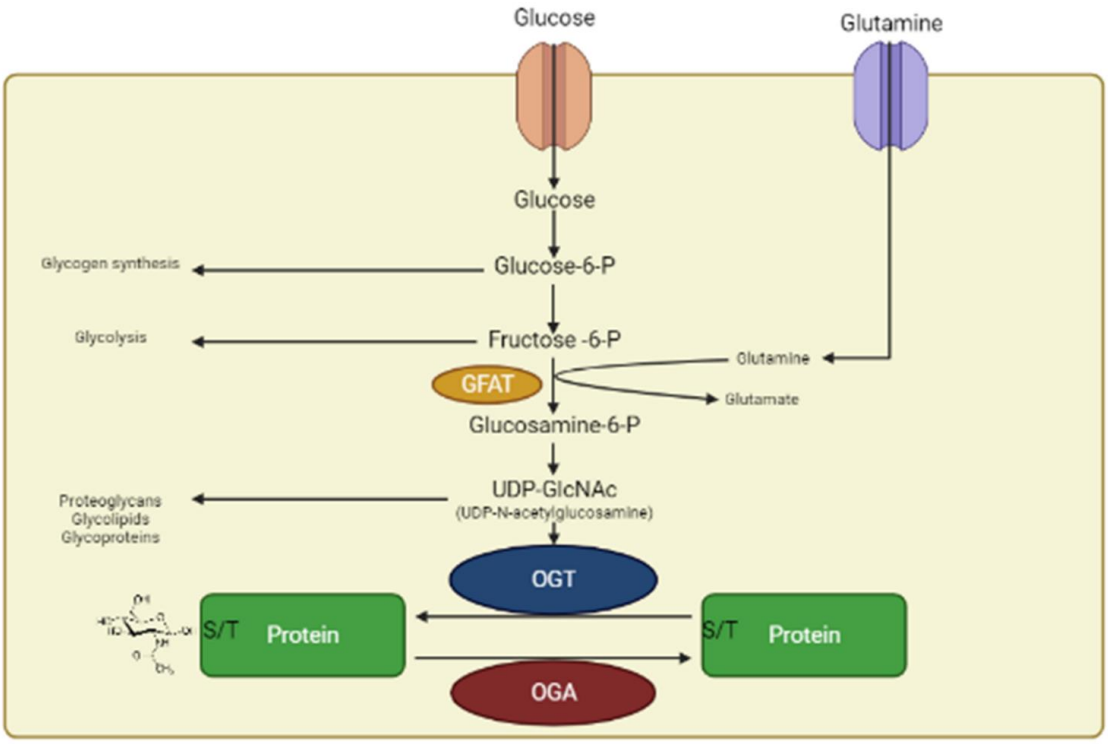

Glucose
Glutamine
Glucose
Glycogen synthesis
Glucose-6-P
Glycolysis
Fructose -6-P
Glutamine
GFAT
Glutamate
Glucosamine-6-P
Proteoglycans
Glycolipids
Glycoproteins
UDP-GlcNAc
(UDP-N-acetylglucosamine)
OGT
S/T
Protein
S/T
Protein
OGA

Figure 4. The hexosamine biosynthesis pathway in mammalian cells.

The above figure shows the synthesis of UDP-GlcNAc through the HBP and the enzymatic regulation of *O*-GlcNAcylation (adapted from Fardini et al., 2013). The rate limiting step of the HBP is the conversion of fructose-6-phosphate to glucosamine-6-phosphate via the GFAT enzyme. The product of the HBP is UDP-GlcNAc which is added to serine or threonine residues of proteins with the OGT enzyme, removing the UDP in the process to allow the N-acetylglucosamine to bind through the oxygen molecule of the residues. The removal of *O*-GlcNAc is catalyzed by the OGA enzyme.

inhibitors of OGT and GFAT (Perez-Cervera et al., 2013; Asthana et al., 2018). Vimentin phosphorylation is enhanced when OGT or OGA are overexpressed, suggesting an interaction with *O*-GlcNAc bearing polymers which promote the phosphorylation at Ser-71 (Komura et al., 2012; Liu and Li, 2018).

OGT plays an important role in insulin signaling (Perez-Cervera et al., 2013). The IR is glycosylated by the OGT enzyme and allows signaling through the IR which is a receptor tyrosine kinase and initiates many downstream activities. There appears to be a positive feedback loop regulating the interaction between the IR and OGT. OGT is relocated to the plasma membrane by phosphatidylinositol triphosphate which must first be phosphorylated by the phosphatidylinositol-3-kinase enzyme which is only active when signaled by the IR. The relocation of the OGT to the plasma membrane allows the *O*-GlcNAcylation of the IR, and continued flux through this pathway. Literature (Perez-Cervera et al., 2013) shows that inhibition of either the IR or OGT, has a negative effect on the other. When *O*-GlcNAcylation can be inhibited by chemical inhibitors or by RNA interference techniques, signaling through the IR is ablated, limiting the downstream actions of the IR signaling pathway. This lends credence to the *O*-GlcNAc being important for the infinite proliferatively of cancerous cells. OGT can itself be *O*-GlcNAcylated (Perez-Cervera et al., 2013; Martinez et al., 2017), showing a positive feedback loop and demonstrating that *O*-GlcNAc is very important even for its own sake. OGT is linked to Yamanaka factors, maintaining pluripotency and self-renewal (Jang et al., 2017). Specifically, Sox2 and Oct4 are *O*-GlcNAcylated, and this is associated with the embryonic stem cell state (Jang et al., 2017). As embryonic stem cells differentiate into embryoid bodies, the *O*-GlcNAcylation on these oncogenes decreases rapidly (Jang et al., 2017). Blocking the *O*-GlcNAcylation of proteins in these cells inhibited self-renewal whereas increasing the *O*-GlcNAcylation maintained embryonic stem cell pluripotency (Jang et al., 2017)

O-GlcNAc homeostasis is an important regulator of cancer biology. Many different types of cancers in a variety of cell types in humans display aberrant *O*-GlcNAcylation associated with the cancerous phenotype of the cell (Sherazi et al., 2018; Akella et al., 2020). This is displayed *in vitro*, and *in vivo* as previous researchers showed that healthy

peripheral blood mononuclear cells have less *O*-GlcNAcylated proteins than peripheral blood mononuclear cells isolated from leukemia patients (Asthana et al., 2018). *In vitro,* cancer cells that are terminally differentiated show reduced *O*-GlcNAcylation compared to the cancerous cells. This also works *in vivo* as mice peritoneally injected with APL tumors and treated with inhibitors of GFAT or OGT to reduce the *O*-GlcNAcylation showed a cessation in the growth of the tumors (Asthana et al., 2018).

Many pathways have been implicated in *O*-GlcNAc mediated signaling. For example, the pentose phosphate pathway (PPP) (Rao et al., 2015) requires *O*-GlcNAcylation for the proper functioning of intermediates in the pathway. The PPP produces biomolecules necessary for proliferation and mitosis (Patra and Hay, 2014). The end products of the pathway are only made when glucose-6-phosphate dehydrogenase (G6PD) is *O*-GlcNAcylated and activated, thus catalyzing the reactions and allowing flux through the PPP. When G6PD is not *O*-GlcNAcylated, cells do not grow, proliferate, or divide as much. This shows how runaway proliferation which is a defining characteristic of cancer cells can be mitigated by targeting *O*-GlcNAc and *O*-GlcNAc mediated pathways.

Increased *O*-GlcNAcylation is shown to be protective and is associated with many cellular stress states (Martinez et al., 2017). Some models suppose that while elevated *O*-GlcNAc acutely is protective, chronic elevation is toxic. This is not unique to *O*-GlcNAcylation as acute heat shock protein upregulation plays a role in protecting cells from heat-shock, yet chronic heat shock protein upregulation is toxic in cells (Roth et al., 2014). *O*-GlcNAc reduces ER stress, regulates the heat shock response, disrupts protein aggregation in neurodegenerative diseases, mediates the inflammatory driven stress response, and enables cancer cells to resist stressful tumor microenvironments (Zachara et al., 2004; Akimoto et al., 2007; Pathak et al., 2012; Yi et al., 2012; Yuzwa et al., 2014; Jang et al., 2015; Rao et al., 2015).

O-GlcNAcylation is also implicated in cell differentiation. Stem cells frequently have higher protein *O*-GlcNAcylation than cells in more differentiated states in numerous cell types (Shi et al., 2010; Andres et al., 2017; Sherazi et al., 2018). How *O*-GlcNAcylation, or the lack thereof signals cells through the differentiation process is not entirely

understood. It is known that cancer cells, including leukemias, display aberrant *O*-GlcNAcylation and that genetic manipulations and drugs that target the *O*-GlcNAc cycle are effective in the induction of terminal differentiation, however the exact mechanism of this regulation is not fully characterized in HL-60 cells. It is known that reduction in *O*-GlcNAcylation can limit cell proliferation (Rao et al., 2013) which could prove useful to limit uncontrolled cell division characteristic of many cancers. Currently, how neutrophilic differentiation of HL-60 cells and the transcription of genes associated with this process is related to reduction of global *O*-GlcNAcylation remains to be fully elucidated.

O-GlcNAcylation detection has evolved over time. Many methods of *O*-GlcNAcylated protein detection are available and used by researchers today. Ma and Hart, (2017) detail a mass spectrometric approach to the detection of modified proteins. Collision induced dissociation allows for confirmation of *O*-GlcNAcylation but does not allow for site mapping due to breakage of the glycosidic bond holding the sugar moiety to the peptide due to the high energy in the ionization process by the electro-spray ionizer whereas a fragmentation method known as electron transfer dissonance can be used for site mapping. Non-mass spectrometry methods of detecting *O*-GlcNAcylated proteins have been developed in recent years. There are at least two commercial antibodies available. The RL2 antibody available from ThermoFisher Scientific and the CTD110.6 available from Santa Cruz Biotechnologies. The RL2 antibody can be used in both western blot and immunodot blot to detect modified proteins (Asthana et al., 2018; Sherazi et al., 2018).

O-GlcNAcylation of galectins is not well understood. Only two of the sixteen galectins are known to be *O*-GlcNAcylated, although bioinformatic analysis using the YinOYang 1.2 server suggested that all galectins have at least one predicted site of *O*-GlcNAcylation (Tazhitdinova and Timoshenko, 2020). Galectin-1 was confirmed to be a target of this post translational modification in previous literature (Hart et al., 2011). Only recently has there been evidence of *O*-GlcNAcylation on another galectin, which has been reported in a 2021 pre-print confirming *O*-GlcNAcylation of galectin-3 (Mathew et al., 2021).

O-GlcNAcylation is associated with cancerous cells (Shi et al., 2010) and is reduced in HL-60 cells that have undergone granulocytic differentiation (Asthana et al., 2018, Sherazi et al., 2018). Differentiation of HL-60 cells also changes the expression of various galectins in response to the differentiation stimulus (Abedin et al., 2003; Vinnai et al., 2017). *O*-GlcNAcylation is thought to inhibit the secretion of proteins including galectins. Secretion is hypothesized to first necessitate de-glycosylation of the GlcNAc moiety. In this study, we will investigate the relationship between the expression and secretion of galectins, neutrophilic differentiation, and *O*-GlcNAcylation.

1.4 Hypothesis

Most studies on galectins in cell biology focus on only a single galectin with galectin-3 being very commonly studied. Previously, the Timoshenko lab has characterized the regulation of galectins in response to oxidate stress and DMSO-induced granulocytic differentiation in HL-60 cells (Vinnai et al., 2017) as well as a global inhibition of *O*-GlcNAcylation in differentiated cells (Sherazi et al., 2018). Others have also investigated the differentiation in response to disruption of *O*-GlcNAc homeostasis (Asthana et al., 2018). To date, no other published studies have attempted to combine galectin regulation in neutrophilic differentiation with *O*-GlcNAc mediated regulation of galectins. A new concept has been introduced recently that *O*-GlcNAcylation may regulate trafficking and secretion of galectins (Tazhitdinova and Timoshenko, 2020) (Figure 5). My study was designed within the framework of this concept to examine the effects of *O*-GlcNAc modulation on the expression and secretion of galectins in the context of neutrophilic differentiation.

I will test the hypothesis that by inhibiting *O*-GlcNAcylation, HL-60 cells will undergo terminal differentiation and secrete galectins in a manner similar to the effects of a known differentiation stimulus. The rationale behind this study is that reduction in *O*-GlcNAcylation is important in the differentiation process and that by targeting enzymes that regulate *O*-GlcNAc homeostasis, we can induce terminal differentiation in the absence of a traditional differentiation stimulus such as ATRA.

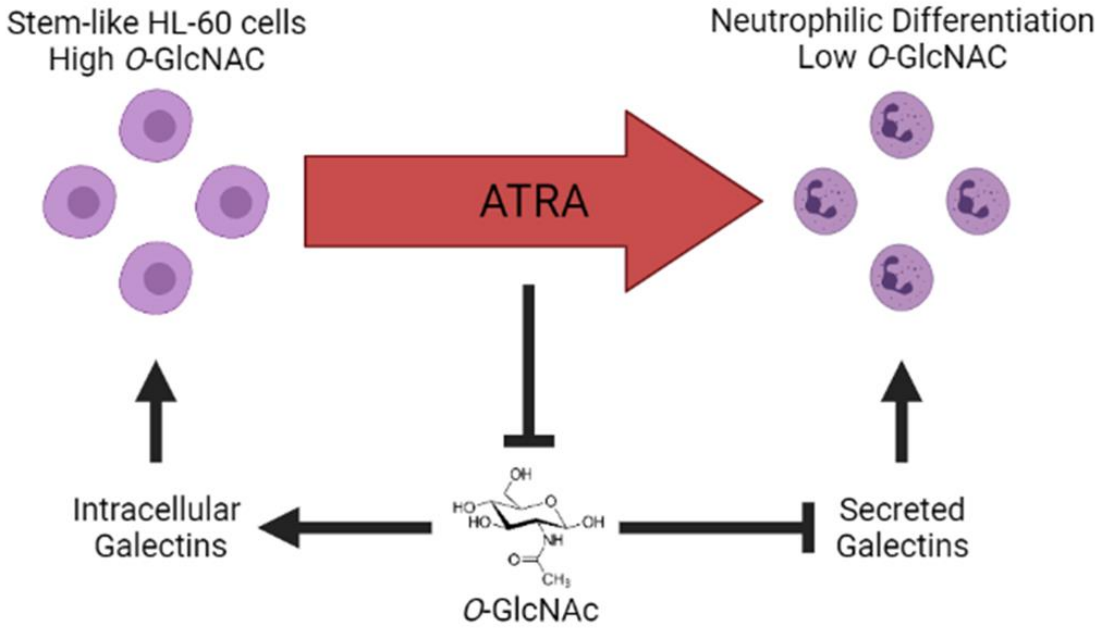

Stem-like HL-60 cells
High O-GlcNAC
Neutrophilic Differentiation
Low O-GlcNAC
ATRA
Intracellular
Galectins
Secreted
Galectins
O-GlcNAc

Figure 5. *O*-GlcNAc sensitive neutrophilic differentiation of HL-60 cells.

The above figure illustrates the hypothesis of this study, detailing the proposed mechanism of *O*-GlcNAc mediated HL-60 differentiation (adapted from Tazhitdinova and Timoshenko, 2020). ATRA is a known inducer of neutrophilic differentiation and is associated with a decrease in global *O*-GlcNAcylation of intracellular proteins. *O*-GlcNAc cycle enzyme inhibition is proposed to induce differentiation and regulate galectin expression bypassing the ATRA-RARα interaction.

Objective 1: To characterize and compare ATRA-induced neutrophilic differentiation of HL-60 cells in serum-containing and serum-free media as a platform for modeling galectin-free conditions in cell culture.

Objective 2: To study the expression profile and distribution of galectins between intracellular and extracellular compartments and compare this distribution between progenitor and differentiated HL-60 cells.

Objective 3: To study the effects of *O*-GlcNAc cycle enzyme inhibitors on the expression profiles and distribution of galectins between intracellular and extracellular compartments of HL-60 cells.

Chapter 2

2 Materials and Methods

2.1 Chemicals and solutions

All-*trans*-retinoic-acid (ATRA) (R2625), 6-diazo-5-oxo-L-norleucine (DON) (D2141), horseradish peroxidase (P-8250), thiamet G (TG) (SML0244), DMSO (D26500), Dulbecco′s Phosphate Buffered Saline (DPBS) with $MgCl_2$ and $CaCl_2$ (D8862), Immobilon Classico Western HRP substrate (WBLUC500), and scopoletin (S2500) were purchased from Sigma-Aldrich Canada (Oakville, ON). DPBS without calcium and magnesium (311-425-CL), 100x ITS Universal Culture Supplements (315-081-QL), and Iscove's Modification of Dulbecco's Modified Eagle Medium (IMDM) (319-105-CL) were purchased from Wisent Bio Products (Saint-Jean-Baptiste, QC EDTA (EB0185), Mammalian Protease Inhibitor Cocktail (BS386), 2x RIPA Buffer IIII with EDTA and EGTA (pH 7.4) (RB4477), sodium azide (NaN_3) (S2002), bovine serum albumin (BSA) (AD0023), and sodium orthovanadate (Na_3VO_4) (SB0869) were purchased from BioBasic (Markham, ON). SsoAdvanced Universal SYBR® Green Supermix (1725274), and non-fat dry milk blotting-grade blocker (1706404) were purchased from Bio-Rad (Mississauga, ON). VECTASHIELD Vibrance Mounting Medium with DAPI (H-1800) was purchased from Vector Laboratories (Burlingame, CA). Phorbol 12-myristate 13-acetate (PMA) (PMA168) was purchased from BioShop (Toronto, ON). TRIzol® (15596018) was purchased from Life Technologies (Toronto, ON). Fetal Bovine Serum (FBS) (12484-028), and SYBR™ Safe DNA Gel Stain (S33102) were purchased from ThermoFisher (Mississauga, ON). Acryl/Bis™ 29:1 ULTRA PURE 40% (w/v) Solution (0311) was purchased from VWR Life Science (Mississauga, ON). Froggarose LE Molecular Biology Grade Agarose (A87) was purchased from FroggaBio (Concord, ON). OGT inhibitor 2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-5-thio-a-D-glucopyranose (Ac-5SGlcNAc) was synthesized in Dr. Vocadlo laboratory (Gloster et al., 2011) and kindly provided as per Material Transfer Agreement between Simon Fraser University and the University of Western Ontario.

2.2 Cell culture and treatments

Human acute promyelocytic leukemia cell line HL-60 (ATCC® CCL-240™) was obtained from ATCC. Cells were cultured as a suspension in IMDM without antibiotics and supplemented with either 10% FBS (IMDM-FBS) or ITS (5 μg/mL human insulin, 5 μg/mL human transferrin, 5 ng/L selenous acid) (IMDM-ITS) in a humidified incubator at 37°C, 5% CO_2. Cells were sub-cultured two to three times weekly in 100 mm suspension culture dishes (83.3902.500) from Sarstedt and concentration was kept below 10^6 cells/mL. Cell viability was determined using the trypan blue (0.4%) exclusion test and was not less than 90% except where indicated. Population doubling time (PDT) of cell cultures was calculated using the following equation:

$$PDT = (T2-T1) / (3.322\log10(N2/N1))$$

where, N1 an N2 are cell concentrations at T1 and T2 time points within the exponential phase of cell growth.

To induce granulocytic differentiation, HL-60 cells were grown in either 60 mm or 100 mm suspension culture dishes from Sarstedt and treated with 1 μM ATRA for 72 hours as described elsewhere (Zhang et al., 2018). To change global *O*-GlcNAcylation, the cells were treated with 10 μM TG (OGA inhibitor) (Sherazi et al., 2018), 25 μM AC (OGT inhibitor) (Sherazi et al., 2018), or 12.5 μM DON (GFAT inhibitor) (Asthana et al., 2018) for up to 72 hours using either a single dose or daily supplementation every 24 hours.

2.3 RNA isolation, cDNA synthesis, and PCR gene expression assays

Following treatments, cells were centrifuged for 5 min at 300 × g, washed once with ice-cold DPBS without Mg^{2+} and Ca^{2+}, and the total mRNA was isolated from cell pellets using TRIzol® reagent according to manufacturer's protocol. The mRNA samples were dissolved in sterile nuclease-free water and their purity and quantity were assessed with the Thermo Scientific™ Nanodrop 2000c UV-Vis spectrophotometer considering an

$A_{260/280}$ ratio of > 1.8 as a minimum threshold for use in later assays. cDNA was synthesized from 500 ng of mRNA using the High-Capacity cDNA reverse transcription Kit from Applied Biosystems (4368813) as per the manufacturer's protocol.

All primers for polymerase chain reaction (PCR) assays were synthesized by *BioCorp* UWO *Oligo*Factory (Western University, Department of Biochemistry, Ontario) and verified by BLAST (Table 1). The T100 Thermo Cycler (Bio-Rad) was used to perform end-point PCR amplification in 20 μL reaction volumes as described elsewhere (Vinnai et al., 2017). Reaction mixtures consisted of 2X Taq FroggaMix from FroggaBio (FBTAQM), 1 μM forward primer, 1 μM reverse primer and 0.5 μL of undiluted cDNA. PCR amplicons were separated on a 2% agarose gel prepared in TAE buffer (20 mM Tris, 40 mM Acetic Acid, 1.2 mM EDTA) containing SYBR® Safe and imaged using a Molecular Imager GelDoc XRT (BioRad) with Image Lab software, version 6.0 (Bio-Rad). The SsoAdvanced Universal SYBR® Green kit (cat. 1725274) from Bio-Rad was used for real-time quantitative PCR (qPCR) amplification of samples (20 μL) containing 1 μM forward primer, 1 μM reverse primer, and 0.5 μL of undiluted cDNA. Quantification of mRNA transcripts was performed in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) using 2-step cycling regime following polymerase activation for 2 min at 95°C: 40 cycles of denaturation (5 s, 95°C) and annealing (25 s, 60-65°C). Temperatures varied based on primer pair and are listed in Table 1. Specificity of qPCR amplification was verified by the presence of a single melt peak at a specific temperature for each amplicon. Relative transcript levels were calculated by the Livak method ($2^{-\Delta\Delta CT}$) using β–actin (*ACTB*) as a reference gene (Schmittgen and Livak, 2008).

2.4 Protein isolation and immunoblotting

Following treatments, cells were centrifuged at 300 × g for 5 min and the supernatants (8-10 mL) were collected and frozen at -80°C to use later for analyses of extracellular proteins. The cell pellets were washed twice with ice-cold PBS and lysed in 300 μL of RIPA buffer (50 mM Tris-HCl, pH 7.4, 0.1% sodium dodecyl sulfate, 0.5% w/v sodium deoxycholate, 5 mM EDTA, 1 mM EGTA and 150 mM NaCl) supplemented with 100

Table 1. PCR primer sequences and characteristics

Gene name		Sequence 5'-3'	Size bp	2 step cycling	PMID reference
LGALS1	F	CCTGGAGAGTGCCTTCGAGTG	220	95°C (5 s) 60°C (25 s)	23108139
	R	CTGCAACACTTCCAGGCTGG			
LGALS3	F	CAGAATTGCTTTAGATTTCCAA	108	95°C (5 s) 60°C (25 s)	18202194
	R	TTATCCAGCTTTGTATTGCAA			
LGALS8	F	TGGGGACGGGAAGAGATCAC	172	95°C (5 s) 62°C (25 s)	30504378
	R	TGCCATAAATGCCCAGAGTGTC			
LGALS9	F	CTTTCATCACCACCATTCTG	91	95°C (5 s) 62°C (25 s)	18202194
	R	ATGTGGAACCTCTGAGCACTG			
LGALS10 (CLC)	F	GGATGGCCAAGAATTTGAACTG	82	95°C (5 s) 62°C (25 s)	30504378
	R	GGTGTAAGAGGATTGGCCATTG			
LGALS12	F	TGTGAGCCTGAGGGACCA	111	95°C (5 s) 65°C (25 s)	18202194
	R	GCTGAGATCAGTTTCTTCTGC			
NCF1	F	GTCAGATGAAAGCAAAGCGA	93	95°C (5 s) 60°C (25 s)	23147401
	R	CATAGTTGGGCTCAGGGTCT			
NCF2	F	CGAGGGAACCAGCTGATAGA	767	95°C (5 s) 60°C (25 s)	10754283
	R	CATGGGAACACTGAGCTTCA			
MYC	F	CCTGGTGCTCCATGAGGAGAC	95	95°C (5 s) 62°C (25 s)	Designed
	R	CAGACTCTGACCTTTTGCCAGC			
ACTB	F	TCAGCAAGCAGGAGTATGACGAG	95	95°C (5 s) 60°C (25 s)	30504378
	R	ACATTGTGAACTTTGGGGGATG			

μM phenylmethylsulfonyl fluoride, 100 μM Na_3VO_4, 1 μM bestatin, 1.4 μM E-64, 1 μM leupeptin, 0.08 μM aprotinin and 1.5 μM pepstatin A. The cell lysates were incubated on ice for 10 min and centrifuged at 12,000 × g for 15 min at 4^{o}C. Total protein concentration was quantified using the DC^{TM} Protein Assay Kit II (5000112) from Bio-Rad and BSA as a standard by measuring absorbance at 655 nm in a model 3550 Microplate Reader (Bio-Rad).

To analyze global levels of selected galectins and *O*-GlcNAc, the protein samples were immobilized onto nitrocellulose membrane (0.22 μm pore size, GE Healthcare 1060006) using a Bio-Dot® Microfiltration apparatus (Bio-Rad) as previously described (Sherazi et al., 2018). Briefly, the nitrocellulose membrane was prewetted in Tris-buffered saline (TBS: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl) and placed into the apparatus. Each well was loaded with 200 μl of whole cell protein extract (20 μg/mL in DPBS) and proteins were transferred to the membrane by gravity filtration for 90 min. Afterwards, the membrane was blocked with a 3% non-fat dry milk reconstituted in TBST buffer (TBS plus 0.05% Tween 20) for 60 min at room temperature, washed three times with TBST and incubated overnight at 4^{o}C with an appropriate primary antibody solution (15 mL) in TBST supplemented with 5% BSA and 0.05% sodium azide. The membrane was washed again three times with TBST and then incubated with either goat-anti-mouse or goat-anti-rabbit IgG-HRP conjugated secondary antibody diluted 1:10,000 in a 5% non-fat dry milk reconstituted in TBST for 1 hour at room temperature with light agitation. Lastly, the TBST-rinsed membrane with immunodots was evenly covered with 1.5 mL of Immobilon Classico chemiluminescent reagent (Sigma-Aldrich, WBLUC0500) and imaged using a ChemiDoc XRS system with Quantity One Software, version 4.6.6. For western blot analysis, samples of cell lysates were mixed with a loading buffer (2% (w/v) SDS, 0.04% BME), boiled for 5 min, loaded on 4% polyacrylamide stacking gel (25 μg protein per lane), and electrophoretically separated by SDS-PAGE in 12% polyacrylamide resolving gel (100 V, until blue tracker dye reaches the bottom of the gel). The proteins were transferred from the gel to 0.22 μm PVDF membrane (Sigma-Aldrich, 03010040001) at a constant voltage of 20 V at 4^{o}C overnight using wet transfer in a 25 mM Tris, 190 mM glycine, 20% (v/v) methanol (pH 8.3) buffer. Blocking,

antibody incubation, and imaging steps for western blots were performed as described above for immunodot blots. The primary and secondary antibodies were obtained from Santa Cruz Biotechnology (Dallas, Texas), Abcam Canada (Toronto, Ontario), and ThermoFisher Scientific (Mississauga, ON) (Table 2).

2.5 Scopoletin assay to measure PMA-induced H_2O_2 production

H_2O_2 production by HL-60 cells was measured by scopoletin/peroxidase fluorescence assay using an AMINCO-Bowman Series 2 luminescence spectrometer as described earlier (Vinnai et al., 2017) with minor modifications. Briefly, cell culture suspension was centrifuged for 5 min at 300 × g, and cells were washed twice with and resuspended in DPBS containing Mg^{2+} and Ca^{2+} to yield a concentration of $6x10^5$ cells/mL. Scopoletin (500 nM) and HRP (20 μg/mL) were added to 2 mL of the cell suspension, which was prewarmed for 5 min in a cuvette of the fluorimeter at 37°C before adding 1 μM of PMA. A decrease in fluorescence of scopoletin was monitored at 460 nm (excitation at 350 nm) and the maximal slope of the recorded traces was calculated in RStudio (version 4.0.5) to quantify the rate of H_2O_2 generation by cells (Vinnai et al., 2017).

2.6 Fluorescence microscopy and nuclear staining

To prepare slides for nuclear staining, HL-60 cells were diluted to a concentration of $5x10^5$ cells/mL in 200 μL and were centrifuged onto glass slides for 5 min at 500 rpm using the Shandon Cytospin 2 centrifuge (Vinnai, 2016). The attached cells were fixed with chilled methanol for 5 min and staining of nuclei was achieved by mounting the slides with DAPI-contained VECTASHIELD Vibrance Mounting Medium. An AxioImager A1 fluorescent microscope (Carl Zeiss) equipped with DAPI filter cube was used to view the slides. The images were taken with a high-resolution monochrome XCD-X700 CCD camera (Sony Corporation) using Northern Eclipse 8.0 software from Empix Imaging (Mississauga, Ontario).

Table 2. Primary and secondary antibodies used for immunodot blot and western blot assays.

Antigen	Host	Type	Conjugates	Dilution	Source	Catalog #
Galectin-1	Mouse	monoclonal	N/A	1:800	Santa-Cruz	sc-166618
Galectin-3	Rabbit	polyclonal	N/A	1:200	Santa-Cruz	sc-20157
Galectin-9	Rabbit	monoclonal	N/A	1:1,000	Abcam	ab227046
Galectin-10	Rabbit	monoclonal	N/A	1:10,000	Abcam	ab157475
O-GlcNAc (RL2)	Mouse	monoclonal	N/A	1:1,000	ThermoFisher	MA1-072
Mouse IgG (H+L)	Goat	polyclonal	HRP	1:10,000	ThermoFisher	A16066
Rabbit IgG (H+L)	Goat	polyclonal	HRP	1:10,000	ThermoFisher	A16096

2.7 Enzyme-linked immunosorbent assay (ELISA)

Extracellular concentrations of human galectin-1, galectin-3, and galectin-9 were measured using SimpleStep ELISA® kits from Abcam as described by the manufacturer. For all three kits, cell culture supernatant samples were diluted 4-fold in the supplied sample diluent that was found to fall within the range of concentrations that correspond to the standard curve. Absorbance at 450 nm was measured using a Model 3550 Microplate Reader (Bio-Rad).

2.8 Statistical analysis

One-way ANOVA followed by Tukey's multiple comparisons test, two-way ANOVA followed by Tukey's multiple comparison test, Student's t-test and Pearson's correlation test were performed using GraphPad Prism, version 6.01 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). All experiments were conducted using a minimum of three biological replicates. Data were presented as means ± SD and differences between means were considered significant at $p < 0.05$.

Chapter 3

3 Results

3.1 Comparison of HL-60 growth and neutrophilic differentiation in serum-containing and serum-free media

HL-60 acute promyelocytic leukemia cells were grown in IMDM supplemented with either 10% FBS or ITS supplement. A growth curve was constructed for cells grown in both media by taking measurements for cell concentration every 12 hours for 5 days. Cell viability was also assessed simultaneously and was consistently above 90% live cells for IMDM-ITS cultured cells. IMDM-FBS cultured cells were also consistently above 90% live cells except for the final measurement at 5 days after passaging when the viability dropped to 87% while the number of cells was still increasing (Figure 6A). From an initial seed concentration of 10^5 for cells culture in both media, the cell concentration of the cultures steadily increased at all measurement times. After 120 hours had passed, the IMDM-FBS cultured cells reached a final concentration of $(1.93 \pm 0.30) \times 10^6$ cells/mL, while the IMDM-ITS cultured cells reached a final concentration of $(1.44 \pm 0.72) \times 10^6$ cells/mL (Figure 6B). The population doubling time was calculated using the doubling time equation (Equation 1) found in section 2.2, considering two consecutive time points within the log phase. The growth rate was not significantly different between IMDM-FBS cells and IMDM-ITS cells which doubled in 22.3 ± 2.7 and 23.4 ± 4.9 hours respectively (Figure 6C).

ATRA is a well characterized and widely used method of inducing granulocytic differentiation of promyeloid cells. To assess the degree of differentiation of HL-60 cells, a range of concentrations was used to treat HL-60 cells for 72 hours. Nuclear morphology analysis of HL-60 cells treated with 1 μM ATRA showed many segmented nuclei characteristic of granulocytic differentiation (Figure 7A). IMDM-FBS cells that were treated with 1 μM ATRA were significantly more differentiated than untreated controls, where 89.4% of cells displayed neutrophil-like nuclear morphology compared to 6.6% of untreated cells ($p < 0.0001$) HL-60 cells grown in a serum-free culture showed a similar

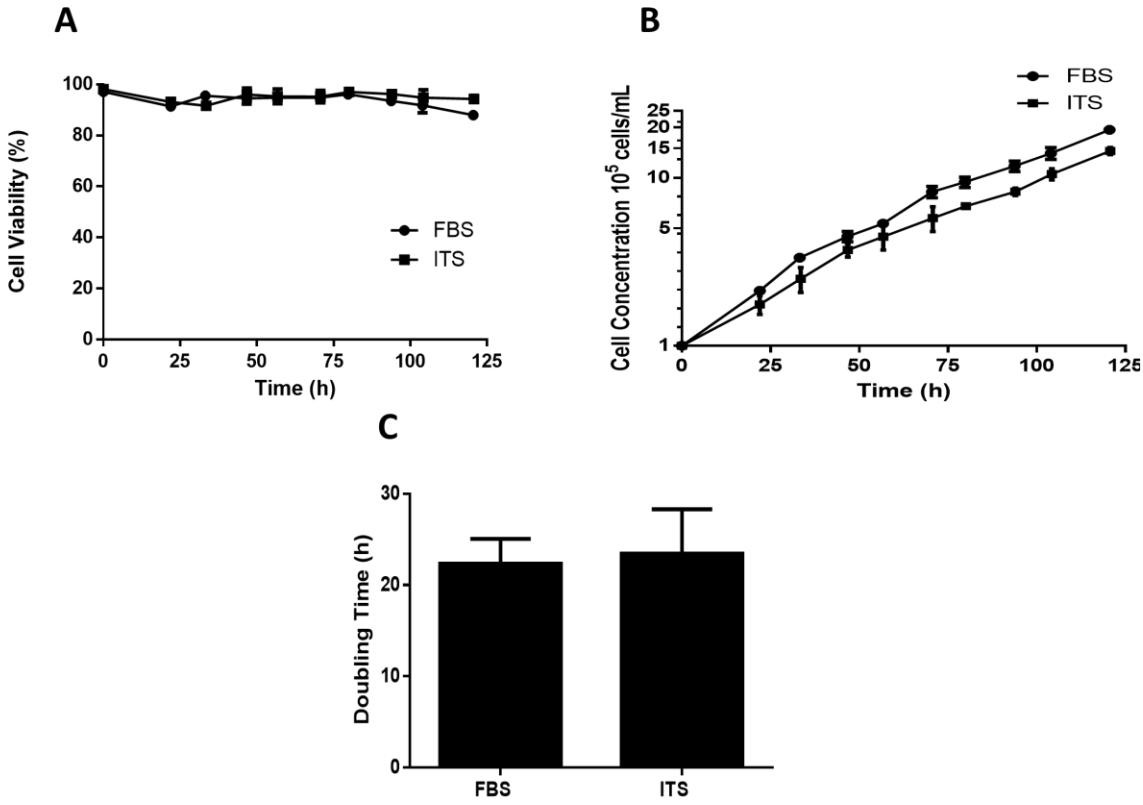

A
Cell Viability (%)
100
80
60
40
20
0
0
25
50
75
100
125
Time (h)
FBS
ITS
B
Cell Concentration 10^5 cells/mL
25
20
15
10
5
1
0
25
50
75
100
125
Time (h)
FBS
ITS
C
Doubling Time (h)
30
20
10
0
FBS
ITS

Figure 6. HL-60 cells grown in serum-containing and serum-free media show similar growth and viability.

HL-60 cells were cultured in 60 mm suspension culture dishes at an initial concentration of 10^5 cells/ml and were grown for 5 days in a humidified incubator set to 37^{o}C and 5% CO_2. Every 12 hours, cell concentration and viability were recorded. Cell viability (A) and concentration (semi-log scale) (B) are presented as the mean ± SD, n=3. The population doubling time of HL-60 cells was calculated considering two consecutive measurements within the log phase of the growth curve (C), mean ± SD, n=3. Significant differences were determined using Student's t-test. * $p < 0.05$

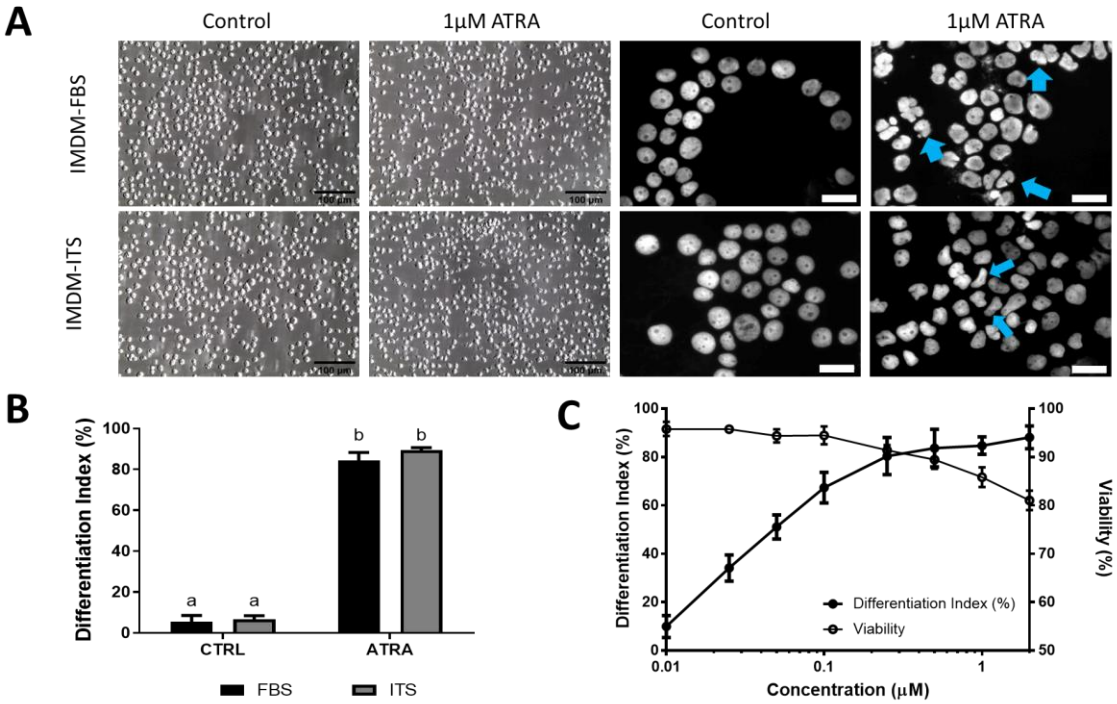

A
Control
1μM ATRA
Control
1μM ATRA
IMDM-FBS
IMDM-ITS
B
Differentiation Index (%)
100
80
60
40
20
0
a
a
b
b
CTRL
ATRA
FBS
ITS
C
Differentiation Index (%)
Viability (%)
Differentiation Index (%)
Viability
0.01
0.1
1
Concentration (μM)

Figure 7. Confirmation of ATRA-induced differentiation of HL- 60 cells by nuclear morphology in the presence and absence of serum.

HL-60 cells were cultured in IMDM supplemented with 10% FBS, or with ITS supplement and were induced to differentiate by the treatment of cells with vehicle (CTRL), or 1 μM ATRA for 72 hours. (A) HL-60 cell morphology (left panels) and nuclei staining by DAPI (right panels). (B) Differentiation Index was determined as the percentage of nuclei that displayed abnormal nuclear shape (black bars: serum containing medium; grey bars; serum-free medium) (C). Scale bars represent 100 μM (bright field) and 20 μM (fluorescence). The differentiation index was a dose dependent response and is correlated negatively with cell viability. Significant differences were determined using two-way ANOVA followed by Tukey's HSD test and are represented as different letters, considering $p < 0.05$.

response to ATRA as 84.3% of cells displayed neutrophil-like nuclear morphology relative to only 5.3% of untreated cells ($p < 0.0001$). The differentiation index did not significantly differ between medium type for either treatment (Figure 7A-B). Initially, the amount of differentiated cells was proportional to the concentration of ATRA used in culture as ATRA concentrations between 0.01 and 0.1 μM induced significantly more differentiation step wise with each increase in concentration. However, a plateau was reached at concentrations above 0.25 μM, as the amount of differentiated cells was not significantly different as all such concentrations (0.25, 0.5, 1.0, and 2.0 μM ATRA) induced a high level of differentiation (80.4, 83.7, 84.7, and 88.1 % respectively) ($F_{7, 16} = 191.0$, $p < 0.0001$). Cell viability was assessed with trypan blue (0.4%) and a hemocytometer. ATRA concentration was inversely proportional to the cell viability (Figure 7C). Interestingly, the significance pattern for the cell viability across different ATRA concentrations was the opposite of that seen for the differentiation index. All ATRA concentrations of 0.1 μM or below were not statistically different however, for each concentration above (0.25, 0.5, 1.0, and 2.0 μM ATRA) the viability of cell cultures was significantly less at each increasing concentration interval ($F_{7, 16} = 58.47$, $p < 0.0001$) with the lowest viability (81%) seen at an ATRA concentration of 2.0 μM (Figure 7C)

The expression of genetic biomarkers of differentiation was determined using RT-PCR and RT-qPCR. HL-60 cells grown in both serum-supplemented and serum-free conditions express significantly higher amounts of neutrophil specific genes in response to 1 μM of ATRA for 72 hours (Figure 8A). Semiquantitative analysis of endpoint RT-PCR products separated on a 2% agarose gel show that *NCF1* was upregulated 12.1-fold in IMDM-FBS grown cells relative to untreated cells ($p < 0.0001$) and 60.5-fold in IMDM-ITS grown cells relative to untreated cells ($p < 0.0001$) (Figure 8B). *NCF2* was upregulated 4.1-fold and 8.7-fold in IMDM-FBS and IMDM-ITS respectively after treatment with 1 μM ATRA for 72 hours (Figure 8C). The expression of the oncogenic marker *MYC* was also investigated for its response to ATRA. Upon treatment with ATRA, and subsequent differentiation, expression of this oncogene was determined by RT-qPCR and was significantly decreased in both IMDM-FBS cells (22.4-fold, $p < 0.001$) and in IMDM-ITS cells (3.6-fold, $p < 0.01$) (Figure 8D).

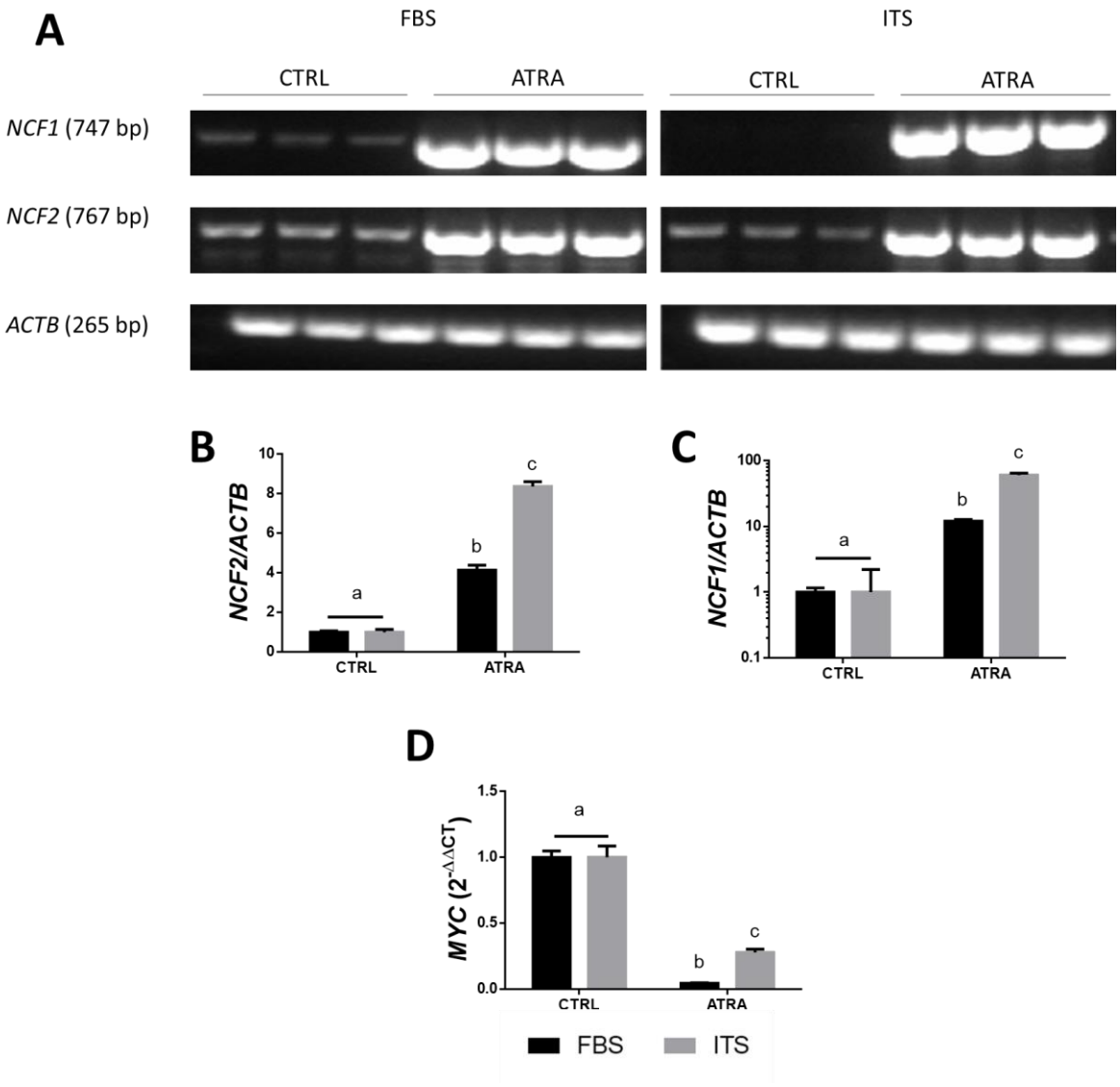

A
FBS
ITS
CTRL
ATRA
CTRL
ATRA
NCF1 (747 bp)
NCF2 (767 bp)
ACTB (265 bp)
B
NCF2/ACTB
10
8
6
4
2
0
a
b
c
CTRL
ATRA
C
NCF1/ACTB
100
10
1
0.1
a
b
c
CTRL
ATRA
D
MYC (2^-ΔΔCT)
1.5
1.0
0.5
0.0
a
b
c
CTRL
ATRA
FBS
ITS

Figure 8. HL-60 cells fated to the granulocytic lineage express significantly more neutrophil associated genes.

HL-60 cells were treated with vehicle (CTRL) or 1 μM ATRA for 72 hours to induce neutrophilic differentiation. (A) Expression of neutrophil specific genes *NCF1* and *NCF2* was assessed by RT-PCR and products were separated on a 2% agarose gel. (B-C) Expression of *NCF1* and *NCF2* was quantified with a semi-quantitative method using *ACTB* as an internal control. (D) Oncogenic marker of pluripotency *MYC* was quantified using RT-qPCR with the Livak Method ($2^{-\Delta\Delta Ct}$) using *ACTB* as an internal control (black bars: serum-containing medium; grey bars: serum-free medium). Data are presented as the mean ± SD, n=3-5. Significant differences were determined using two-way ANOVA followed by Tukey's HSD test and are represented as different letters, considering $p < 0.05$.

Global *O*-GlcNAcylation was assessed by immunodot blot assay using the pan-specific monoclonal antibody RL2 (Figure 9A). Densitometric analysis showed a significant decrease in the *O*-GlcNAcylation ($F_{1,8} = 110.3$, $p < 0.0001$) of cytosolic proteins isolated from differentiated cells relative to promyeloid cells (Figure 9B). This reduction in *O*-GlcNAcylation was seen in both media types and was not significantly different between the two types of media.

3.2 Galectins are differentially regulated in response to ATRA-induced HL-60 differentiation in both serum-containing and serum-free media

Genetic regulation of six galectins was assessed using RT-qPCR to compare their expression between neutrophil-like cells and promyeloid stem-like cells. HL-60 cells were grown in either a serum-containing medium, or a serum-free medium to assess the effect of serum growth factors on galectin regulation. *LGALS1* was significantly downregulated after neutrophilic differentiation in both media ($F_{1,8} = 379.3$, $p < 0.0001$.) The choice of medium had no effect on the down regulation of *LGALS1* as cells grown in both media showed a significant drop in *LGALS1* mRNA after treatment with 1 μM ATRA (Figure 10A). Two-way ANOVA shows that three galectin genes *LGALS3* ($F_{1,8} = 243.8$, $p < 0.0001$), *LGALS10* ($F_{1,8} = 522.9$, $p < 0.0001$), and *LGALS12* ($F_{1,8} = 243.4$, $p < 0.0001$) were significantly upregulated in HL-60 cells after neutrophilic differentiation. Interestingly for *LGALS10* ($F_{1,8} = 477.8$, $p < 0.0001$) and *LGALS12* ($F_{1,8} = 71.14$, $p < 0.0001$), there were stronger inductions of these genes in serum-containing medium whereas for *LGALS3* ($F_{1,8} = 0.1945$, $p > 0.05$), there was no statistically significant effect of medium type on the upregulation. The genetic expression of *LGALS9* increased upon ATRA treatment only in the serum-free medium (Figure 10D). Two-way ANOVA showed that there was a significant increase in *LGALS9* expression when cells were treated with ATRA and grown in a serum-free medium ($F_{1,8} = 9.094$, $p < 0.05$), a pattern opposite of that seen with *LGALS10* and *LGALS12*. The expression of *LGALS8* was unaffected by both medium type and by treatment with ATRA (Figure 10C).

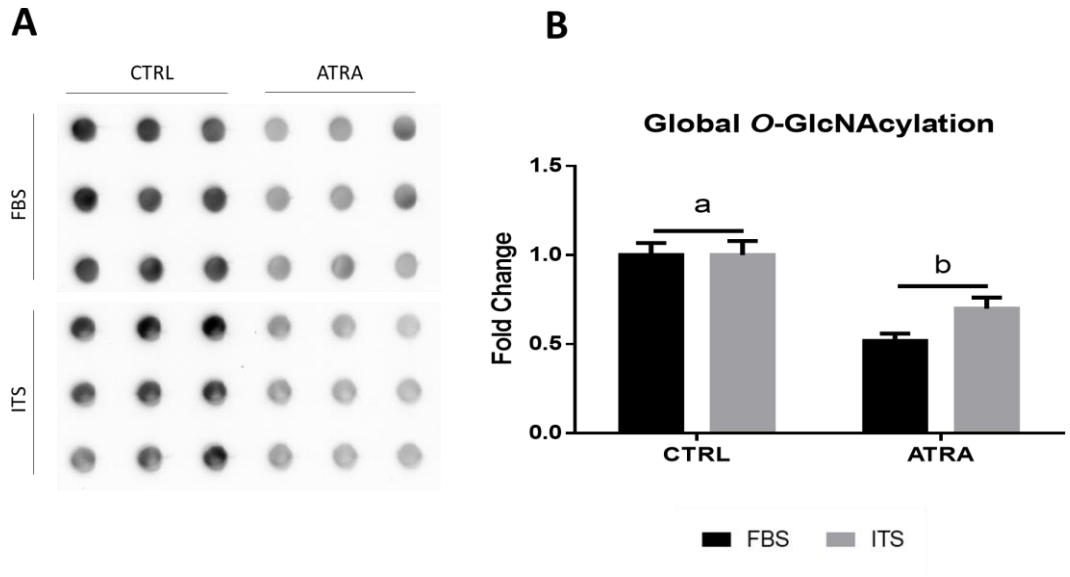

A
CTRL
ATRA
FBS
ITS
B
Global O-GlcNAcylation
Fold Change
1.5
1.0
0.5
0.0
a
b
CTRL
ATRA
FBS
ITS

Figure 9. Global *O*-GlcNAcylation as a biomarker of neutrophilic differentiation.

HL-60 cells grown in IMDM with (black bars) and without serum (grey bars) were differentiated to neutrophil-like cells with 1 μM ATRA for 72 hours and protein lysates were assessed for *O*-GlcNAcylation using the RL2 antibody (A). Densitometric analysis shows that the global *O*-GlcNAcylation is significantly decreased ($p < 0.0001$) regardless of media supplement using two-way ANOVA followed by Tukey's HSD test (B).

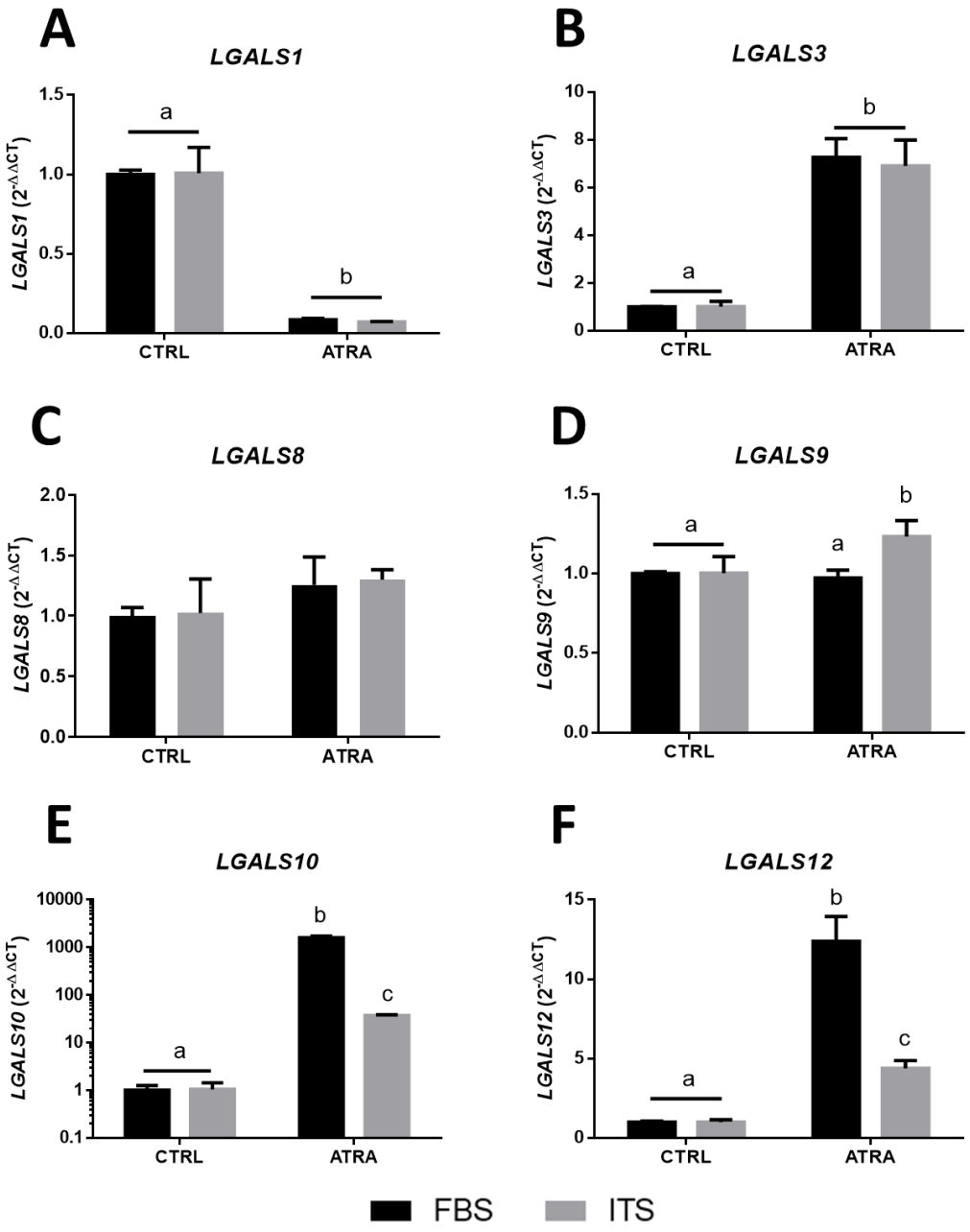

A
LGALS1
LGALS1 (2^-ΔΔCT)
1.5
1.0
0.5
0.0
a
b
CTRL
ATRA
B
LGALS3
LGALS3 (2^-ΔΔCT)
10
8
6
4
2
0
a
b
CTRL
ATRA
C
LGALS8
LGALS8 (2^-ΔΔCT)
2.0
1.5
1.0
0.5
0.0
CTRL
ATRA
D
LGALS9
LGALS9 (2^-ΔΔCT)
1.5
1.0
0.5
0.0
a
a
b
CTRL
ATRA
E
LGALS10
LGALS10 (2^-ΔΔCT)
10000
1000
100
10
1
0.1
a
b
c
CTRL
ATRA
F
LGALS12
LGALS12 (2^-ΔΔCT)
15
10
5
0
a
b
c
CTRL
ATRA
FBS
ITS

Figure 10. Neutrophilic differentiation induced changes in the transcription of galectin genes.

ΔΔCt for galectin mRNA transcript expression in HL-60 cells grown with (black bars) and without serum (grey bars) after 72 hours treatment with 1 μM ATRA relative to vehicle-treated (CTRL) cells for both conditions were determined using RT-qPCR. (A) *LGALS1*, (B) *LGALS3*, (C) *LGALS8*, (D) *LGALS9*, (E) *LGALS10*, and (F) *LGALS12*. *ACTB* was used as an internal control. Data are presented as the mean ± SD, n=3-5. Significant differences were determined using two-way ANOVA followed by Tukey's HSD test and are represented as different letters, considering $p < 0.05$.

The differences of transcript expression were correlated with western blot analyses (Figure 11A). Densitometric analysis of bands showed a significant decrease in the expression of galectin-1 in either medium after treatment with ATRA ($F_{1,8} = 44.85$, $p < 0.001$), but that the medium used did not significantly affect the expression of galectin-1 (Figure 11B). No significant differences in the expression of galectin-9 were seen between ATRA-treated and untreated cells ($F_{1,8} = 3.642$, $p > 0.05$), nor between serum-containing and serum-free medium ($F_{1,8} = 3.883$, $p > 0.05$) (Figure 11D). Two-way ANOVA determined that the expression of galectin-3 was significantly higher after treatment with ATRA ($F_{1,8} = 377.8$, $p < 0.0001$) and was also higher in cells grown in a serum-free medium ($F_{1,8} = 72.59$, $p < 0.0001$) (Figure 11C). The expression of galectin-10 was also significantly higher in cells treated with ATRA ($F_{1,8} = 333.9$, $p < 0.0001$). The expression of galectin-10 was also affected by the medium type, as seen by the greater fold change in expression in the serum-containing medium ($F_{1,8} = 3157.7$, $p < 0.0001$) (Figure 11E).

3.3 Alterations in the *O*-GlcNAcylation homeostasis of HL-60 cells affects the expression of galectins

HL-60 cells were treated with inhibitors of *O*-GlcNAc cycle related enzymes to determine the role of *O*-GlcNAcylation in the granulocytic differentiation process of promyeloid cells. Cell nuclei were stained with DAPI, and images were visually inspected to assess the percent of cells displaying segmented nuclei characteristic of neutrophilic differentiation (Figure 12A). Few untreated cells (5.4 % in IMDM-FBS, 6.6% in IMDM-ITS) had nuclear morphology characteristic of differentiation, suggesting that spontaneous differentiation is rare. Inhibition of OGA with TG also had no significant effect on the nuclear morphology, indicative of little to no differentiation occurring. Inhibitors that lower the global *O*-GlcNAcylation by inhibiting either OGT or GFAT induced changes in the nuclear morphology. With the inhibition of either of these enzymes, there were higher percentages of differentiated cells as measured by nuclear morphology relative to untreated cells. Inhibition of OGT by AC was associated with an increase of the differentiated appearing cells which was statistically significant whereas

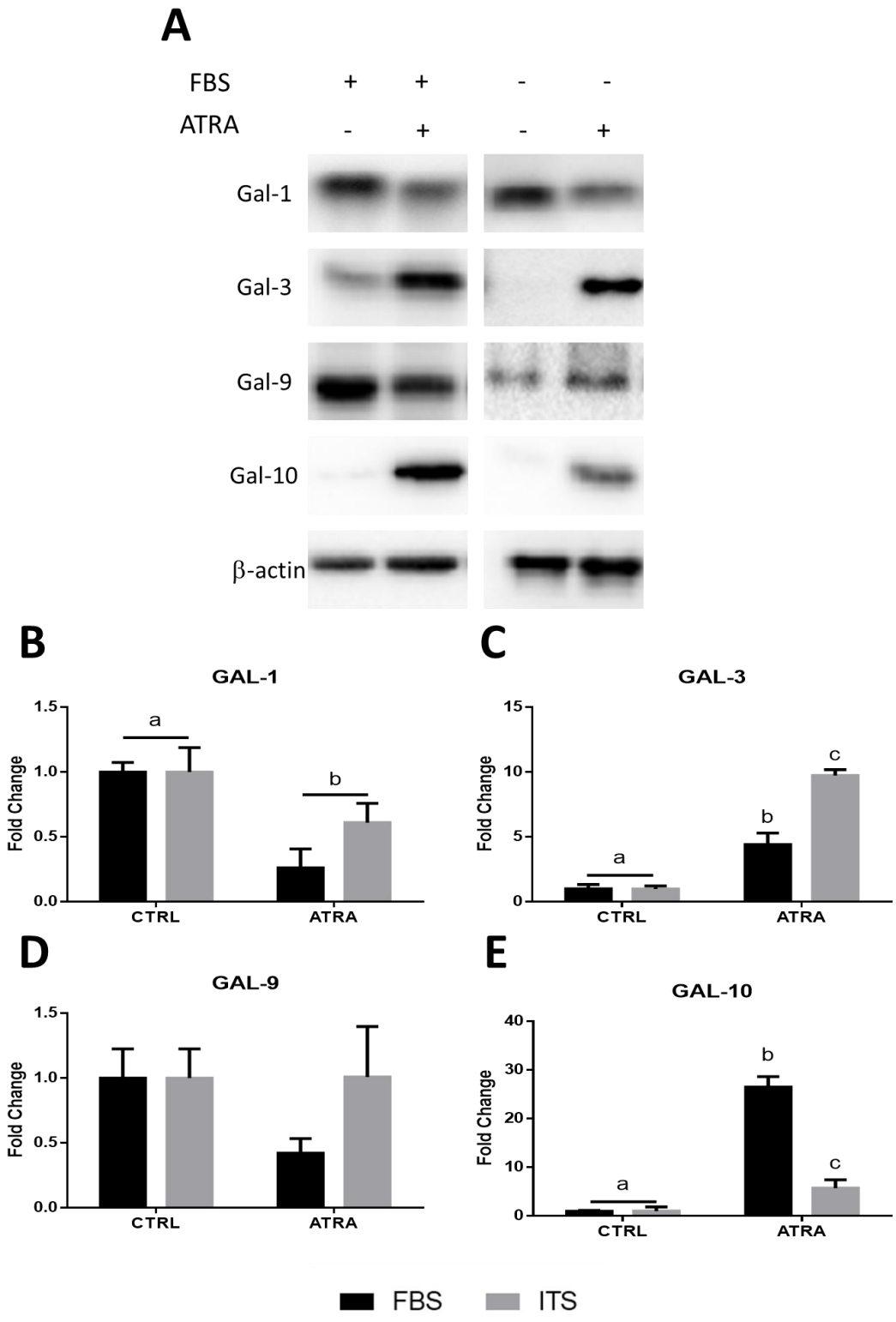
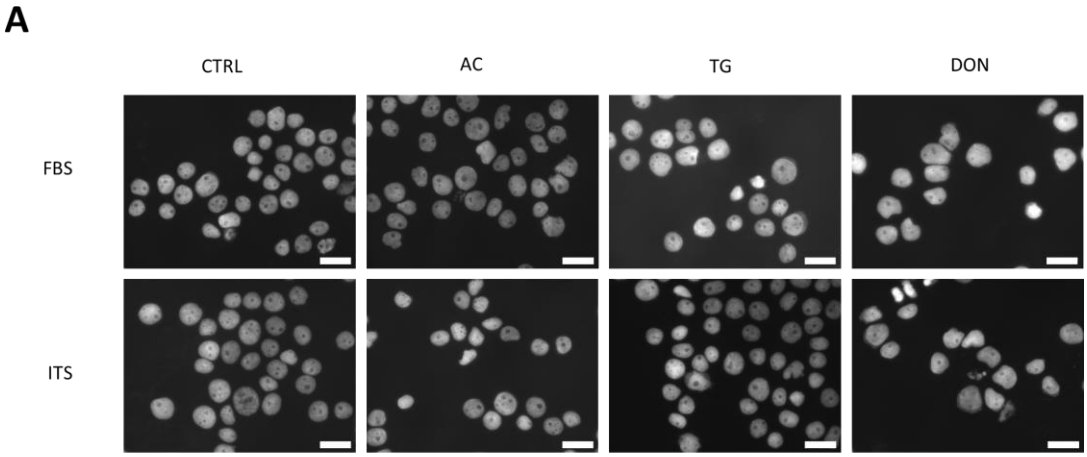

A
FBS + + - -
ATRA - + - +
Gal-1
Gal-3
Gal-9
Gal-10
β-actin
B
GAL-1
Fold Change
1.5
1.0
0.5
0.0
a
b
CTRL
ATRA
C
GAL-3
Fold Change
15
10
5
0
a
b
c
CTRL
ATRA
D
GAL-9
Fold Change
1.5
1.0
0.5
0.0
CTRL
ATRA
E
GAL-10
Fold Change
40
30
20
10
0
a
b
c
CTRL
ATRA
FBS
ITS

Figure 11. Galectins are differentially regulated between differentiated neutrophil-like cells and stem-like precursor HL-60 cells.

Immunoblots for galectins (A) in HL-60 cells grown with either 10% FBS (black bars), or ITS supplement (grey bars), treated with vehicle (CTRL), or 1 μM ATRA for 72 hours. Quantification of galectin-1 (B), galectin-3 (C), galectin-9 (D), and galectin-10 (E) by densitometric analysis of immunoblots) by densitometric analysis with ImageLab V6.0. β-actin was used as a loading control for western blot. Data are presented as the mean ± SD, n=3-5. Significant differences were determined using two-way ANOVA followed by Tukey's HSD test and are represented as different letters, considering $p < 0.05$.


A
CTRL
AC
TG
DON
FBS
ITS

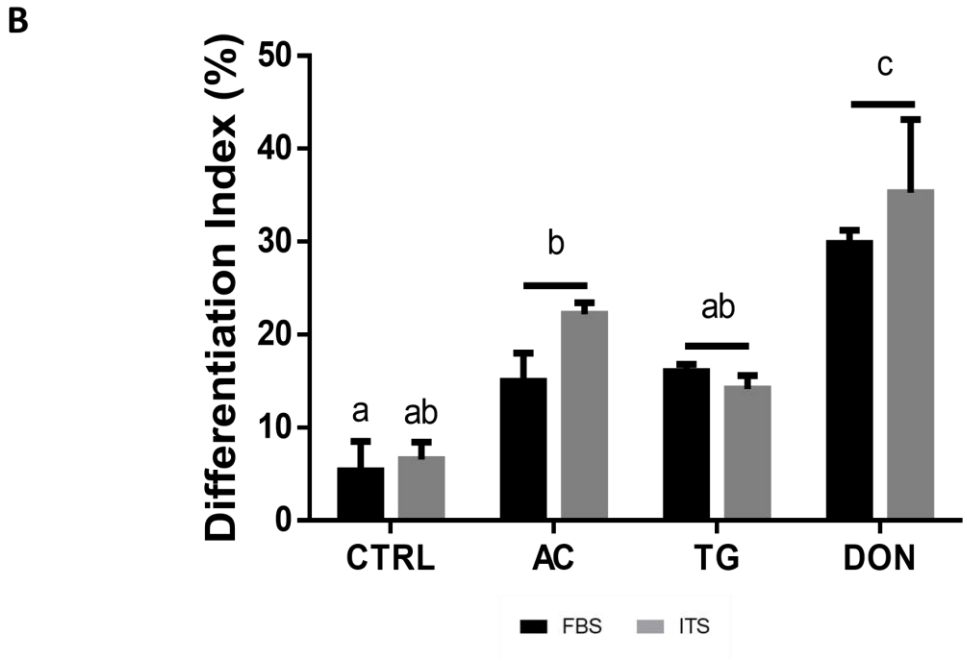

B
Differentiation Index (%)
50
40
30
20
10
0
a
ab
b
ab
c
CTRL
AC
TG
DON
FBS
ITS

Figure 12. The disruption of the *O*-GlcNAc homeostasis alters the nuclear morphology of HL-60 cell nuclei.

HL-60 cells were culture in IMDM supplemented with 10% FBS (black bars), or with ITS (grey bars) supplement and were induced to differentiate by the treatment of cells with vehicle (CTRL), 25 μM AC, 10 μM TG, or 12.5 μM DON for 72 hours with daily drug supplementation. (A) Nuclear morphology of treated and untreated cells were visualized by DAPI staining. Scale bars represent 20 μM. (B) Differentiation Index was determined as the percentage of nuclei that displayed abnormal nuclear shape. Data are presented as the mean ± SD, n=3-5. Significant differences were determined using two-way ANOVA followed by Tukey's HSD test and are represented as different letters, considering $p < 0.05$.

GFAT inhibitor DON saw an even higher percent of differentiated nuclei ($F_{3,16} = 64.02$, $p < 0.0001$), significantly more than both untreated cells and those cells treated with an OGT inhibitor (Figure 12B).

All galectins expressed in HL-60 cells showed significantly altered expression levels among various treatments disrupting the *O*-GlcNAc homeostasis. For both *LGALS3* ($F_{3,16} = 250.8$, $p < 0.0001$) and *LGALS8* ($F_{3,16} = 60.06$, $p < 0.0001$), there were significant increases in the expression of these gene in cells treated with DON for 72 hours. The medium used did not affect the expression of *LGALS3* nor *LGALS10* (Figure 13 B-C). *LGALS9* also showed no measurable difference between the two media used but did show a significant increase in expression in cells treated with TG ($F_{3,16} = 6.193$, $p < 0.01$) (Figure 13D). All other galectin genes were significantly altered in response to different treatments and media types. *LGALS1* expression was significantly increased in cells treated with AC and DON ($F_{3,16} = 446.6$, $p < 0.0001$), and it was significantly higher in DON-treated cells in serum-containing medium compared to serum-free medium ($F_{1,16} = 90.13$, $p < 0.01$) (Figure 13A). For both of *LGALS10* ($F_{3,22} = 5.063$, $p < 0.01$) and *LGALS12* ($F_{3,16} = 61.2$, $p < 0.0001$), two-way ANOVA revealed significantly higher expression of *LGALS10* (Figure 13E) in response to DON treatment, while *LGALS12* (Figure 13F) expression was significantly higher in both TG- and DON-treated cells. However, the DON-induced increase in expression of *LGALS10* ($F_{1,16} = 14.33$, $p < 0.01$), was only seen in serum-containing medium. The TG and DON-induced increase in expression of *LGALS12* ($F_{1,16} = 126.0$, $p < 0.0001$) similarly was significantly higher in cells grown in serum-containing medium.

The protein levels of galectins also showed changes when treated with *O*-GlcNAc cycle enzyme inhibitors corresponding roughly to their genetic regulation (Figure 14A). The expression of galectin-1 was significantly increased in response to DON-treatment ($F_{3,16} = 7.042$, $p < 0.01$), but only in serum-containing medium ($F_{1,16} = 7.936$, $p < 0.05$) (Figure 14B). The expression of galectin-9 was significantly decreased in response to DON- and AC-treatment ($F_{3,16} = 15.96$, $p < 0.0001$), however, this decrease was only seen in serum-containing medium with AC-treated cells ($F_{1,16} = 5.326$, $p < 0.05$) whereas galectin-9 expression was reduced in both media types upon treatment with DON (Figure 14D). The

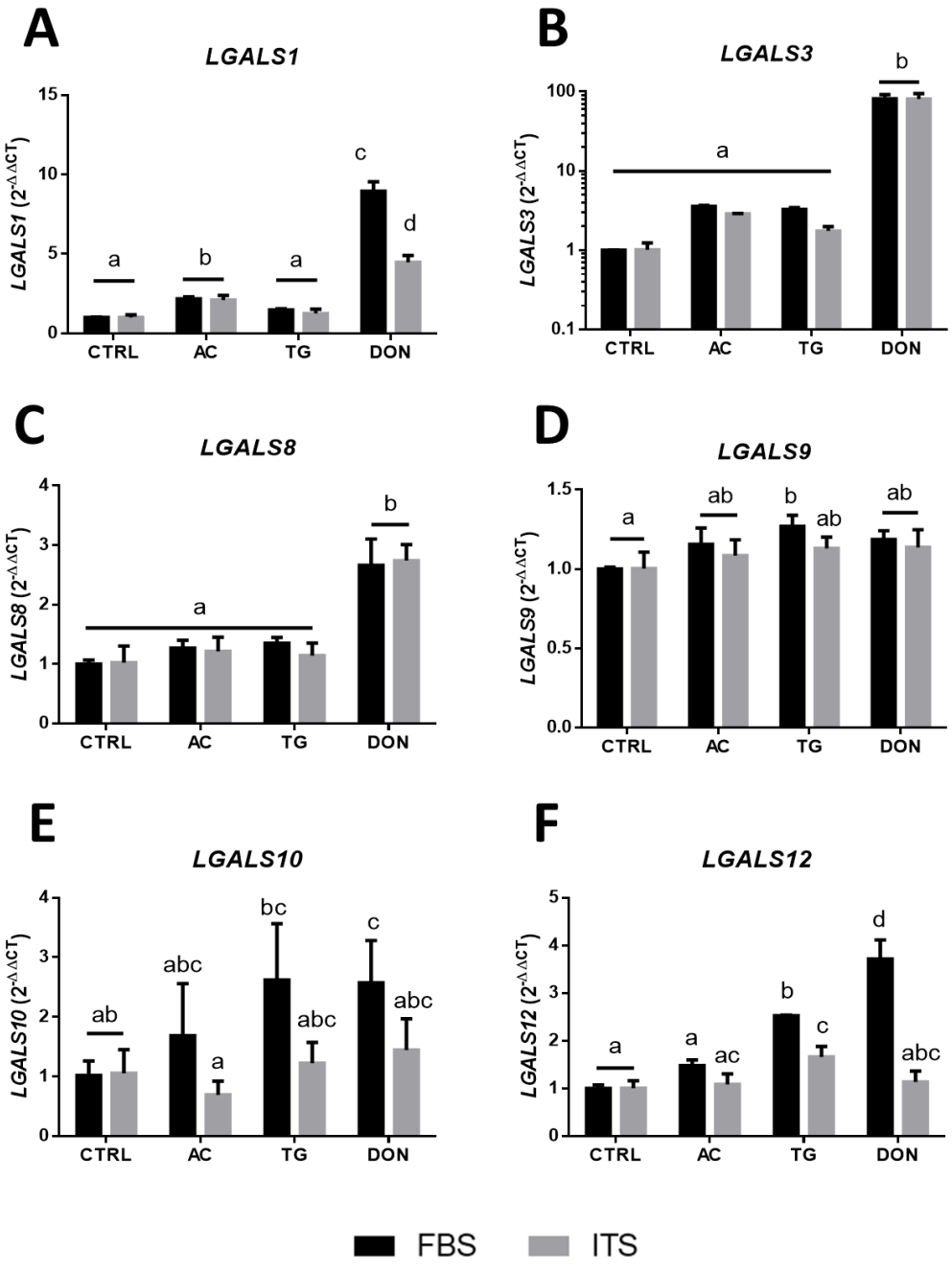

A
LGALS1
LGALS1 (2-ΔΔCT)
15
10
5
0
a
b
a
c
d
CTRL
AC
TG
DON
B
LGALS3
LGALS3 (2-ΔΔCT)
100
10
1
0.1
a
b
CTRL
AC
TG
DON
C
LGALS8
LGALS8 (2-ΔΔCT)
4
3
2
1
0
a
b
CTRL
AC
TG
DON
D
LGALS9
LGALS9 (2-ΔΔCT)
1.5
1.0
0.5
0.0
a
ab
b
ab
ab
CTRL
AC
TG
DON
E
LGALS10
LGALS10 (2-ΔΔCT)
4
3
2
1
0
ab
abc
a
bc
abc
c
abc
CTRL
AC
TG
DON
F
LGALS12
LGALS12 (2-ΔΔCT)
5
4
3
2
1
0
a
a
ac
b
c
d
abc
CTRL
AC
TG
DON
FBS
ITS

Figure 13. Galectin genes are differentially regulated by drugs disrupting the *O*-GlcNAc cycle in serum-free vs serum-containing media.

ΔΔCt for galectin mRNA transcript expression in HL-60 cells grown with (black bars) or without serum (grey bars) treated with either vehicle (CTRL), 25 μM AC, 10 μM TG, or 12.5 μM DON for 72 hours with daily drug supplementation for both media was determined using RT-qPCR. (A) *LGALS1*, (B) *LGALS3*, (C) *LGALS8*, (D) *LGALS9*, (E) *LGALS10*, and (F) *LGALS12*. *ACTB* was used as an internal control. Data are presented as the mean ± SD, n=3-5. Significant differences were determined using two-way ANOVA followed by Tukey's HSD test and are represented as different letters, considering $p < 0.05$.

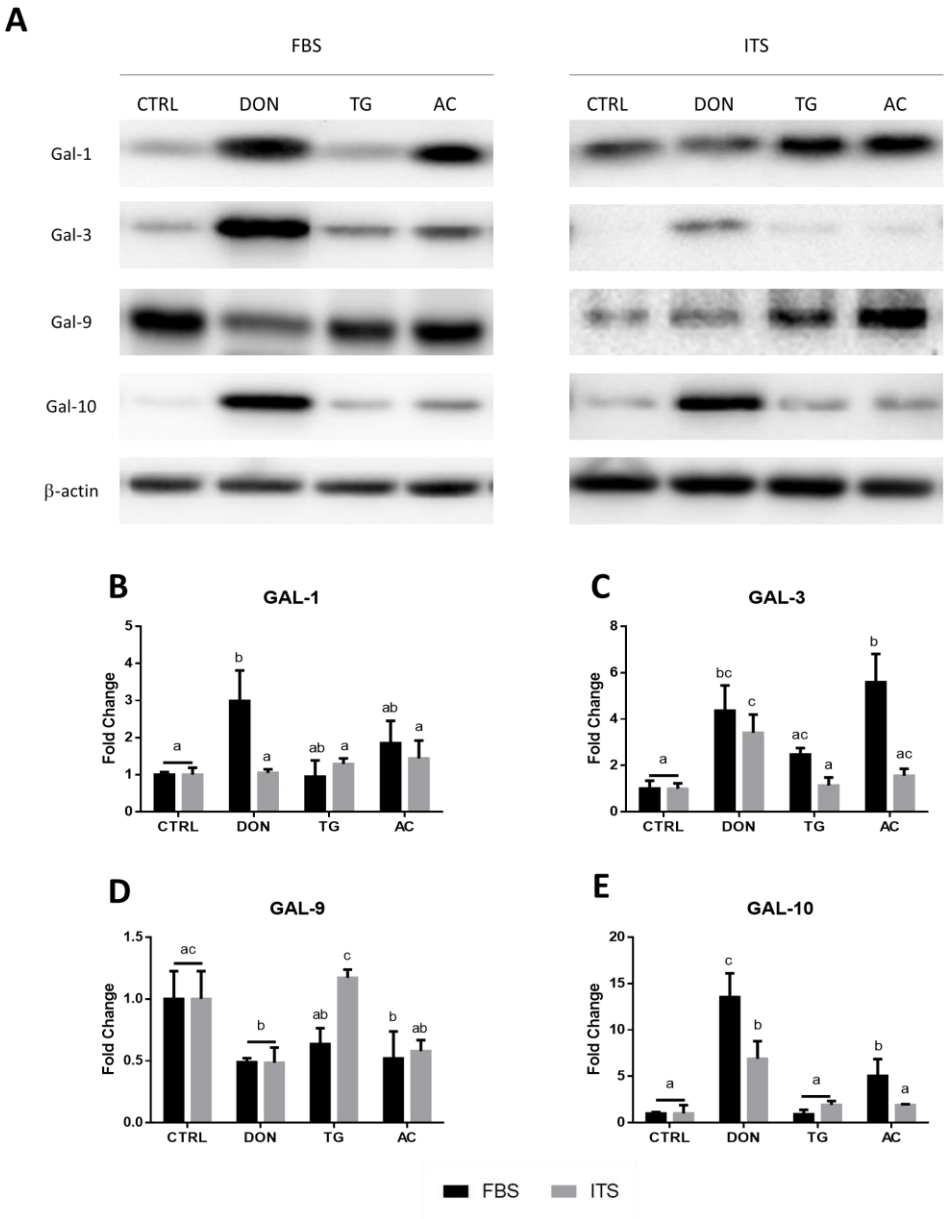

A
FBS
ITS
CTRL
DON
TG
AC
Gal-1
Gal-3
Gal-9
Gal-10
β-actin
B
GAL-1
C
GAL-3
D
GAL-9
E
GAL-10
Fold Change
FBS
ITS

Figure 14. Galectins are differentially regulated by drugs disrupting the *O*-GlcNAc cycle in a serum-containing media.

Immunoblots for global *O*-GlcNAcylation by pan-specific RL2 monoclonal antibody in (A) HL-60 cells grown with either 10% FBS, or ITS supplement, treated with either vehicle (CTRL), 25 μM AC, 10 μM TG, or 12.5 μM DON for 72 hours with daily drug supplementation relative to untreated cells. Quantification of galectin-1 (B), galectin-3 (C), galectin-9 (D), and galectin-10 (E) by densitometric analysis of immunoblots with ImageLab V6.0. β-actin was used as a loading control for western blot. Data are presented as the mean ± SD, n=3-5. Significant differences were determined using two-way ANOVA followed by Tukey's HSD test and are represented as different letters, considering $p < 0.05$.

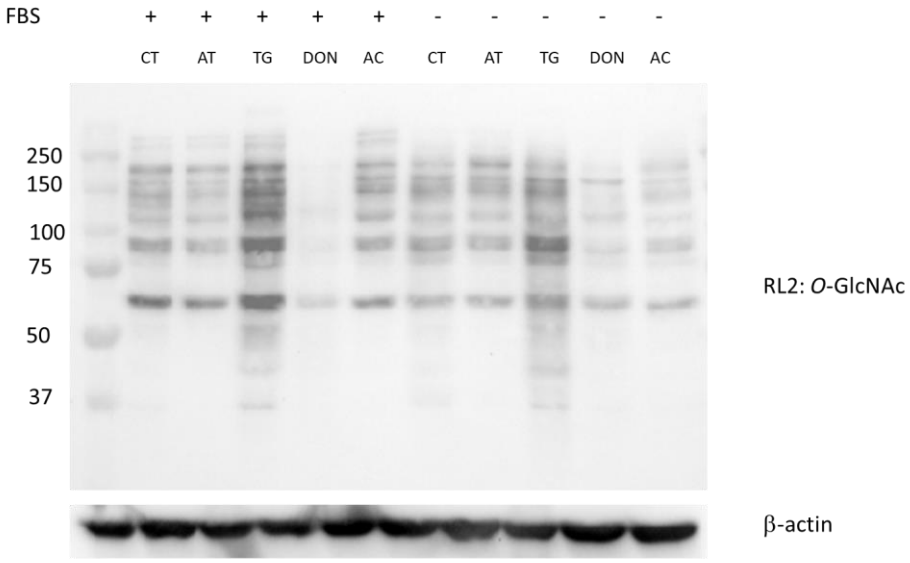
protein expression of galectin-3 and galectin-10 displayed similar patterns to each other. DON treatment resulted in significantly higher expression of both galectin-3 ($F_{3,17}$ = 26.27, $p < 0.0001$) and galectin-10 ($F_{3,16}$ = 59.68, $p < 0.0001$) (Figure 14B, D), as did AC-treatment, but only in cells grown in serum-containing medium for both galectin-3 ($F_{1,17}$ = 34.49, $p < 0.0001$) and galectin-10 ($F_{1,16}$ = 15.83, $p < 0.01$). Furthermore, the protein fold change in the expression of galectin-10 after DON-treatment was also significantly higher in cells grown in a serum-containing media.

To confirm the action of drugs disrupting the *O*-GlcNAc cycle, global *O*-GlcNAcylation was assessed by western blot analysis using the RL2 antibody (Figure 15A). Treatment with AC significantly reduced *O*-GlcNAcylation relative to untreated cells in a manner comparable to ATRA-treated cells, while DON-treated cells showed a much stronger reduction in *O*-GlcNAcylation. Cells treated with TG had many more *O*-GlcNAcylated proteins relative to all other treatment groups ($F_{4,20}$ = 188.9, $p < 0.0001$). The effects of drugs that disrupt the *O*-GlcNAc cycle enzymes exhibit their effects regardless of the medium type as there was no significant difference between these media types (Figure 15B).

3.4 Effects of ATRA and *O*-GlcNAc cycle inhibitors on the secretion of galectins from HL-60 cells

A previous study by Asiamah et al. (2018) indicated that galectins are contained within bovine serum products. As such, only cells cultured in a serum-free medium were used to assess the secretion of galectins in order to eliminate background galectins from the cultured medium. Cell culture supernatants were collected and tested for the extracellular concentrations of galectin-1, galectin-3, and galectin-9 using ELISA. There was only a significant increase in the extracellular galectin-1 concentration in cultures of cells treated with DON ($F_{4,10}$ = 33.53, $p < 0.0001$) (Figure 16A). With regards to both galectin-3 ($F_{4,24}$ = 10.12, $p < 0.0001$) and galectin-9 $F_{4,10}$ = 41.16, $p < 0.0001$, there were significant increases in the secretion of these proteins when treated with either ATRA or DON compared to all other treatments for both proteins (Figure 16B-C). Surprisingly, the OGT inhibitor AC although very potent in its ability to reduce *O*-GlcNAcylation of intracellular proteins, did not significantly increase the secretion of any galectins assayed

A


FBS
+ + + + + - - - - -
CT AT TG DON AC CT AT TG DON AC
250
150
100
75
50
37
RL2: O-GlcNAc
β-actin

B

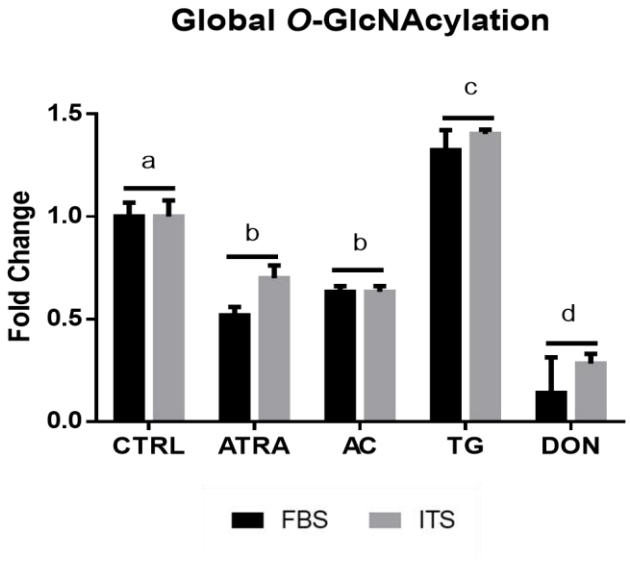

Global O-GlcNAcylation
Fold Change
1.5
1.0
0.5
0.0
a
b
b
c
d
CTRL
ATRA
AC
TG
DON
FBS
ITS

Figure 15. Global *O*-GlcNAcylation is perturbed by drugs targeting *O*-GlcNAc cycle enzymes in HL-60 cells grown with and without serum.

Immunoblots for global *O*-GlcNAcylation by pan-specific RL2 monoclonal antibody in (A) HL-60 cells grown with either 10% FBS, or ITS supplement, treated with either 1 μM ATRA, 25 μM AC, 10 μM TG, or 12.5 μM DON for 72 hours with daily drug supplementation relative to untreated cells. Quantification of *O*-GlcNAcylation was determined by the integral intensity of bands within each individual lane by densitometric analysis of immunoblots. Quantification of *O*-GlcNAcylated proteins (G) by densitometric analysis with ImageLab V6.0. β-actin was used as a loading control for western blot. Data are presented as the mean ± SD, n=3-5. Significant differences were determined using two-way ANOVA followed by Tukey's HSD test and are represented as different letters, considering $p < 0.05$.

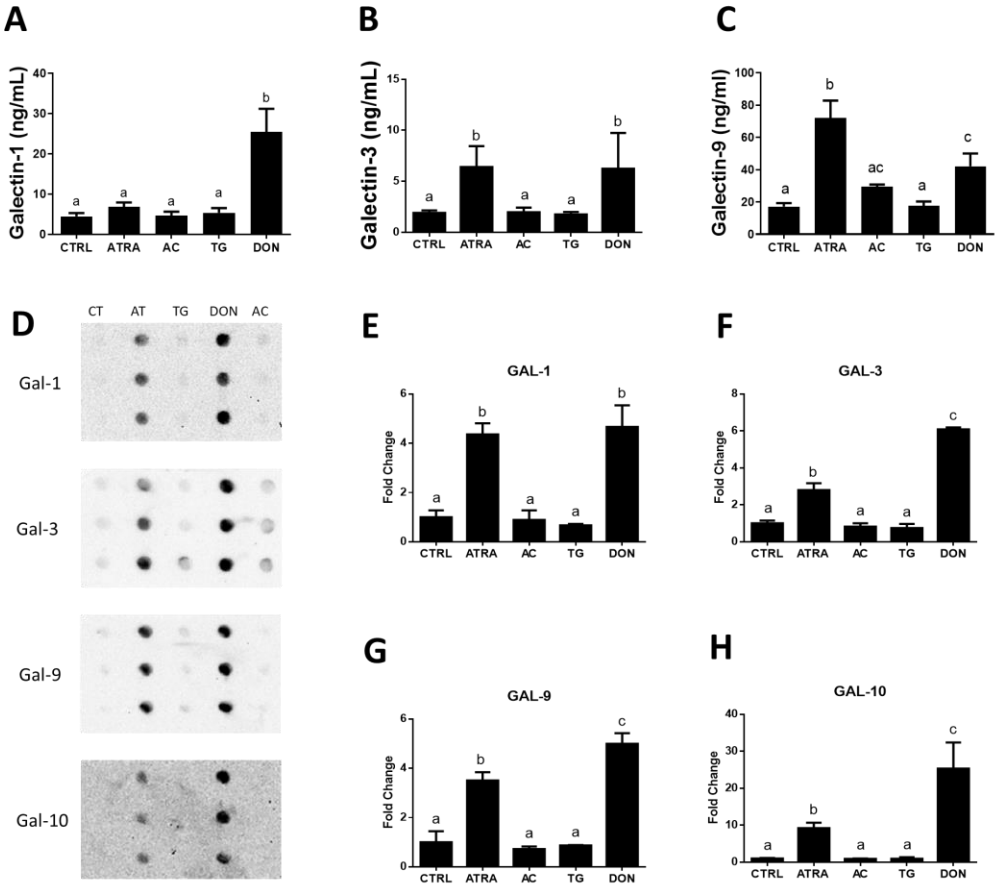

A
Galectin-1 (ng/mL)
CTRL ATRA AC TG DON
B
Galectin-3 (ng/mL)
CTRL ATRA AC TG DON
C
Galectin-9 (ng/ml)
CTRL ATRA AC TG DON
D
CT AT TG DON AC
Gal-1
Gal-3
Gal-9
Gal-10
E
GAL-1
Fold Change
CTRL ATRA AC TG DON
F
GAL-3
Fold Change
CTRL ATRA AC TG DON
G
GAL-9
Fold Change
CTRL ATRA AC TG DON
H
GAL-10
Fold Change
CTRL ATRA AC TG DON

Figure 16. The secretion of galectins is related to the *O*-GlcNAc status of the cell.

The extracellular galectin concentrations for Gal-1, Gal-3, and Gal-9 were assessed for HL-60 cells grown with ITS treated with either vehicle (CTRL), 1 μM ATRA, 25 μM AC, 10 μM TG, or 12.5 μM DON for 72 hours with daily drug supplementation relative to untreated cells was determined using ELISA. The concentration (ng/mL) of galectins -1, -3, and -9 (A-C) found in the cell culture supernatant varied among treatments. The extracellular galectins were quantified by densitometric analysis of immunoblots (D). The relative intensities of antibody signals were normalized to untreated HL-60 cells and normalized to the cell concentration (E-H). Data are presented as the mean ± SD, n=3-5. Significant differences were determined using one-way ANOVA followed by Tukey's HSD test and are represented as different letters, considering $p < 0.05$.

by ELISA. Extracellular proteins from HL-60 cell culture supernatants were immobilized on nitrocellulose membranes and blotted for galectins (Figure 16 D). The secretion of galectin-1 ($F_{4,10} = 50.47$, $p < 0.0001$), galectin-3 ($F_{4,10} = 313.1$, $p < 0.0001$), galectin-9 ($F_{4,10} = 111.4$, $p < 0.0001$), and galectin-10 ($F_{4,10} = 39.80$, $p < 0.0001$) were significantly increased in response to ATRA and DON treatments for all galectins (Figure 16E-H).

3.5 Reduction of *O*-GlcNAc in HL-60 cells leads to PMA-induced generation of H_2O_2 in both serum-containing and serum-free media

Granulocytic differentiation of HL-60 cells resulted in the expression of proteins that comprise the NADPH-oxidase complex which is responsible for the production of hydrogen peroxide. Differentiation of HL-60 cells into neutrophil-like cells in a serum-containing medium was confirmed in both ATRA and DON treatments for 72 hours ($F_{4,10} = 464.7$, $p < 0.0001$) by the high relative expression of *NCF1* (Figure 17A). In the absence of serum in the growth medium, ATRA and DON treatments similarly show significant increased expression of *NCF1* ($F_{4,10} = 222.8$, $p < 0.0001$) (Figure 17 B).

The upregulation of neutrophil-associated biomarkers of differentiation corresponded to the increased production of hydrogen peroxide via the NADPH oxidase complex. Hydrogen peroxide production was induced by PMA exposure and was measured by the reduction of scopoletin fluorescence. HL-60 cells treated with ATRA or DON oxidized scopoletin faster than untreated cells and reached minimal fluorescence before then end of the time trace (Figure 17 C-D). The rate of hydrogen peroxide production was calculated using RStudio V4.0.5 software and all measurements were normalized to the ATRA positive control. HL-60 cells treated with DON produced hydrogen peroxide upon induction by PMA at rates not significantly different than the ATRA positive control in both serum-containing ($F4_{,18} = 17.42$, $p < 0.0001$) and serum-free ($F_{4,18} = 21.88$, $p < 0.0001$) media (Figure 17 E-F). In both media, the cells treated with ATRA or DON produced more hydrogen peroxide than cells treated with other drugs or untreated cells as there was no significant difference in the rates of hydrogen peroxide production in cells treated with TG or AC from untreated, stem-like promyeloid cells.

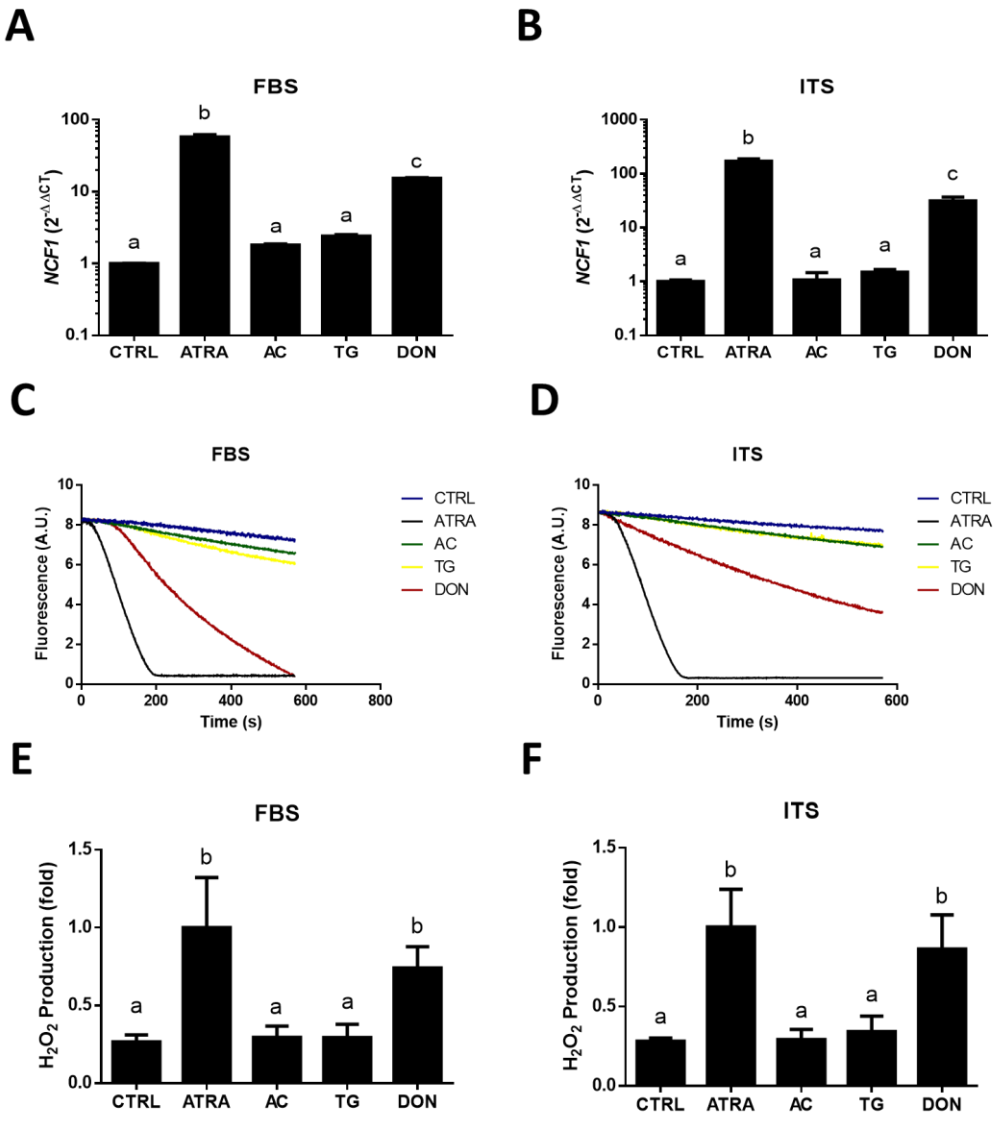

A
FBS
NCF1 (2^-ΔΔCT)
100
10
1
0.1
a
b
a
a
c
CTRL
ATRA
AC
TG
DON
B
ITS
NCF1 (2^-ΔΔCT)
1000
100
10
1
0.1
a
b
a
a
c
CTRL
ATRA
AC
TG
DON
C
FBS
Fluorescence (A.U.)
Time (s)
CTRL
ATRA
AC
TG
DON
D
ITS
Fluorescence (A.U.)
Time (s)
CTRL
ATRA
AC
TG
DON
E
FBS
H2O2 Production (fold)
1.5
1.0
0.5
0.0
a
b
a
a
b
CTRL
ATRA
AC
TG
DON
F
ITS
H2O2 Production (fold)
1.5
1.0
0.5
0.0
a
b
a
a
b
CTRL
ATRA
AC
TG
DON

Figure 17. H_2O_2 production via NADPH oxidase activity is a key component of neutrophilic pathogen response.

The relative expression of *NCF1* transcript abundance for serum-containing and serum-free HL-60 cells treated with vehicle (CTRL), 1 μM ATRA, 25 μM AC, 10 μM TG, or 12.5 μM DON for 72 hours with daily drug supplementation was determined using RT-qPCR considering *ACTB* as a housekeeping gene (A-B). The oxidation of scopoletin occurs in the presence of hydrogen peroxide and is determined by a decrease in fluorescence over time (C-D). Rate of H_2O_2 production (standardized to fold expression) as a measure of cellular differentiation, induced by the PKC activator PMA, in HL-60 cells. Data are presented as the mean ± SD, n=5. Significant differences among treatments were determined using one-way ANOVA followed by Tukey's HSD test and are represented as different letters considering $p < 0.05$.

3.6 Correlative analysis validates galectins and *O*-GlcNAcylation as biomarkers of cell differentiation

To identify a specific correlation between galectins, global *O*-GlcNAcylation, and cellular differentiation, a Pearson's correlation was conducted based on treatments with 25 μM Ac-5sGlcNAC, 10 μM thiamet G, and 12.5 μM 6-diazo-5-oxo nor -L-leucine. Pearson's correlation measures the strength of linear association between two variables, in this case relative gene expression. *NCF1* and *MYC* were used as positive and negative markers of neutrophilic differentiation of HL-60 cells. The quantification of chemiluminescent signal produced by western blot with the RL2 primary antibody recognizing *O*-GlcNAcylated proteins measures the relative global *O*-GlcNAcylation. The Pearson's correlation test showed a significant negative correlative between global *O*-GlcNAcylation and the expression of three galectin genes: *LGALS1* ($p < 0.0001$, $R^2 = 0.6427$, n = 24), *LGALS3* ($p < 0.0001$, $R^2 = 0.6624$, n = 24), and *LGALS8* ($p < 0.0001$, $R^2 = 0.5977$, n = 24) (Figure 18 A-C).

A Pearson's correlation test was also used to determine how markers of neutrophilic differentiation were correlated with the expression of galectin genes. With respect to the neutrophil specific marker *NCF1*, two genes showed a significant positive relationship with *NCF1* expression, those being *LGALS1* ($p < 0.001$, $R^2 = 0.4137$, n = 24), *LGALS3* ($p < 0.0001$, $R^2 = 0.8406$, n = 24), and *LGALS8* ($p < 0.0001$, $R^2 = 0.8033$, n = 24) (Figure 19 A-C). The oncogenic marker *MYC* is associated with the maintenance of stem-like features in HL-60 cells. Five galectins showed significant negative correlation with expression of *MYC*: *LGALS1* ($p < 0.0002$, $R^2 = 0.7236$, n = 24), *LGALS3* ($p < 0.0001$, $R^2 = 0.5490$, n = 24), *LGALS8* ($p < 0.001$, $R^2 = 0.4475$, n = 24), *LGALS10* ($p < 0.05$, $R^2 = 0.1723$, n = 24), *LGALS12* ($p < 0.05$, $R^2 = 0.1779$, n = 24) (Figure 20 A-C, E, F).

A final correlation analysis was conducted to assess the relationship between global *O*-GlcNAcylation and the expression of biomarkers *NCF1* and *MYC*. *NCF1* is significantly negatively correlated with global *O*-GlcNAcylation ($p < 0.001$, $R^2 = 0.4897$, n = 24). Whereas *MYC* showed a significant positive correlation with global *O*-GlcNAcylation ($p < 0.0001$, $R^2 = 0.6676$, n = 24) (Figure 21 A-B).

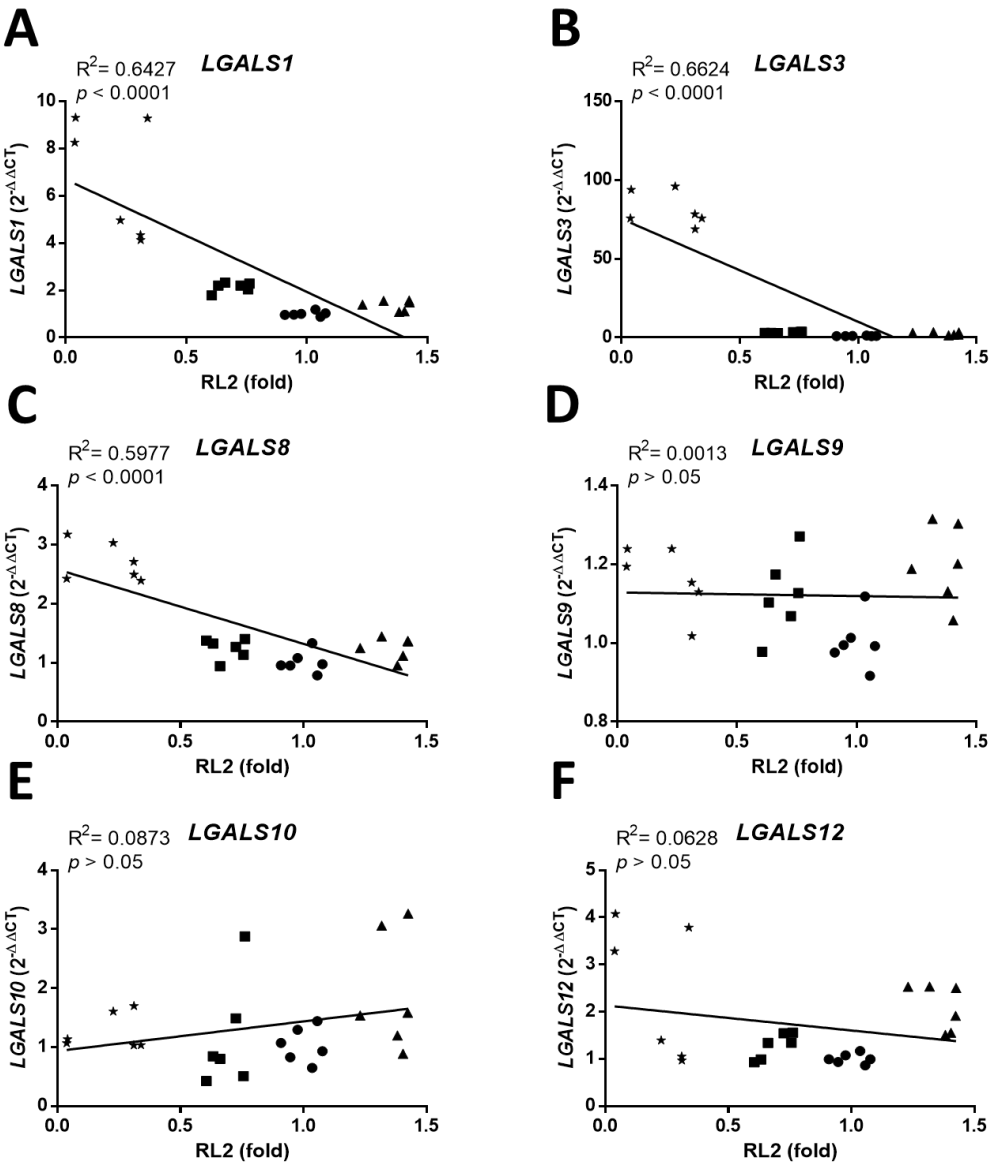

A
R2= 0.6427
p < 0.0001
LGALS1
LGALS1 (2-ΔΔCT)
RL2 (fold)
B
R2= 0.6624
p < 0.0001
LGALS3
LGALS3 (2-ΔΔCT)
RL2 (fold)
C
R2= 0.5977
p < 0.0001
LGALS8
LGALS8 (2-ΔΔCT)
RL2 (fold)
D
R2= 0.0013
p > 0.05
LGALS9
LGALS9 (2-ΔΔCT)
RL2 (fold)
E
R2= 0.0873
p > 0.05
LGALS10
LGALS10 (2-ΔΔCT)
RL2 (fold)
F
R2= 0.0628
p > 0.05
LGALS12
LGALS12 (2-ΔΔCT)
RL2 (fold)

Figure 18. Correlation in transcript levels between galectins and relative global *O*-GlcNAcylation (RL2).

Relative global *O*-GlcNAcylation of intracellular proteins in HL-60 cells measured using semi quantitative Western blot analysis, plotted against ΔΔCt for galectin genes. HL-60 cells were treated with 25 μM AC (■), 10 μM TG (▲), 12.5 μM DON (★) or vehicle (CTRL) (●). A) *LGALS1*, (B) *LGALS3*, (C) *LGALS8*, (D) *LGALS9*, (E) *LGALS10*, (F) *LGALS12*. *ACTB* was used as an internal control. Significant correlation between genes and global *O*-GlcNAcylation was determined using the Pearson's correlation test and a threshold set at $p < 0.05$.

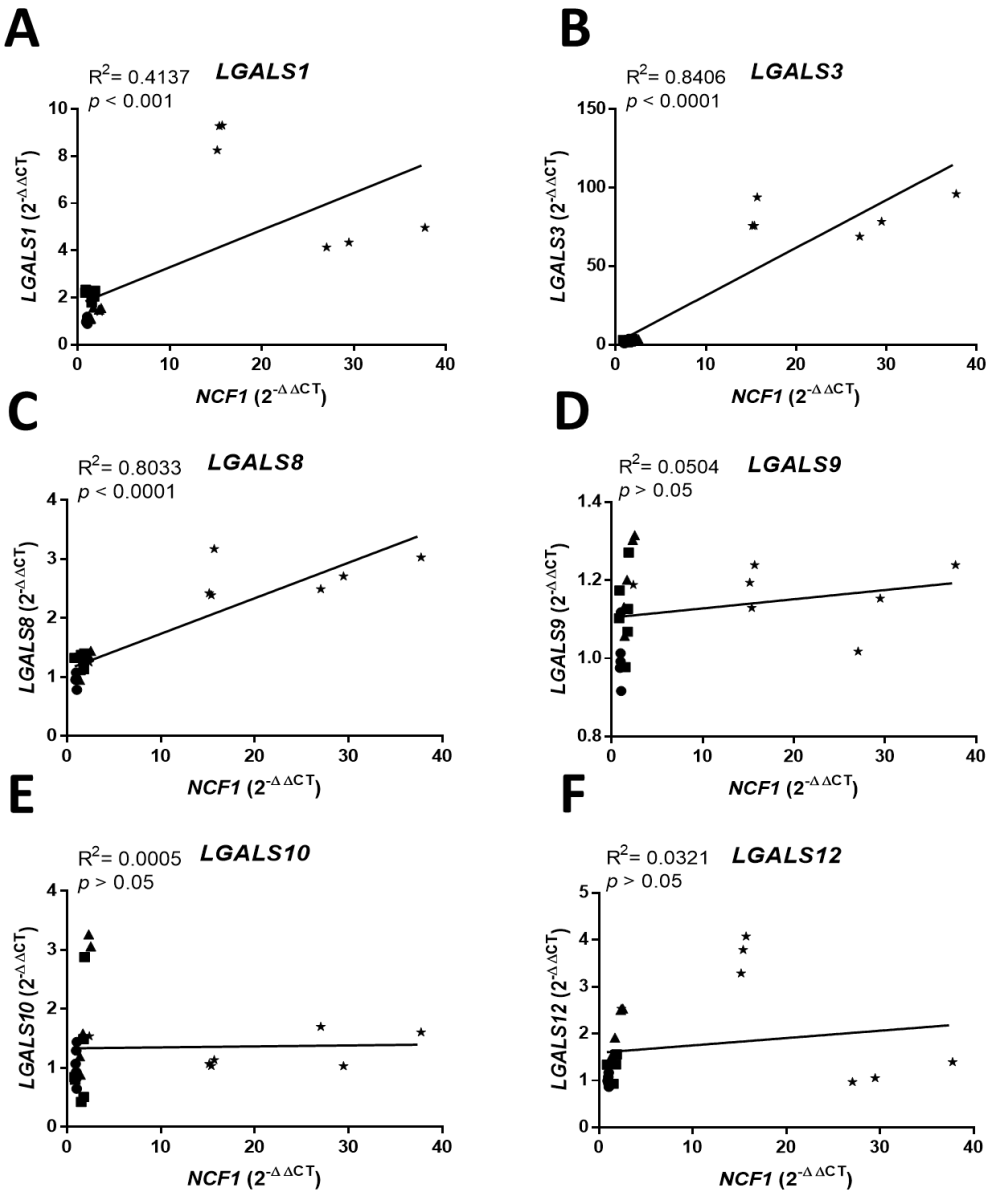

A
R2= 0.4137
p < 0.001
LGALS1
LGALS1 (2-ΔΔCT)
NCF1 (2-ΔΔCT)
B
R2= 0.8406
p < 0.0001
LGALS3
LGALS3 (2-ΔΔCT)
NCF1 (2-ΔΔCT)
C
R2= 0.8033
p < 0.0001
LGALS8
LGALS8 (2-ΔΔCT)
NCF1 (2-ΔΔCT)
D
R2= 0.0504
p > 0.05
LGALS9
LGALS9 (2-ΔΔCT)
NCF1 (2-ΔΔCT)
E
R2= 0.0005
p > 0.05
LGALS10
LGALS10 (2-ΔΔCT)
NCF1 (2-ΔΔCT)
F
R2= 0.0321
p > 0.05
LGALS12
LGALS12 (2-ΔΔCT)
NCF1 (2-ΔΔCT)

Figure 19. Correlation in transcript levels between galectins with neutrophil specific genetic biomarker *NCF1*.

ΔΔCt of neutrophil specific genetic biomarker *NCF1*, plotted against ΔΔCt galectin genes. HL-60 cells were treated with 25 μM AC (■), 10 μM TG (▲), 12.5 μM DON (★) or vehicle (CTRL) (●). (A) *LGALS1*, (B) *LGALS3*, (C) *LGALS8*, (D) *LGALS9*, (E) *LGALS10*, (F) *LGALS12*. *ACTB* was used as an internal control. Significant correlation between genes and global *O*-GlcNAcylation was determined using the Pearson's correlation test and a threshold set at $p < 0.05$.

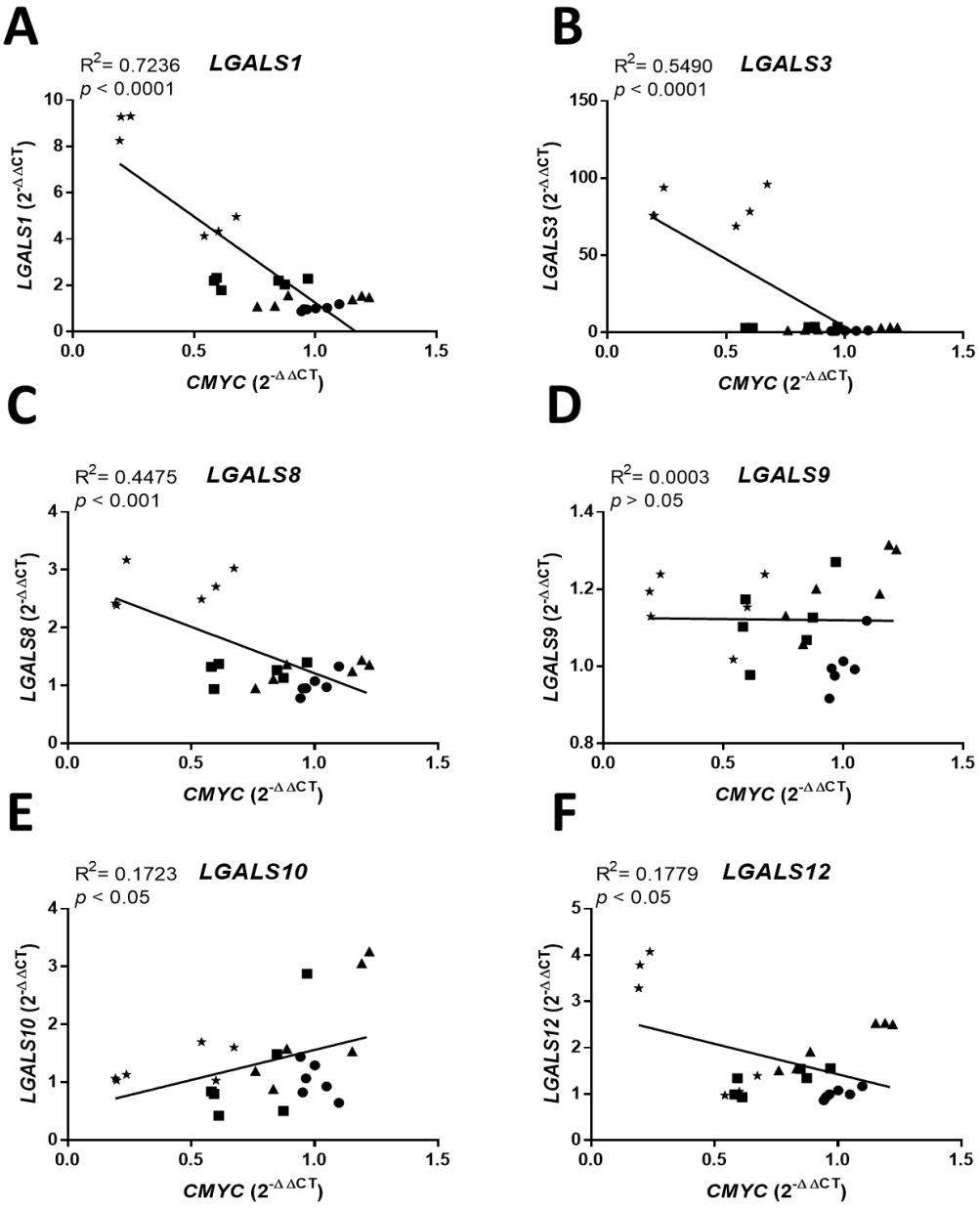

A
R2= 0.7236
p < 0.0001
LGALS1
LGALS1 (2-ΔΔCT)
CMYC (2-ΔΔCT)
B
R2= 0.5490
p < 0.0001
LGALS3
LGALS3 (2-ΔΔCT)
CMYC (2-ΔΔCT)
C
R2= 0.4475
p < 0.001
LGALS8
LGALS8 (2-ΔΔCT)
CMYC (2-ΔΔCT)
D
R2= 0.0003
p > 0.05
LGALS9
LGALS9 (2-ΔΔCT)
CMYC (2-ΔΔCT)
E
R2= 0.1723
p < 0.05
LGALS10
LGALS10 (2-ΔΔCT)
CMYC (2-ΔΔCT)
F
R2= 0.1779
p < 0.05
LGALS12
LGALS12 (2-ΔΔCT)
CMYC (2-ΔΔCT)

Figure 20. Correlation in transcript levels between galectins with oncogenic biomarker of cell pluripotency *MYC*

ΔΔCt of oncogenic biomarker of cell pluripotency *MYC*, plotted against ΔΔCt galectin genes. HL-60 cells were treated with 25 μM AC (■), 10 μM TG (▲), 12.5 μM DON (★) or vehicle (CTRL) (●). (A) *LGALS1*, (B) *LGALS3*, (C) *LGALS8*, (D) *LGALS9*, (E) *LGALS10*, (F) *LGALS12*. *ACTB* was used as an internal control. Significant correlation between genes and global *O*-GlcNAcylation was determined using the Pearson's correlation test and a threshold set at $p < 0.05$.

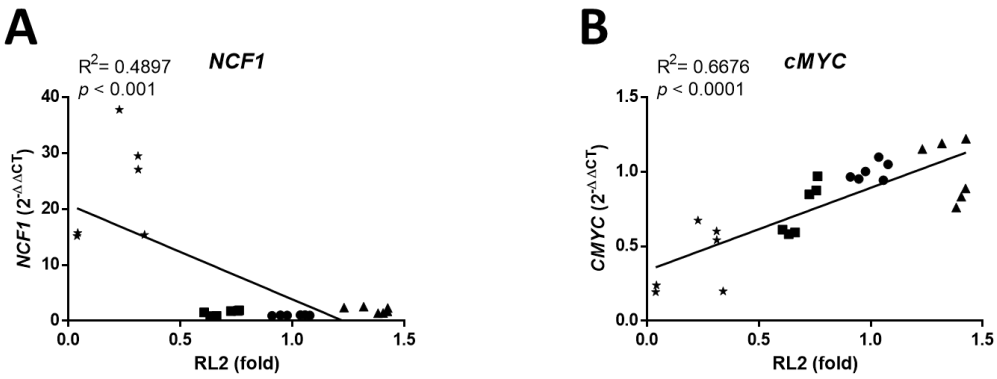

A
NCF1
R2= 0.4897
p < 0.001
NCF1 (2-ΔΔCT)
RL2 (fold)
B
cMYC
R2= 0.6676
p < 0.0001
CMYC (2-ΔΔCT)
RL2 (fold)

Figure 21. The correlation between *O*-GlcNAcylation and neutrophilic differentiation.

Relative global *O*-GlcNAcylation of intracellular proteins in HL-60 cells measured using semi quantitative Western blot analysis, plotted against ΔΔCt for genetic markers of differentiation. HL-60 cells were treated with 25 μM AC (■), 10 μM TG (▲), 12.5 μM DON (★) or vehicle (CTRL) (●). (A) *NCF1*, (B) *MYC*. *ACTB* and its protein β-actin were used as housekeeping controls for RT-qPCR and Western blots, respectively. Significant correlation between genes and global *O*-GlcNAcylation was determined using the Pearson's correlation test and a threshold set at $p < 0.05$.

Chapter 4

4 Discussion

The findings of my research demonstrate that galectins are involved in neutrophilic differentiation of HL-60 promyeloid leukemia cells and that this is an *O*-GlcNAc sensitive relationship. The first objective showed that HL-60 cells readily differentiate to the granulocyte lineage upon stimulation with ATRA both in the presence of, and absence of serum factors. This was confirmed morphologically by examining the segmentation of nuclei, and genetically by the expression of associated biomarkers or, by lack of expression of markers associated with the stem-like precursor cells. The second objective verified the expression profile of galectins, the genetic changes of neutrophil associated genes, and functional activity of neutrophil like cells. RT-qPCR, western blot, and the NADPH-oxidase functional assay were used to construct a profile to characterize the neutrophil. Galectins -3 and -10 were identified as inducible by the differentiation stimulus. In my third objective, I modulated the global *O*-GlcNAcylation of HL-60 cells with biochemical inhibitors of enzymes in the *O*-GlcNAc cycle to compare to the profile of neutrophils as per objective two and verify the link between *O*-GlcNAcylation and neutrophilic differentiation. Reduction of global *O*-GlcNAcylation resulted in increased expression of galectin-3 and galectin-10, consistent with changes that were observed in ATRA-induced differentiation as well as increased expression of neutrophil specific genetic marker *NCF1*. The similar changes in the expression of neutrophil markers, galectin expression profiles, and reduction in global *O*-GlcNAcylation suggest that granulocytic differentiation is achievable by targeting either the canonical differentiation pathway or the hexosamine biosynthesis pathway. I also revealed that granulocytic differentiation of HL-60 cells induced by either ATRA, or DON was accompanied by increased secretion of galectins and decreased *O*-GlcNAcylation of intracellular proteins. As such, my experimental findings confirm a novel concept of *O*-GlcNAc-mediated regulation of endogenous and exogenous networks of galectins in the context of cellular differentiation.

4.1 Interpretation

4.1.1 Neutrophilic differentiation is achieved with and without serum, altering galectin expression in the process

The use of ATRA as a differentiation agent has long been used in research and in clinical treatment of APL (Drach et al., 1993; Johnson and Redner, 2015). Terminal differentiation is a treatment for cancer because the mature leukocytes lose their infinite proliferative capacity due to genetic changes resulting in the loss of signaling through pathways that drive the cell to mitosis (Chaplinski et al., 1986; Mollinedo et al., 1998). HL-60 cells were grown in both serum-containing and serum-free media, supplemented with ITS to keep cells proliferative, to assess what if any impact serum has on the terminal differentiation of HL-60 cells and what effect this may have on galectin expression profiles. HL-60 cells grow at approximately the same rate regardless of media supplement, and differentiation is easily induced by ATRA in both cases as well.

O-GlcNAcylation is a marker of differentiation. Highly abundant in cancerous precursor cells, the *O*-GlcNAcylation is significantly reduced in cells which have been terminally differentiated by ATRA. Differentiation down regulates the IR which is necessary for glucose metabolism (Chaplinski et al., 1986). Cancer cells make liberal use of the Warburg effect (Ferrer et al., 2014; Akella et al., 2020), preferentially producing energy through glycolysis rather than through oxidative phosphorylation and the electron transport chain (Cervantes-Madrid et al., 2015). The increased flux through pathways, which are offshoots of glycolysis, such as the PPP (Patra and Hay, 2014) and the HBP (Asthana et al., 2018), result from this. Reductions of global *O*-GlcNAcylation have previously been reported in HL-60 granulocyte differentiation (Asthana et al., 2018) and result in lowered flux through the PPP (Rao et al., 2013) negatively regulating cell growth and proliferation. We show here that the interplay between protein *O*-GlcNAcylation and granulocytic differentiation is unaffected by the presence or absence of serum in HL-60 cell culture.

The expression of some galectins is induced by ATRA-induced differentiation whereas other galectins are suppressed. While some galectins displayed increased expression after

differentiation, like *LGALS3*, *LGALS10*, and *LGALS12*, others remain either unaffected, like *LGALS8* and *LGALS9*, while one galectin showed significant reduction in transcript abundance, namely *LGALS1*. While the existence of many galectins with similar structure and functionality suggests an evolutionary redundance, clearly not all galectins are the same and preform the same function and do so in the same cell types. The trends exhibited in the induction or suppression of galectins were not affected by the presence of absence of serum in the growth media. Markers of granulocyte differentiation such as increased expression of *NCF1* and *NCF2*, reduced *O*-GlcNAcylation, and NADPH-oxidase H_2O_2 production were also induced regardless of serum supplementation consistent with previous studies detailing granulocyte differentiation in a serum-free model (Breitman et al., 1980b). The changes in galectin expression profiles both in the presence and absence of serum indicate that galectins can be used as biomarkers of differentiation.

Galectin-1 was significantly downregulated during ATRA-induced neutrophilic differentiation. This differs from previous studies which used DMSO as a differentiation agent and reported an upregulation of galectin-1 (Abedin et al., 2003; Vinnai et al., 2017). However, it has also been reported that ATRA can induce a downregulation of galectin-1 in HL-60 cells (Vakrushev et al., 2019). Chiarotti et al. (1994) also reported a decrease in galectin-1 gene expression upon treatment with ATRA although this was in a thyroid cell model. The results of this study confirm retinoic acid-induced suppression of *LGALS1* expression as has been previously reported. Taken together, this suggests a differential regulation for *LGALS1* using different induction agents.

Galectin-3 was significantly upregulated during neutrophilic differentiation. This is consistent with previous literature (Abedin et al, 2003; Vinnai et al., 2017). Galectin-3 has been widely studied in neutrophil models and has many reported functions including stimulating superoxide production (Yamaoka et al., 1995), promoting neutrophil adhesion to laminin (Kuwabara et al., 1996), and promoting neutrophil extravasation (Sato et al., 2002). Here, we show that galectin-3 is upregulated when cells are induced to neutrophils by ATRA, similar to observations in previous studies which used DMSO as a differentiation agent (Abedin et al., 2003; Vinnai et al., 2017). The consistency of

galectin-3 upregulation in response to two different agents implicates galectin-3 as an important protein in neutrophil functions and unlike galectin-1 is upregulated by both ATRA and DMSO, making galectin-3 a good marker of differentiation.

LGALS10 was significantly upregulated in response to ATRA treatment. PU.1 is a known transcription factor (Nowak et al., 2009) upregulated during granulocyte differentiation. Dyer and Rosenberg (2001) reported a PU.1 binding site in the promoter region of the *LGALS10* gene. Galectin-10 was upregulated in cells fated to the neutrophilic lineage. Previous literature (Dvorak and Ishizaka, 1995; Dyer and Rosenberg, 2001) reported high galectin-10 expression in eosinophils and basophils, particularly in eosinophils where the galectin-10 protein comprises up to 7-10% of eosinophil protein content (Dvorak et al., 1998). As high galectin-10 expression is reported in other leukocytes, it is unsurprising to see high expression in neutrophils which are leukocytes similar to eosinophils and basophils, and indeed, this has been reported, albeit sparingly, in the literature (Vinnai et al., 2017). Galectin-10 is under the transcriptional control of two PU.1 binding sites in the promoter region (Dyer and Rosenberg, 2001). PU.1 is a transcription factor upregulated during neutrophilic differentiation driving the cell towards terminal differentiation (Yan et al., 2016). Although a difference in the magnitude of induction was seen between HL-60 cells treated with or without serum (Figure 10E) the promoter region as reported elsewhere does not contain anything resembling a serum response element leaving this difference in induction unexplained (Dyer and Rosenberg, 2001).

The expression profile for galectin-12 was assessed only on the genetic level by use of RT-qPCR. The galectin-12 gene, *LGALS12*, has previously been studied by our lab (Vinnai et al., 2017) in the context of DMSO-induced neutrophilic differentiation. We however used ATRA to induce neutrophilic differentiation. The increased transcript abundance of *LGALS12* seen upon differentiation by ATRA is at odds with previous research (Xue et al., 2016) which reported that inhibition of *LGALS12* by a doxycycline induced knockdown of *LGALS12* enhanced ATRA-induced differentiation. However, while Xue et al. (2016) showed that *LGALS12* knockdown increased differentiation markers by producing more ROS through the NADPH oxidase complex, they showed that cells with wildtype *LGALS12* expression also displayed significantly higher levels of

neutrophilic biomarkers even in the presence of *LGALS12*. The researchers did not assess the galectin expression profile among differentiation status by RT-qPCR and to my knowledge there are no papers which report the changes in *LGALS12* expression in such a model with ATRA specifically. This suggests that *LGALS12* does not need to be inhibited in order to induce differentiation. The expression of *LGALS12* was decreased in DMSO-treated HL-60 cells (Figure S1) which is consistent with previous reports using DMSO as a differentiation agent. Yang et al. (2001) reported that galectin-12 expression was upregulated in cells experiencing cell cycle arrest. This may be consistent with my results wherein cells that had inhibited growth and proliferation by ATRA or DON, in both cases increased their expression of galectin-12. This group also show that transfection with galectin-12 increased the proportion of cells arrested at the G1 phase. Galectin-12 transfected cells showed higher proportions of cells arrested at the G1 phase even in the presence of nocodazole (Yang et al., 2001), a drug which arrests cells in the M phase by disrupting spindle pole formation (Agami and Bernards, 2000) demonstrating that that growth is arrested at G1 rather than there being an accelerated transition from M to G1 (Yang et al., 2001). These results may be consistent with the observation of this study which showed that galectin-12 is more highly expressed in differentiated cells which have slowed growth rates knowing that differentiated cells arrest their growth and proliferation concurrently with differentiation (Asthana et al., 2018). Indeed, galectin-12 could be a marker of neutrophilic differentiation, although the differential expression between DMSO-induced neutrophils and ATRA-induced neutrophils suggests that upregulation of galectin-12 may not be essential for growth arrest. These results in combination with previous findings (Yang et al., 2001) suggest that growth arrest in DMSO-induced neutrophils is likely triggered through a different mechanism.

Increased activity of the HBP is observed in all AML subtypes (Melnick and Licht, 1999; Asthana et al., 2018) however, the APL fusion protein that allows for ATRA induced differentiation is found only in the M3 subtype (Johnson and Redner, 2015). As demonstrated by pervious research and in my research, the targeting of the HBP is an effective method of inducing differentiation of HL-60 cells, suggesting a potential for therapies targeting this pathway in the other subtypes of AML which are unable to be induced by ATRA to terminally differentiate. Hyper-*O*-GlcNAcylation is hypothesized to

keep cells in an undifferentiated state (Asthana et al., 2018; Zhang et al., 2018). Previous literature showed that a differentiation agent administered in conjunction with an OGA inhibitor like TG could reduce the proportion of differentiated cells (Zhang et al., 2018). This shows how important the *O*-GlcNAc homeostasis is in the differentiation process of HL-60 cells. Zhang et al. (2018) also demonstrated this *O*-GlcNAc dependent regulation in the erythroid linage of the hematopoietic stem cell linage.

4.1.2 Inhibition of global *O*-GlcNAcylation induced the expression of biomarkers of neutrophilic differentiation and altered galectin expression.

O-GlcNAcylation is a dynamic post-translational modification implicated in the regulation of many diseases such as diabetes (Akimoto et al., 2007), Alzheimer's (Yuzwa et al., 2014), Parkinson's (Yuzwa et al., 2014) and cancer (Shi et al., 2010; Yi et al., 2012). The modification is controlled by only two enzymes, OGT and OGA which add and remove the GlcNAc moiety, respectively. Many reports show aberrant *O*-GlcNAcylation in cancer (Shi et al., 2010; Asthana et al., 2018). As this is controlled by only two enzymes and the precursor molecule is produced only by the HBP, manipulation of the *O*-GlcNAc homeostasis is easily achieved in cell culture by chemical inhibitors, and by RNA interference techniques (Perez-Cervera et al., 2013; Ferrer et al., 2014). Here I used biochemical inhibition of *O*-GlcNAc cycle enzymes to determine the relationship between differentiation, *O*-GlcNAcylation, and galectin expression.

DON is a powerful GFAT inhibitor which limits the availability of UDP-GlcNAc, the precursor substrate necessary for *O*-GlcNAcylation. Following a protocol established by Asthana et al. (2018), I showed an over 90% decrease in the chemiluminescent signal detectable by the *O*-GlcNAc specific antibody RL2. This *O*-GlcNAc cycle enzyme inhibitor very effectively induced neutrophilic differentiation as assessed by all but one measure used in this study, namely the segmented nuclei assessed by DAPI staining. All other markers of differentiation, *NCF1*, H_2O_2 production by PMA-induced NADPH-oxidase activity, and galectin secretion were significantly increased when this drug was used, and these results follow the same pattern as ATRA induced differentiation. Death of AML cells was preceded by differentiation as has also been described elsewhere

(Asthana et al., 2018). This suggests that terminal differentiation in addition to mitigating the infinite proliferative capacity of AML cells also serves to kill these cells making HBP-targeting therapies excellent candidates for potential future chemotherapeutics. Surprisingly, the OGT inhibitor AC did not induce any of the markers of differentiation. Although AC was a good inhibitor of OGT as indicated by the reduction of RL2 signal, which was comparable to ATRA treatments, there was no upregulation of *NCF1*, no H_2O_2 production, and the galectin secretion was not significantly increased by this drug. Previous reports suggest that inhibition of OGT should induce neutrophilic differentiation, as previously those groups using OSMI-1 (Asthana et al., 2018) and alloxan (Ding et al., 2018), both of which are OGT inhibitors albeit with some off target effects and with more cytotoxic effects, were able to show at least some upregulation of biomarkers associated with differentiation.

The inhibition of cell growth was most prominently seen in HL-60 cells treated with the GFAT inhibitor DON. UDP-GlcNAc is an important and widely used biomolecule in cells, being a precursor for the production of proteoglycans, glycolipids and glycoproteins (Grønning-Wang et al., 2011). Rao et al. (2015) showed that G6PD, an enzyme in the PPP, must be glycosylated to allow flux through the pathway. The PPP is vital to meet the demands for anabolic biosynthesis and provided antioxidant defense (Patra and Hay, 2014). Mitotic arrest like what was seen in my research is explained by the decreased flux through this PPP, which limited the *de novo* synthesis of DNA and RNA as the PPP produces ribose-6-phospahte, a necessary precursor for nuclei acid synthesis (Rao et al., 2015). The lack of necessary UDP-GlcNAc substrate for *O*-GlcNAcylation of G6PD would be expected to lead to the results seen in my study. This differs from ATRA- and DMSO-induced differentiation wherein the growth arrest of HL-60 cells when induced by ATRA or DMSO takes days to occur (Mollinedo et al., 1998) and this is supported by my observations that the cell concentration did increase over the 72-hour treatment frame, albeit the growth was slower than untreated cells (Table S1). However, the growth arrest in DON-treated HL-60 cells was near-immediate as the number of cells in culture over 72 hours was almost the same as the number of cells before the treatment (Table S1). It seems to be the case that induction of genes involved in differentiation precedes the growth arrest observed in ATRA- and DMSO-induced

differentiation, but with the DON treatments, this linearity of occurrences doesn't appear obvious. Growth arrest preceding differentiation also was reported by Soddu et al. (1994) who recovered wild-type p53 expression in HL-60 p53-null cells and consequently reported granulocytic differentiation. The reassertion of cell cycle controls could be a contributing factor to the observed differentiation. Cell growth arrest by *O*-GlcNAc inhibition was also reported by (Li et al., 2017) who showed that inhibitors of *O*-GlcNAcylation had effects on other post-translational modifications, specifically they noted that reduced *O*-GlcNAcylation led to reduced phosphorylation of vimentin at Ser-71, a vital modification for severing the vimentin filaments during cytokinesis which resulted in failed cytokinesis. Indeed, it appears that *O*-GlcNAc is implicated in many key pathways and processes of normal cellular function and that targeting the *O*-GlcNAc pathway and regulation of *O*-GlcNAc *in vitro* impacts many of these cellular processes, dysregulating them, with negative side effects on cell growth and proliferation (Rao et al., 2015; Martinez et al., 2017; Asthana et al., 2018). It remains uncertain if the effects of reduced *O*-GlcNAcylation on any of these pathways and processes can be rescued by the "reactivation" of any given one of the dysregulated pathways.

Galectin-3 and galectin-10 were both upregulated in response to global *O*-GlcNAcylation reduction just as they were in response to ATRA-induced neutrophilic differentiation. We showed that inhibition of the HBP by DON significantly reduced the global *O*-GlcNAcylation of HL-60 cells even more than was observed using ATRA. We also showed that targeting the HBP induced neutrophilic differentiation by increasing the expression of *NCF1* and inducing H_2O_2 production by NADPH oxidase activity, characteristic of neutrophil-like cells. Galectins-3 and -10 can therefore be viewed as differentiation markers in neutrophilic models due to their similar upregulation using different agents. There is also a similar trend of increased galectin-12 expression in response to DON as was seen with ATRA. These results similarly suggest a role for galectin-12 in neutrophilic differentiation and neutrophil function. This result too, is consistent with Yang et al. (2001) who reported an association between galectin-12 and cell cycle arrest at the G1 phase. DON almost immediately induced growth arrest as evidenced by the minimal increase in cell concentration after 72 hours of treatment with DON (Table S1). It is possible that the increased galectin-12 expression is related to the

slower growth induced by both DON and ATRA, however the DMSO-induced neutrophilic differentiation which resulted in decrease galectin-12 expression (Vinnai et al., 2017) is inconsistent with my findings.

Galectin-8 expression was consistently high in HL-60 cells across all differentiating treatments used, showing significantly increased expression in cells treated with DON (Figure 13C). Galectin-8 plays a key role in neutrophil migration (Nishi et al., 2003). While the results of this study demonstrate that galectin-8 is highly expressed among all treatments, it only increased upon treatment with DON. The function that galectin-8 plays in mediating neutrophil function is reported as being dependent on interaction with neutrophilic markers of differentiation, such as CD11b also known as integrin αM (Nishi et al., 2003). Indeed, it appears that galectin-8 shows constitutive expression among cells belonging to different differentiation states of the myeloid lineage, waiting for the expression of its binding partners to mediate its function (Nishi et al., 2003).

Galectin-9 transcript abundance remained relatively constant across all treatments in all experiments, consistent with previous literature (Vinnai et al., 2017; Abedin et al., 2003). However, the intracellular protein levels decreased in treatments with ATRA, AC, and DON, all of which significantly reduce the global *O*-GlcNAcylation of intracellular proteins. While this might suggest some proteasomal degradation associated with differentiation or reduced *O*-GlcNAcylation, I do not consider this to be the case as the concentration of galectin-9 detected in cell culture supernatants was significantly increased in those treatments as well. These observations suggest that galectin-9 expression is constitutive in HL-60 cells among varying differentiation states and stress states, and that galectin-9 is preferentially secreted in differentiated cells and nutrient stressed cells, suggesting that localization and function are affected by the treatments, not the expression itself. Galectin-9 is well studied in its relationship to Tim-3. The binding of galectin-9 and Tim-3 was demonstrated to be essential for the growth of leukemic cells (Kikushige et al., 2015). I showed that HL-60 cells express galectin-9 at both the mRNA and protein level. Other researchers (Gonçalves Silva et al., 2015; Prokhorov et al., 2015) reported that galecin-9 interaction with Tim-3 triggers signaling pathways involving the activation of phosphatidylinositol-3-kinase and downstream mTOR activity which signals

cell growth and proliferation (Gibbs et al., 2011). The results of the present study are consistent with these previous findings. Indeed, as the galectin-9 protein appears to be preferentially secreted by cells that have undergone differentiation, suggestive of reduced signaling through the mTOR pathway explaining the growth arrest in HL-60 cells treated with ATRA or DON.

4.1.3 Differentiated HL-60 cells secreted more galectins than precursor cells.

The secretion of galectins was assessed only in cells grown in a serum-free culture media. Galectins are widely reported as signaling molecules present in serum in many models from human to mouse to cow (Tribulatti et al., 2007; He et al., 2017; Asiamah et al., 2018; Asiamah et al., 2019; Asiamah et al., 2020). In the supplemental material, I show that using galectin antibodies specific to human galectins, I was able to detect these galectins in the unconditioned IMDM+10% FBS medium and was unable to do the same in the serum-free ITS-supplemented IMDM (Figure S2). To eliminate the background detection of galectins for the purpose of ensuring that any galectin detection in the extracellular environment was due to secretion from HL-60 cells, we adapted the serum-free culture method pioneered by the Breitman group in the 1980s (Breitman et al., 1980b).

HL-60 cells induced to the granulocyte lineage by ATRA secreted significantly more of all tested galectins compared to precursor stem-like cells. Interestingly, the increased secretion of galectins from HL-60 cells was also achieved using a GFAT inhibitor DON. DON was by far the most powerful drug in terms of reduction of *O*-GlcNAcylated proteins as assessed by both western blot and dot blot assays. The ELISA data showed that DON-treated HL-60 cells secreted significantly more galectin-1, galectin-3, and galectin-9. These neutrophil-like cells induced by DON display galectin expression and secretion trends similar to ATRA-treated HL-60 cells. Extracellular galectin-3 is an important regulator of neutrophil function, acting both with and independently of immunoglobin E to stimulate superoxide production via NADPH oxidase activity characteristic of phagocytes (Hsu et al., 1992; Herrmann et al., 1993; Yamaoka et al., 1995; Sundqvist et al., 2018). Secreted galectin-3 also promotes neutrophil phagocytosis

of pathogens (Kohatsu et al., 2006; Linden et al., 2013) and non-pathogens (Fernández et al., 2005). ROS production is also inducible by galectins-8 and -9. Galectin-8 stimulates ROS production through the C-terminal domain (Nishi et al., 2003; Nishi et al., 2006) whereas galectin-9 accomplishes this through a Tim-3 independent pathway (Vega-Carrascal et al., 2014; Steichen et al., 2015). Extracellular galectin-10 is well characterized in eosinophil models (Chua et al., 2012), but the roles in neutrophils and when secreted by neutrophils is not well described in the literature. However, galectin-10 has been shown to drive IL-1β production in macrophages which results in neutrophil accumulation (Rodríguez-Alcázar et al., 2019).

Galectin-3 is a chemoattractant for monocytes (Sano et al., 2000) and endothelial cells (Nangia-Makker et al., 2000) while galectin-9 is a chemoattractant for eosinophils (Matsumoto et al., 1998). Immune cell types often work together in the immune response (Karlsson et al., 2009., Robinson et al., 2019) thus for neutrophil-like cells to secrete these galectins which bring in other cell types to aid in the pathogen clearance response is expected based on previous literature and is observed in this study as well.

The findings with galectin-1 are both consistent and inconsistent with findings by other researchers as galectin-1 has many reported roles and expression patterns, some of which appear contradictory. I showed here that galectin-1 secretion is significantly increased only when treated with DON. Treatment with DON in this study was the only drug treatment that led to a substantial increase in the proportion of dead cells. Galectin-1 is reported to induce phosphatidylserine exposure in neutrophils and HL-60 cells (Dias-Baruffi et al., 2003). Phosphatidylserine exposure signals apoptosis and cell death so it is only logical that the treatment that caused much galectin-1 secretion also had significantly more dead cells by inducing phosphatidylserine exposure through an autocrine signaling method. Galectin-1 has also been shown to induce ROS production in neutrophils (Elola et al., 2005; Ashraf et al., 2018) and is involved in neutrophil chemotaxis (Auvynet et al., 2013). High *LGALS1* expression is correlated with lower survival in clear cell renal carcinoma patients and patients with low *LGALS1* expression showed higher survival rates in Kaplan-Meier curves (Li et al., 2019). Similar results were also seen *in vivo*. Mice injected peritoneally with OCI-AML3 cells which are

another immortalized M3 leukemia cell line were followed for 14 weeks after being transfected with *LGALS1* shRNA. Those mice with the *LGALS1* knockdown survived significantly longer, with 40% of mice surviving to 100 days whereas mice injected with OCI-AML3 cells expressing wild-type *LGALS1* all died by day 50 (Ruvolo et al., 2020). This is consistent with my results which showed high *LGALS1* expression in rapidly dividing HL-60 cells, and low expression in differentiated cells that show growth arrest. OCI-AML3 cells with suppressed *LGALS1* showed significantly increased expression of apoptotic markers p21, Mdm2, and APAF1 (Ruvolo et l., 2020). High galectin-1 is associated with high programmed death ligand 1 levels in AML cells and in head and neck cancers (Li et al., 2019; Nambiar et al., 2019). Knockdowns that reduce *LGALS1* expression lower the programmed death ligand 1 levels in AML cells and made these cells less susceptible to anti-programmed death ligand 1 treatment (Li et al., 2019). Indeed, *LGALS1* should be considered a biomarker of the cancer cell and not the differentiated cell as might be concluded from the result shown here with DON, and in previous literature (Vinnai et al., 2017).

4.1.4 Galectin gene expression correlates with *O*-GlcNAcylation and differentiation.

The correlation analyses conducted in this study indicate strong correlations, in some cases positive and in others negative, between *O*-GlcNAcylation, neutrophilic differentiation, and expression of galectins. The correlations between neutrophilic marker *NCF1* and *O*-GlcNAc with galectins show galectins are involved in differentiation and that their expression may be regulated by *O*-GlcNAc-mediated mechanisms. Specifically, *LGALS1*, *LGALS3,* and *LGALS8* are all negatively correlated with *O*-GlcNAcylation (Figure 17 A-C), the only galectins to show any correlation with *O*-GlcNAc homeostasis. This represents a novel finding as the regulation of galectins in response to manipulation of global *O*-GlcNAcylation is not well documented.

The same galectins that were correlated with *O*-GlcNAcylation are also correlated with the expression of a neutrophilic specific biomarker *NCF1*, albeit the correlation here is strongly positive for all three galectins, *LGALS1*, *LGALS3*, and *LGALS8* (Figure 18A-C). All galectins except for *LGALS9* were negatively correlated with the expression of

oncogenic marker *MYC*. While the correlation is negative and mildly significant between *LGALS10* and *LGALS12* with *MYC*, the correlation of *MYC* with the *O*-GlcNAc sensitive galectins *LGALS1*, *LGALS3*, and *LGALS8* is a stronger negative correlation with a higher degree of significance (Figure 19).

Unsurprisingly, *NCF1* is strongly negatively correlated with *O*-GlcNAcylation, while *MYC* is strongly positively correlated with *O*-GlcNAcylation (Figure 21). These findings indicate a strong association between *O*-GlcNAcylation of cytosolic proteins with the differentiation state of the cells and many galectins correlate with *O*-GlcNAcylation and with markers of differentiation. Although growth arrest is not uniformly affected by differentiation and by *O*-GlcNAc stress, galectin expression and secretion follow the same trends indicating that galectins are biomarkers for both processes, but galectins may also be necessary for neutrophilic differentiation given their upregulation in HL-60 cells.

There is an association between *O*-GlcNAcylation and the secretion of galectins by HL-60 cells. Only two galectins are verified to be *O*-GlcNAcylated in the literature, galectin-1, and galectin-3 (Hart et al., 2011; Mathew et al., 2021). My study clearly demonstrates an association between the reduction of global *O*-GlcNAcylation and increased secretion of all galectins tested (-1, -3, -9, and -10) suggesting that galectin secretion is regulated by *O*-GlcNAcylation and that other galectins are subject to *O*-GlcNAcylation.

4.2 Conclusions and applications

In conclusion, while differentiation appears to be induced both by ATRA and by DON, not all galectin profiles are similarly inducible by the various drugs capable of stimulating differentiation. Specifically, galectin-1 and its gene *LGALS1* displayed opposite patterns with ATRA and DON where both the gene and protein where significantly lower in HL-60 cells induced by ATRA yet were significantly higher in HL-60 cells induced by DON. This differs from previous reports (Zhao et al., 2011; Vinnai et al., 2017) that galectin-1 ought to increase with neutrophilic differentiation by DMSO yet is consistent with the findings of Chiarotti et al. (1994), and with Vakrushev et al. (2018) who reported that *LGALS1* expression decreased upon ATRA-treatment specifically in HL-60 cells.

While the expression profiles of some galectins appear similar throughout my present studies using ATRA as a differentiation stimulus and is consistent with previous literature (Abedin et al., 2003., Vinnai et al., 2017) which used DMSO to induce differentiation, there are some inconsistencies such as that of *LGALS1* and *LGALS12*. It must be considered that DMSO and ATRA induce differentiation by different means and as such the biochemical and physiological changes of the cell induced by each drug may not be in perfect alignment. Manda-Handzlik (2018) examined differentiation in HL-60 cells induced by three different drugs, ATRA, DMSO, and dimethyl formamide. While some markers of differentiation are highly upregulated regardless of stimuli, other markers are more strongly induced with a specific stimulus but show weaker induction with other stimuli (Sham et al., 1995; Manda-Handzlik et al., 2018). Manda-Handzlik et al. (2018) also showed that some characteristics of neutrophil like cells are missing altogether from differentiated *in vitro* HL-60 cells. For example, calcium-ionophore induced calcium influx varies depending on the differentiation-inducing agent used. Specifically, these researchers reported ATRA-differentiated cells were resistant to calcium ionophore-induced calcium influx whereas DMSO and dimethyl formamide differentiated cells showed no statistical difference in this measure from healthy peripheral blood neutrophils.

Little information exists on the expression patterns of galectins in the context of neutrophilic differentiation of HL-60 cells (Abedin et al., 2003; Vinnai et al., 2017), and there appears to be no published study on how different inducers differentially regulate the expression patterns of these galectins. My findings are consistent with previous work by Sham et al. (1995) and Manda-Handzlik et al. (2018) in that the fold change of markers may not be consistent across differentiation stimuli and in that the direction of change, upregulation, or downregulation, are not consistent across different stimuli. Indeed, much work remains to be done in the future to explain the roles of galectins in neutrophilic differentiation and in relation to *O*-GlcNAc homeostasis. What transcription factor(s) are activated or suppressed in any given drug treatment? What roles do galectins play in the differentiation of the cell? Or in the function of the differentiated cell? Future work could expand on the approach of Manda-Handzlik et al. (2018) using different induction stimuli and incorporate the work from our laboratory with regards to

glycobiology to elucidate how galectins and *O*-GlcNAc participate in the neutrophilic differentiation and in the functions of mature neutrophils.

The role of galectin-12 in neutrophilic differentiation remains elusive. The seemingly contradictory results here with *LGALS12* are also seen with the galectin-1 gene *LGALS1*. However, the contradictory expression profile of *LGALS1* in neutrophil-like differentiated cells can be supported by previous reports where the downregulation of *LGALS1* by ATRA reported elsewhere (Vakrushev et al., 2018) differs from reports of the upregulation of *LGALS1* by DMSO (Vinnai et al., 2017). What roles do these galectins play in differentiation? It is possible that the expression of these genes and their proteins may not be related to the neutrophilic differentiation of HL-60 cells or that different pathways that regulate these genes could be acted upon by these different drugs.

4.3 Study limitations and future directions

While it is certainly nice that we can show neutrophilic differentiation of HL-60 cells by targeting the HBP and bypass the ATRA-RAR interaction, we must remember that this *O*-GlcNAc mediated differentiation occurs in M2 leukemia cells which are notably susceptible to ATRA-induced differentiation. It remains to be seen if the *O*-GlcNAc-mediated neutrophilic differentiation can be induced in ATRA-resistant AML. This could be accomplished in a number of ways. There is the possibility to knock out the RAR to see if the signaling through this receptor is important even in the absence of ATRA when using inhibitors of OGT or GFAT. RNA interference techniques could also be used although these would be more transient. The obvious next step I would take is to acquire known ATRA-resistant cell lines like the NB4/RA cell line (Takeshita et al., 2000) and look to see if this *O*-GlcNAc-mediated differentiation bypassing the ATRA-RAR interaction is possible. This would provide much insight into the clinical treatment of ATRA-resistant AML.

To change *O*-GlcNAc levels in HL-60, we used biochemical inhibitors which may also have non-target effects on cells. This aspect was not addressed in this study and potentially could be responsible for variations in the effects of OGT and GFAT inhibitors we used on galectin expression and neutrophilic differentiation, although both treatments

induced an inhibition of *O*-GlcNAc. Optimization of the treatment conditions as well as genetic manipulations with relevant genes might be a good strategy for future studies.

The inability to confirm *O*-GlcNAcylation of specific galectin proteins represents a limitation of this work. We were also unable to confirm the *O*-GlcNAcylation of specific galectins. Due to time constraints, we were unable to work with the Biological Mass Spectrometry Lab (Biochemistry Department, UWO) to assess the *O*-GlcNAcylation of peptides. While we attempted to use western blot to look for overlaps in bands corresponding to galectins with the *O*-GlcNAc specific antibody RL2, the signal elicited by this antibody is very weak at lower molecular weights, and there is much signal from higher molecular weight proteins. As galectins are low molecular weight proteins, future studies most certainly should aim to do what I initially intended to do in my proposal by enriching cell lysates for galectins as per the protocol described in Timoshenko et al. (2014) and using UPLC-MS/MS to identify *O*-GlcNAcylated peptides. A secondary goal with be to assess whether galectins must be de-glycosylated prior to secretion. I showed that four galectins (-1, -3, -9, and -10) are secreted at significantly higher concentrations when cells were treated with a GFAT inhibitor DON relative to untreated cells. A study designed to assess galectin *O*-GlcNAcylation and subsequent de-glycosylation should look at both intracellular and extracellular galectins to examine what if any differences in the amount of, or site of this modification exist and how this relates to secretion.

Future work will also investigate the apparent differences in induction and suppression of *LGALS1* and *LGALS12*. My results differ from previous literature by Vinnai et al. (2017) who reported that upon DMSO-induced differentiation, *LGALS1* and *LGALS12* exhibit increased expression and decreased expression, respectively. Since my project looked at all galectins expressed in HL-60 cells and focused on *O*-GlcNAc mediated differentiation, I did not specifically look at the genetic regulation of any one galectin. Future studies may focus on a single galectin expressed in HL-60 and attempt to better explain the genetic regulation while accounting for differences in expression when treated with different granulocyte-inducing chemicals.

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Appendix A: Supplementary Material

Table S1: Initial and final densities, and cell viability of HL-60 cell cultures grown in IMDM + 10% FBS treated with differentiation agents and *O*-GlcNAc cycle enzyme inhibitors for 72 hours.

	CTRL	**ATRA**	**AC 25 μM**	**AC 50 μM**	**DON**	**TG**	**DMSO**
Initial Cell Concentration (10^5 cells/mL)	1.0	1.5	1.0	1.5	4.0	1.0	4.5
Ending Cell Concentration (10^5 cells/mL)	9.5	7.9	8.6	4.6	4.4	8.2	5.3
Cell Viability (%)	96.7	93.4	94.6	69.7	68.7	95.0	85.0

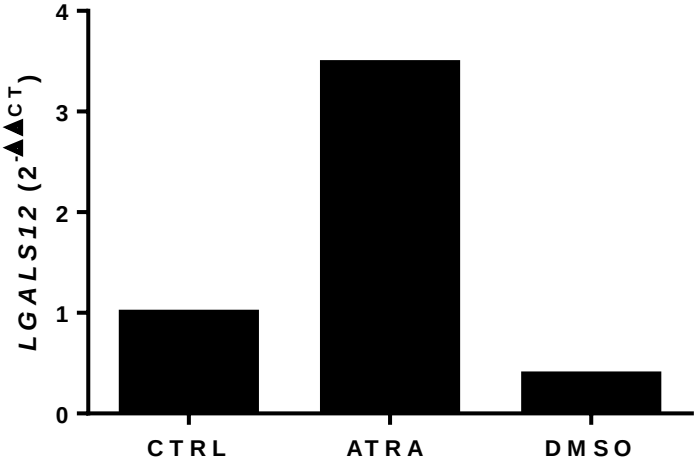

Figure S1: HL-60 cells were treated with two known inducers of neutrophilic differentiation, 1 μM ATRA and 1.3% DMSO for 72 hours and were tested for *LGALS12* transcript abundance relative to untreated cells, n=1.

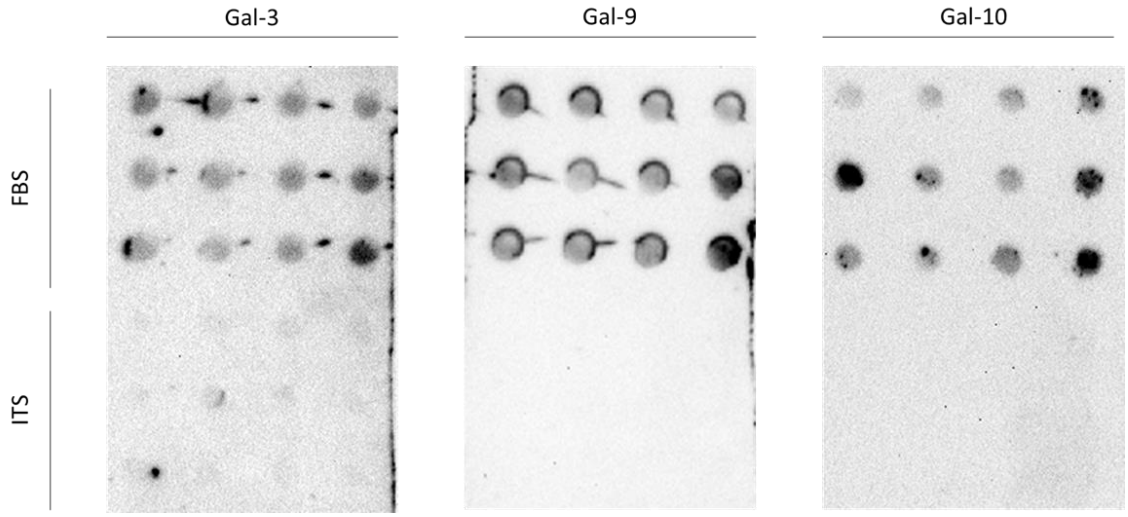

Figure S2: Immunodot blots for serum galectins in IMDM media supplemented with 10% FBS (top) or 1X ITS (bottom). Unconditioned media was gravity filtered through an Immunodot blot micro-apparatus and the presence of galectins was confirmed using anti-galectins antibodies.

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Abstracts and Posters:

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- McTague A, Tazhitdinova R, and Timoshenko AV (2021) *O*-GlcNAc homeostasis regulates expression and secretion of galectins in HL-60 cells. Ontario Cell Biology Symposium (online), July 12-16, 2021.