Investigating the Role of Lectins in LAG3 Function and T cell Activity

by
Shifa Fatima Mohideen

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Department of Medical Microbiology & Infectious Diseases
University of Manitoba
Winnipeg, Canada

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Abstract

During prolonged antigen exposure, as in cancer or HIV infection, surface inhibitory receptors called immune checkpoints (ICs), such as Lymphocyte Activation Gene-3 (LAG3), are upregulated on T cells and induce immunosuppression resulting in evasion of the immune system. Despite well-characterized LAG3-induced T cell exhaustion inhibiting cell function and cytokine responses, much about LAG3 remains elusive.

LAG3's extensive N-glycosylation, constitutes a recognition interface for LAG3-expressing cells to communicate and engage with binding partners in their microenvironment. Accordingly, its N-glycosylated residues can coordinate immune interactions and IC function by potentially engaging glycan-binding proteins or lectins such as Galectin-3 or LSECtin, often secreted in these disease contexts. However, the precise mechanisms of this interaction and its functional consequences on LAG3-mediated T cell activity remain a significant knowledge gap. This thesis clarifies that LSECtin and Galectin-3 exhibit dose-dependent specificity for binding LAG3 N-glycans through their carbohydrate recognition domains, affirming the necessity of N-glycosylation in LAG3-lectin engagements.

As an additional immune regulatory layer, this study also resolves that Galectin-3 and LSECtin binding stimulates LAG3-mediated T cell suppression, with LSECtin yielding stronger inhibitory effects. Contingent on LAG3, these lectin-driven reductions in pro-inflammatory cytokines, IL-2 and IFNγ, may skew and compromise antiviral and antitumor immunity. Conversely, this lectin-induced T cell inhibition is alleviated with N-glycan removal. Addressing a critical research gap in LAG3 literature, this dissertation uncovers a previously underappreciated mechanism of how post-translational N-glycosylation and lectins modulate and instruct LAG3-mediated T cell suppression.

In broadening the scope of LAG3 biology, such insights can spur further research into the complex molecular interplays engendering immune exhaustion and immune escape to better relieve disease progression. By illuminating promising therapeutic targets regarding LAG3-lectin engagements, the research herein can optimize and inform the efficacy of a lectin or LAG3 blockade clinically. Targeted interventions of this nature, alongside current immunotherapies, can refine current cure strategies in addressing undesired immune dysfunction and reinvigorating host immunity for better patient prognosis.

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Dedication

I honourably dedicate this thesis to my beloved Dad, Mom & Nani. Three special humans who have not only given me life but also co-authored its most cherished chapters. This work attests to the unceasing benevolence, optimism and belief you have showered upon me.

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5.6

Acronyms List

Ab	Antibody
ADAM	A disintegrin and metalloproteinase
AP-1	Activator protein 1
APC	Antigen presenting cells
ART	Antiretroviral treatments
ATP	Adenosine triphosphate
BCR	B cell receptor
BTLA	B- and T-lymphocyte attenuator
CCR5	C-C chemokine receptor type 5
CENPJ	Centromere protein J
CFSE	Carboxyfluorescein succinimidyl ester
CRD	Carbohydrate recognition domain
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte-associated protein-4
CXCR4	C-X-C chemokine receptor type 4
DAG	Diacylglycerol
DC	Dendritic cells
DMSO	Dimethyl sulfide
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FGL-1	Fibrinogen-like protein 1
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
Gal-3	Galectin-3
HESN	HIV-exposed seronegative
HIV	Human immunodeficiency virus
HLA-DR	Human Leukocyte Antigen – DR isotype
IC	Immune checkpoint
ICAM	Intercellular Adhesion Molecule
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
iNKT	Invariant natural killer T
IP_3	Inositol trisphosphate
ITAM	Immunoreceptor tyrosine-based activation motifs
ITIM	Immunoreceptor tyrosine-based inhibitory motif

ITSM	Immunoreceptor tyrosine-based switch motif
KIR	Killer immunoglobulin-like receptor
LAG3	Lymphocyte Activation Gene-3
LAP	LAG3 associated protein
LAT	Linker for activated T cells
Lck	Lymphocyte-specific protein tyrosine kinase
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
LSECtin	Liver and lymph node sinusoidal endothelial cell C-type lectin
LAG3	Lymphocyte activation gene-3
mAb	Monoclonal antibody
MAIT	Mucosal-associated invariant T
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
mIgG	Mouse-derived Immunoglobulin G
mRNA	Messenger RNA
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NLR	Nod-like receptors
ORF	Open reading frame
pAb	Polyclonal antibody
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD-1	Programmed cell death-1
pDC	Plasmacytoid dendritic cells
PE	Phycoerythrin
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PLCγ1	Phospholipase C, gamma 1
PRR	Pattern recognition receptors
RLR	RIG-I-like receptors
RNA	Ribonucleic acid
rSD	Robust standard deviation
SD	Standard deviation
SEB	Staphylococcus enterotoxin B

SED	Staphylococcus enterotoxin D
SEE	Staphylococcus enterotoxin E
SH2	Src homology 2
SHIP	SH2-containing inositol 5-phosphatase
SIV	Simian immunodeficiency virus
sLAG3	Soluble LAG3
SLP-76	SH2 domain containing leukocyte protein of 76kDa
STAT	Signal transducer and activator of transcription
T-bet	T-box transcription factor
TCR	T cell receptor
TFH	T follicular helper
TGFβ	Transforming growth factor beta
Th	Helper T
TIGIT	T-cell immunoreceptor with Ig and ITIM domains
Tim-3	T cell immunoglobulin and mucin domain containing-3
TLR	Toll-like receptors
TME	Tumor Microenvironment
TNFα	Tumor necrosis factor alpha
TOX	Thymocyte selection-associated high mobility group box protein
Treg	Regulatory T cells
α-syn	α-synuclein fibrils

1 Introduction

1.1 HIV

1.1.1 HIV Epidemiology

Human Immunodeficiency Virus (HIV) poses an ongoing health challenge for the 38.4 million people infected with the virus, globally [1]. Despite considerable research endeavors devoted to prevention and treatment, marginalized and vulnerable populations bear a disproportionate burden of new HIV infections. This is largely due to existing socioeconomic inequalities that exacerbate HIV incidence within resource-limited settings, further contributing to the ongoing transmission of the virus.

HIV is transmitted through direct contact with infected blood or mucosal secretions such as semen, vaginal fluids, or breast milk. Consequently, most HIV infections are acquired by unprotected sexual intercourse, sharing contaminated needles and perinatal transmission from mother to infant during birth or breastfeeding.

Without early treatment, HIV primarily infects and depletes CD4+ T-cells, which crucially coordinate the body's immune system. As rapid viral replication progressively destroys immune cells and hampers the immune response, individuals are rendered highly susceptible to opportunistic infections due to increasing immune dysfunction. Compared to the count of ~1000 CD4+ T cells/µL in healthy individuals, the decline in CD4+ T-cells to <200 CD4+ T cells/µL, resulting from chronic HIV infection, marks the diagnosis of Acquired Immunodeficiency Syndrome (AIDS) [2]. Fortunately, the growing arsenal of tools to manage the HIV epidemic, including antiretroviral (ART) therapy, has extended the lifespan of people living with HIV (PLWH) by delaying the progression to AIDS and reducing AIDS-related deaths [2].

1.1.2 HIV Biology

As a member of the genus *Lentiviridae* and family *Retroviridae*, HIV attaches to host cells by binding the CD4 receptor and co-receptor, C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4), expressed primarily on macrophages and CD4+ T-cells. Engagement of both receptors permits viral entry into the cell where HIV utilizes reverse transcription to convert its single-stranded ribonucleic acid (RNA) genome into deoxyribonucleic acid (DNA). After insertion of its DNA into the host's genome, the integrated provirus serves as a template to produce viral proteins and RNA for assembly of new virions.

As such, HIV infection is preferentially established in activated cells as they provide the necessary factors for efficient viral replication and production of new infectious particles i.e. productive infection. These released HIV particles subsequently infect other CD4+ T-cells and susceptible host cells, perpetuating viral infection [3].

Unlike productive infection that actively produces viral particles, HIV can lay dormant as silent provirus within host DNA where there is minimal viral transcription or protein expression, a phenomena known as latency. Establishment of this latent state is facilitated by transcriptionally-inactive or resting cells that deprive HIV of essential host-cell factors for its replication. Remaining undetectable, latent HIV persists inside these cells for extended durations, evading immune surveillance and elimination.

Although ART can effectively control active HIV replication, ART is unable to eliminate latent HIV, allowing its persistence in so-called latent reservoirs at various anatomical sites. Thus, the presence of latent HIV reservoirs poses a major barrier for addressing an HIV cure. For this

reason, elimination of the latent reservoir is a primary focus of HIV treatment and eradication strategies [4,5].

1.1.3 HIV Reservoir Reduction and Eradication Approaches

Ongoing scientific efforts are directed towards the specific targeting and disruption of the hidden reservoir, aiming to develop safe and effective treatment regimens. The "shock and kill" and "block and lock" approaches represent two distinct strategies currently under investigation to control the reservoir in the pursuit of an HIV cure.

1.1.3.1 Shock and Kill

Depletion of the latent reservoir through the “shock and kill”, aims to “shock” the virus out of hiding from its latent reservoirs and subsequently “kill” any HIV-infected cells that actively produce virus. Multiple techniques are employed to expose hidden HIV reservoirs to the immune system and therapeutic interventions, for successful clearance or control of the virus.

The first phase involves using latency-reversing agents (LRAs) such as Vorinostat, Romidepsin or Chaetocin to “shock” or reactivate viral gene expression from latently-infected cells that harbor dormant HIV [4,6]. By target mechanisms that suppress HIV transcription and latency, LRAs reawaken silent provirus, favoring viral replication and production while under ART.

During the “kill” phase, once the virus is reactivated, it becomes vulnerable to immune recognition and the effects of ART. Accordingly, the immune response or ART drugs can eliminate or “kill” the newly-activated HIV-infected cells, ultimately reducing the pool of infected cells and purging of the latent reservoir in PLWH [4–6].

1.1.3.2 Block and Lock

Rather than eliminating latently-infected cells, the “block and lock” approach focuses on long-term suppression of the virus by reinforcing viral latency and preventing viral resurgence.

This first step involves using therapeutic agents to “block” or inhibit various steps of the viral life cycle. This silencing is achieved by targeting key viral proteins or processes at entry, integration and transcription. By blocking viral activity, the replication of HIV is suppressed, preventing the formation of new reservoirs and spread within the body.

The second phase aims to stabilize or “lock” the virus in a non-replicative dormant state, minimizing the potential for viral rebound within the reservoir. Latency-promoting agents (LPAs), such as Spirinolactone, Ruxolitinib or Mycophenolic acid target various molecular and epigenetic signatures in HIV latency regulation [4–7]. The resulting transcriptional and histone modifications induced by LPAs can drive the HIV promoter in a deep latent state. Additionally, gene editing techniques that modify the viral genome or host cells can also hinder viral replication, ensuring permanent silencing of the virus in the absence of ART.

Unlike several repurposed shock and kill therapeutics that demonstrate preclinical and clinical promise in reducing HIV reservoir size in PLWH, block and lock therapeutics comprise highly novel molecules. These molecules have not received prior FDA approval, and hence, the clinical benefit of block and lock therapeutics is yet to be assessed following experimental development. Challenges remain in optimizing the safety and feasibility of these approaches for clinical use. Nevertheless, collective efforts evaluating their efficacy in reducing the latent reservoir towards a functional cure for HIV or sustained viral remission necessitates further research and clinical investigation [4–7].

1.2 Introduction to the host immune system

The human body is regularly exposed to a plethora of bacteria, fungi and viruses in the environment. To ensure survival, humans rely on a robust immune system that recognizes and responds to the wide array of invading pathogens, while minimizing harm to host. The host immune system also helps eliminate cancerous cells as they develop. The immune system comprises two lines of defenses: innate and adaptive immunity. The intricate network of specialized immune cells and factors within these two arms collectively safeguard the body against infection and disease.

1.2.1 Innate Immune System

The innate immune system serves as the first line of defense, swiftly detecting invading pathogens. As initial defense, physical and chemical barriers such as the skin, microbiota, mucosal surfaces, secretions and stomach acid block pathogen entry or create unfavorable conditions for their survival and proliferation. When pathogens breach this initial barrier, the cells of the innate immune system identify a diverse range of threats, non-specifically recognizes common patterns that typify pathogens. Although lacking immunological memory, the innate system operates uniformly in response to non-self (foreign) proteins, subsequently triggering and shaping adaptive immunity [8].

For immune recognition, pattern recognition receptors (PRRs) on innate cells detect invaders by their conserved pathogen-associated molecular patterns (PAMPs). Of the major human PRRs such as nod-like receptors (NLRs), RIG-I-like receptors (RLRs) and C-type lectin receptors (CLRs), Toll-like receptors (TLRs) are well-characterized PRRs, recognizing many PAMPs like lipopolysaccharide (LPS), flagellin and double stranded RNA. Association of PAMPs with its

cognate PRR prompts intracellular signalling pathways within innate cells to secrete cytokines and chemokines. These soluble proteins, classified as anti-inflammatory or pro-inflammatory, are central for cell trafficking, activation, differentiation and cell-cell communication. Such mediators recruit immune cells to the infection site to engulf and destroy microbes (phagocytosis) for pathogen clearance [8].

The main cells of innate immunity are granulocytes (neutrophils, eosinophils and basophils), mast cells, natural killer (NK) cells and professional antigen presenting cells (APCs) such as dendritic cells and monocytes/macrophages

When engaged, NK cells can identify and lyse cancerous or virally-infected cells and play immunoregulatory roles via cytokine secretion. Their activation relies on interactions between killer immunoglobulin receptors (KIRs) and their inhibitory or activating ligands. Macrophages, dendritic cells and B-cells serve as professional APCs. Following pathogen recognition and uptake via PRRs, APCs will phagocytize microbes into small peptides or antigens (Ags) for loading onto protein complexes called major histocompatibility complexes (MHCs), on the APC surface. Subsequently, APCs present Ags to the adaptive arm via MHCI or MHCII molecules, secreting cytokines and chemokines to recruit, activate and modulate the antigen-specific adaptive immune response [8].

1.2.2 Adaptive Immunity

While initially slow-responding, the adaptive immune system exhibits antigen-specificity, creating enduring immune memory and mounting a swift and vigorous response upon subsequent encounters with a pathogen. This arm of the immune system can be divided into the cell-mediated and humoral responses, governed by T and B lymphocytes, respectively [8].

Humoral immunity relies on B cells producing specific soluble antibodies (Abs) or membrane-bound Abs, known as B cell receptors (BCRs). These Abs recognize and bind cognate Ags on pathogens or toxins, neutralizing their attachment to host cell receptors. Upon recognizing their specific Ags, BCR activation triggers intracellular signalling that induce proliferation and differentiation of B cells into long-lived memory B cells, poised for secondary responses, and plasma cells, that secrete Abs. Circulating throughout the body, antibodies fulfill roles in pathogen neutralization, complement activation, opsonization, phagocytosis enhancement and antibody-dependent cellular cytotoxicity, among others. As professional APCs expressing MHCII, B cells also aid in antigen presentation, activating CD4+ T helper cells [8].

1.2.2.1 Cell-Mediated Immunity

T lymphocytes, or T cells, are vital in orchestrating and coordinating antigen-specific responses against intracellular pathogens as part of cell-mediated/adaptive immunity. To initiate such responses, each T cell contains a single unique type of the T cell receptor (TCR) that recognizes its cognate antigen presented exclusively on MHCs by APCs. This specificity is conferred by the TCR's distal variable domains which enable selective binding to specific Ag:MHC complexes [8].

T cells can be subdivided based on the TCR co-receptors CD4 and CD8, which help stabilize TCR interactions with MHCII and MHCI, respectively. In addition to secreting effector pro-inflammatory cytokines such as IFNγ and TNFα, antigen-specific CD8+ T cells or cytotoxic T lymphocytes (CTLs) also employ the cytotoxins perforin and granzyme B upon activation to directly kill cancerous or infected self-cells. Conversely, CD4+ T cells largely consist of regulatory T cells (Treg) or T helper (Th) cells. Upon binding MHC-restricted antigens, CD4+ T cells are activated with appropriate co-stimulation such as CD28 and differentiate into distinct subsets

based on the local cytokine/chemokine milieu. Th cells subsets such as Th1, Th2, Th17, T follicular helper cells (Tfh) and T regulatory cells (Tregs), each express unique set of surface proteins and cytokines. By releasing distinct chemokine and cytokine profiles, Th cells are vital in coordinating other immune cells, eliciting subset-specific responses upon secondary Ag encounter [8].

When activated, Th1 cells produce cytokines such as IFNγ, IL-2 and TGF-β to eliminate intracellular pathogens by inducing cell-mediated immunity such as CTLs, complement and recruitment of phagocytes (macrophages). In contrast, Th2 cells guide humoral responses by activating B cells to produce Abs that can neutralize allergens and extracellular parasites, such as helminths. Moreover, Th17 cells enhance antimicrobial peptide production and recruitment of neutrophils, making this subset effective against extracellular pathogens. Usually localized in lymphoid follicles, Tfh promote B cell maturation for high-affinity antibody generation within germinal centers [8].

As their name suggests, Tregs modulate immune responses to preserve immune homeostasis, peripheral tolerance, and limit autoimmunity and chronic inflammation. This is achieved by producing anti-inflammatory cytokines (IL-10 and TGFβ), cytolysis, metabolic disruption and expressing co-inhibitory receptors (such as PD1, CTLA4, TIGIT and LAG3: programed cell death (PD)-1, cytotoxic T-lymphocyte antigen (CTLA)-4, Lymphocyte activation gene (LAG)-3 and T cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibitory motif (ITIM) domains (TIGIT)) [8].

Lacking CD4 and CD8, the double negative T (DNT) cell population is heterogenous with immunoregulatory roles in both innate and adaptive immunity. These innate-like or

unconventional T cells consist of mucosal-associated invariant T (MAIT) cells, invariant natural killer T (iNKT) cells and γδ T cells, each with unique functions [9].

The diverse and interdependent components of the immune system must collaborate harmoniously to coordinate effective responses against pathogens. Dysregulation or disbalance of any of these cell populations can hinder the ability to successfully clear infections.

1.2.2.1.1 TCR signaling

Effective communication and signalling between two cells requires stable cell-to-cell contacts. T cell adhesion molecules LFA1 and CD2 to contact their ligands ICAM and CD58, respectively, on APCs to facilitate docking. The subsequent immune synapse formation allows a stable platform for efficient TCR signalling. After recognizing its cognate Ag: MHC complex, TCR signalling cascades are induced leading to T cell activation, proliferation and effector responses [8].

1.2.2.1.2 Initiation of TCR Signaling

In a quest to elucidate key players and signalling events, Jurkat cells, a T cell leukemia cell line, have been a valuable model system in unraveling and characterizing mechanisms of TCR signalling [10].

After TCR engagement, a series of signalling events occur to mount effective effector responses. As the TCR lacks its own signalling domain, it associates with CD3's multi-subunit chains, forming the TCR-CD3 complex. As CD3 is required for TCR function, T cells are identified from other immune cells based on their CD3 expression. TCR activation leads to the phosphorylation of cytoplasmic activation motifs or ITAMs within CD3γ/δ/ε/ζ cha ins. This phosphorylation recruits and activates the protein kinase Lck to phosphorylate CD3's ITAMs. This phosphorylated docking site recruits ZAP-70 for phosphorylation by Lck, amplifying its

activation. Activated ZAP-70 then phosphorylates adaptor proteins LAT and SLP-76 [8]. Crucial to signal transduction, this LAT signalosome serves as a scaffold, simultaneously binding and activating additional signalling molecules to propagate and amplify the cascade. Pathways of note include: RAS/MAPK-ERK, PLCγ1 and calcium pathways. The cleavage of PIP2 into IP3 and DAG by PLCγ1 promotes calcium influx and PKC activation, respectively. Concurrently, the engagement of CD28 on T cells with CD80/CD86 on APCs provides co-stimulatory signals (signal 2), augmenting the intensity of TCR signaling for more robust T cell activation. CD28 co-stimulation, alongside the CD3 complex, activates the PI3K pathway which converts PIP2 to PIP3 by phosphorylation, leading to the eventual recruitment of PLCγ1 and subsequent calcium influxes, as above [8,11]. The combined signaling events lead to activation of different transcription factors (TFs) including NFAT, NFκB and AP-1, amongst others. After translocation to the nucleus, these TFs regulate expression of genes involved in T cell activation, proliferation and cytokine production [8,11].

1.2.2.1.3 T cell Activation and Cytokine Production

Of the cytokines produced by activated T cells, IL-2 and IFNγ hold critical roles in T cell function and modulating subset-specific immune responses. As a key growth factor for T cells, IL-2 is essential for proliferation, survival and differentiation. It promotes the expansion of activated Th cells for coordination of immunity and maintains Tregs for immune tolerance or homeostasis [8,11]. Along with IL-2, IFNγ enhances cytotoxic activity of CTLs, for clearance of cancerous or infected cells within cell-mediated immunity, inflammation and antiviral defense. As such, imbalances in production or signaling of IL-2 and IFNγ collectively disrupts T cell proliferation, generation of effector functions and attenuate cytotoxic and antiviral activities. This disruption can result in insufficient T cell responses and compromised immune regulation against chronic

infections or tumors, common hallmarks of a phenomena known as T cell and immune exhaustion [8,11].

1.2.2.2 T cell and Immune Exhaustion

Unlike acute infection, where infection resolves with Ag clearance, persistent antigen presence, as in cancer or chronic HIV infection, exposes T cells to prolonged high levels of Ag stimulation and inflammation. This repeated activation induces gradual immune exhaustion, whereby effector T-cells progressively lose their proliferative capacity and effector functions. While beneficial in curtailing immunopathology or autoreactivity, exhaustion hinders a robust and sustained immune response desired for disease resolution [8,12–14]. Before HIV, T cell exhaustion was primarily characterized in murine models of chronic lymphocytic choriomeningitis virus (LCMV), hepatitis B virus (HBV) and hepatitis C virus (HCV), to clarify the developmental connections between memory and exhausted T cells, along with their precursors or progenitors [8,12–16]. Exhibiting a continuum of states from terminally differentiated with total [irreversible] effector loss to hypofunctional exhausted T cells, and finally to a subset of highly proliferative and self-renewing progenitor cells. The latter two can still proliferate when stimulated, albeit exerting diminished, but not absent, pathogen control *in vivo.*

Through antiviral treatment for chronic HCV, or T cell transfer from chronically-infected LCMV mice into naïve hosts, research indicates that exhausted T cells regain effector functions following antigen withdrawal [8,12–16]. Terminating chronic antigen stimulation reveals phenotypic, functional and transcriptional changes, re-differentiating HCV or LCMV-specific exhausted CD8+ T cell populations towards an effector memory-like CD8+ T cell profile with restored proliferation. Hence, this T cell hypofunctionality during cellular exhaustion is not permanent,

suggesting a potential for transient reversal and reinvigoration of these cells. Indeed, antigenic burden and duration greatly impact the degree of T cell exhaustion and its ability to recover, post-antigen removal.

At lower levels or shorter durations of antigen exposure during chronic HCV or LCMV, T cells phenotypically resemble stem-like precursors of exhausted T cells post-antigen clearance, in addition to memory T cells of resolved acute infections. Unlike most exhausted T cells with limited functional responses, the less terminally differentiated precursors can be reinvigorated, restoring their hypofunctionality. As these self-renewing progenitor or precursor cells retain higher effector, proliferative and developmental capacity, they critically sustain Ag-specific responses, mediating viral control over prolonged infection [8,12–16]. Conversely, high and sustained antigenic load or prolonged T cell exhaustion precludes development of key memory or exhausted-precursor T cell signatures, as the terminal exhaustion phenotype persists even after antigen withdrawal in both chronic infection and cancer [8,12–17]. Thus, the enduring influence of chronic antigen stimulation on T cell function increases and solidifies the imprinting of exhaustion features, particularly evident in terminally-exhausted T cells.

This hypofunctionality ultimately arises from persistent upregulation of various inhibitory receptors, called immune checkpoints, on the surface of T cells, as characterized in HIV-positive individuals.

1.2.2.3 Immune Checkpoints

In attempts to balance immune activation and suppression, the sustained overexpression of immune checkpoints (ICs), on exhausted T-cells, impair further cellular activation, rendering them hypofunctional [12–14]. The engagement of these IC receptors by their ligands, often

expressed by APCs, tumor cells or the inflammatory milieu, dampens T cell function by suppressing cytokine production. Thus, decreased IL-2 and IFNγ levels impair the exhausted T cell's coordination of an effective immune response to control HIV-replication, exacerbating exhaustion [13].

Through their intracellular motifs, ICs, such as LAG3, PD-1, TIM-3, CTLA-4 and TIGIT, negatively interfere with the TCR signaling pathway by inducing phosphatases. These activated phosphatases dephosphorylate key signaling proteins early in the TCR pathway, impeding signal strength and full T-cell activation for proper effector responses. Hence, ICs are attractive targets for developing novel immunotherapies like immune checkpoint inhibitors to partially rescue immune exhaustion [18]. Such approaches use antibodies to bind to immune checkpoint proteins, inhibiting their activity and reversing T cell exhaustion to reinvigorate immunity. These antibody-mediated blockades show significant success for remediating autoimmunity, certain cancers and infections [18]. By targeting multiple [inhibitory] pathways simultaneously, combinatorial IC inhibitors augment efficacy of cancer immunotherapy.

As the third FDA-approved IC-blockade, LAG3 raises many questions regarding its potential ligands, exact signaling pathways and limited data on LAG3-blockade enhancing HIV-specific T cell responses. Thus, LAG3's elusive mechanism of action warrants further research and therapeutic development, holding promise in countering exhaustion and viral reservoir persistence, for a functional HIV cure [18,19].

1.3 LAG3

1.3.1 LAG3 Structure

Part of the immunoglobulin superfamily (IgSF), LAG3, or CD223, is a negative co-receptor, expressed on the surface of $CD4^+$ T cells, $CD8^+$ T cells, NK cells, iNKTs, Tregs, B cells, plasmocytoid dendritic cells (pDCs) and even neurons. This 70kDa transmembrane protein, structurally similar to its ancestral homolog CD4, houses an intracellular region and 4 Ig-like extracellular domains (D1-D4) housing four N-linked glycosylation sites [19–21].

1.3.2 LAG3 Regulation

1.3.2.1 Transcriptional Control

Several cytokines, such as IL-2, IL-7, IL-12, IL-15, IL-27, and TGF-β, can augment LAG3 transcription, in addition to TCR activation [19–21]. While influenced by some CD4 regulatory loci, LAG3 transcription remains poorly understood. It encompasses various regulatory elements and transcription factor binding sites [22]. Several IC and immune exhaustion-related transcription factors, including Thymocyte selection-associated high mobility group box TOX and NFAT, induce LAG3 expression [23]. Inactivated GSK-3 also downregulates LAG3 expression on CTL and Th cells through increased T-box transcription factor Tbet expression [24]. Furthermore, methylation of different regions of the LAG3 promoter, can acutely upregulate or downregulate LAG3 expression [25].

1.3.2.2 Post-translational Modification and Regulation

LAG3 surface expression is regulated by a disintegrin and metalloproteinase domain-containing protein (ADAM)10 and ADAM17 cleavage on its extracellular side [26]. Cleavage produces a

soluble form of LAG3 (sLAG3), with higher levels found on virally-suppressed PLWH, potentially serving as a biomarker [27].

Nearly half of LAG3 inhabits recycling endosomes within T cells post-activation. This negative feedback by LAG3 endocytosis modulates its surface expression, distribution and inhibitory activity, as seen after α-synuclein fibril interaction. Parallelly, these preformed intracellular LAG3 stores may surface faster to curtail T cell activation easier [28]. However, LAG3 recycling warrants further research, for its other ligands, as well.

As part of another post-translational process, the diverse repertoire of glycans, or the glycome, constitutes an indispensable interface for LAG3-expressing cells to communicate with their environment. Here, post-translational glycosylation influences the localization and function of immune receptors, like LAG3, fine-tuning T cell responses through signaling of stimulatory and inhibitory immune pathways [29]. The coordinated action of glycosyltransferases and glycosidases in the golgi enzymatically links carbohydrates to specific amino acids, particularly nitrogen (N) and oxygen (O), of glycoproteins. The four N-linked glycosylation sites on LAG3 are marked by the sequence Asn-X-Ser/Thr, where X is any amino acid except proline [30]. As a crucial aspect of LAG3 biology, its glycan structures impact the interaction with its potential glycan-binding ligands, like lectins, and ensuing immunomodulatory properties in balancing immune activation and suppression. As detailed herein, I will unveil the underlying mechanisms by which N-linked glycans and its binding partners that instruct LAG3 immunosuppression to inform therapeutic interventions for cancer or chronic HIV.

Given the complexity of multiple levels and mechanisms of LAG3 regulation, direct measurement with flow cytometry is attractive for assessing LAG3 surface expression over transcript or total protein levels.

1.3.3 LAG3 Inhibitory Function

1.3.3.1 LAG3 Cytoplasmic Domain

The inhibitory effects of LAG3 are orchestrated by its intracellular region. Unlike other immune checkpoints with defined phosphatase-recruiting ITIM or ITSM domains that oppose TCR-activating kinases, LAG3 lacks such characterized inhibitory domains. Instead, LAG3's cytoplasmic tail features three conserved motifs, yet their exact effect on LAG3 signaling is debated [19–21].

Located within the RRFSALE region, the potential serine phosphorylation site (S484) has an unspecified role but is correlated with IL-2 production and may function similarly to CD4's PKC binding site. In contrast, alanine mutagenesis of either leucine (L) or phenylalanine (F) in RRFSALE halved LAG3's inhibition of IL-2, emphasizing FxxL motif's essential role in LAG3 function. As well, the lysine (K468) residue of the conserved KIEELE motif vitally mediates LAG3 inhibitory signaling [21,31–34]. Mutational analyses indicate that, deleting the third conserved motif alone, the carboxy-terminal EP (glutamate-proline) rich repeats, maintains LAG3 activity in $CD4^+$ and $CD4^-$ T cells atypical [32]Conversely, deleting both EP and KIEELE motifs, completely nullifies LAG3 activity [35,36]. Interestingly, combined FxxL mutation and EP deletion, converts LAG3 from an inhibitory to a positive co-receptor by nearly doubling IL-2 production, compared to LAG3- cells [37]. Lacking its intracellular domain, LAG3's *stimulatory* role was replicated in 3A9 cells [32,36]. By associating with LAP (LAG3 associated protein), the EP motif enables LAG3

colocalization with CD3, CD4 and CD8 within lipid rafts, indirectly counteracting CD3/TCR activation. Altogether, these findings suggest that the EP motif may impede LAG3 from acting as a stimulatory co-receptor, like CD4, rather than collaborating with KIEELE in inhibitory signaling [35]. Concealing its mechanism, such gaps in LAG3's inhibitory signaling merit resolution, particularly in relation to early T cell signaling events, for effective therapeutic targeting.

1.3.3.2 LAG3 Engagement of Ligands

By binding specific immune receptors, such as LAG3, ligands are crucial components in orchestrating and regulating LAG3-mediated functions. These ligand interactions and consequences thereof are summarized in Figure 1. Upon LAG3-ligand engagement, signal transduction relays outside signals to the intracellular compartment to inhibit T cell activation, proliferation, immune responses and other functional outcomes that impact immune regulation [38–40]. Despite structural similarities to CD4, LAG3 does not engage with the HIV envelope protein gp120 which employs CD4 for cell attachment and entry. However, a 30 amino acid loop in LAG3's D1 ectodomain, in addition to D2, is required for binding MHCII at 100-fold higher affinity than CD4 [41,42].

Accordingly, the 17B4 Ab clone, which targets this D1 loop epitope, successfully blocks this LAG3-MHCII interface by competitive inhibition [41,43]. By precluding necessary LAG3 dimerization and oligomerization, mutations in another D1 segment also hinder MHCII interaction, even for co-expressed wildtype LAG3 [41,44].

Nonetheless, the mechanism through which ligand binding engages and enables LAG3 inhibitory activity remains unclear. Multiple studies refute earlier research proposing LAG3 outcompetes CD4 for MHCII engagement as a major part of LAG3's inhibitory mechanism *in*

vitro [32]. Instead, following LAG3-MCHII binding during T cell activation, LAG3 colocalizes with the immune synapse, bringing it closer to the TCR for T cell inhibition [45]. While some association with immune synapse occurs even without LAG3-ligand binding, the extent of LAG3 colocalization may fall short in effectively mediating T cell suppression [46]. Further, immune synapse docking implies LAG3 acts early in the TCR pathway, as general kinase, PKCθ/δ or calcineurin inhibitors did not affect LAG3 inhibition in LAG3+ Jurkats, unlike a LAG3 blockade[47]. Thus, LAG3-ligand engagement is crucial in colocalizing LAG3 with vital signaling components and fostering stable LAG3 oligomerization, necessary for LAG3 inhibitory function [45,46]. Hence, thorough appreciation of LAG3 biology and function entails understanding the mechanisms of LAG3 binding to its various ligands, which is currently understudied.

Besides the well-characterized primary ligand MHCII, LAG3 has been shown to bind a number of additional ligands such as Fibrinogen-like protein 1 (FGL-1), α-synuclein fibrils, Galectin-3 (Gal-3) and liver and lymph node sinusoidal endothelial cell C-type lectin (LSECtin) [21]. FGL-1 ligation, like MHCII, relies on D1 and D2, yet with a distinct binding interface as shown by anti-LAG3 mAb C9B7W blocking FLG-1 interaction but not MHCII binding [21,48]. Expressed on neurons, LAG3 binds pathologic α-synuclein fibrils through its extra loop D2, D3 and cytoplasmic regions. Despite the unknown immunological significance and impact on LAG3's inhibitory function, this interaction mediates α-synuclein aggregation, endocytosis and transmission between neurons during Parkinson's disease progression [49,50]. Beyond the ligands MHCII, FGL-1 and α-synuclein, characterized as engaging LAG3 via extracellular protein-protein interactions, alternative ligands may also participate in LAG3 binding, function and regulation, via alternative mechanisms such as protein-carbohydrate contacts.

These diverse binding mechanisms expand the spectrum of potential interactions that underly LAG3 function and regulation, illustrating their complexity in LAG3 biology and immune modulation.

1.4 Lectins

Participating in these unique protein-carbohydrate interactions, lectins are a family of proteins, capable of binding carbohydrates or glycans on glycoproteins. Importantly, as implicated ligands, they could broaden the scope of LAG3 biology in disease and immunity. In nature, these carbohydrate-binding proteins are ubiquitously expressed across a broad range of taxa, encompassing plants, animals, fungi, prokaryotes and microorganisms.

Recognizing and binding glycans with their carbohydrate-recognition domains (CRDs), lectins have accrued increasing prominence in immunology, playing pivotal roles in modulating the complex functions of the immune system. These lectin-glycoprotein interactions primarily entail facilitating homeostatic processes and immune cell-APC interactions, aiding in intercellular communications, immune synapse formation, T cell trafficking and differentiation, host-pathogen interactions and influencing downstream T cell signaling pathways for immune recognition and response. In the case of prolonged inflammation, lectins assume integral functions in the pathophysiology, immune dysregulation and exhaustion within the TME or during chronic HIV infection [51].

Among these immunosuppressive lectins, LSECtin and Gal-3 have garnered considerable attention for their potential involvement in T cell function and exhaustion within such disease contexts. Harboring CRDs, LSECtin and Gal-3 interaction with LAG3 likely depends on LAG3's abundant extracellular N-linked glycosylation sites [21,41,52–54]. While the necessity and mechanism of LAG3 glycosylation in lectin binding remains a large unknown due to limited

binding site analysis studies, Gal-3 and LSECtin, expressed in the TME, have both incited LAG3 activity. In a LAG3-dependent manner, both ligands have inhibited IFNγ responses following CTL activation [21,52–54]. Herein, this thesis elucidates the interactions between two lectins, LSECtin and Gal-3, with LAG3, aiming to clarify the mechanisms governing LAG3 engagement and inhibitory function, for full appreciation of LAG3 biology in T cell exhaustion and ensuing tumor and HIV disease progression.

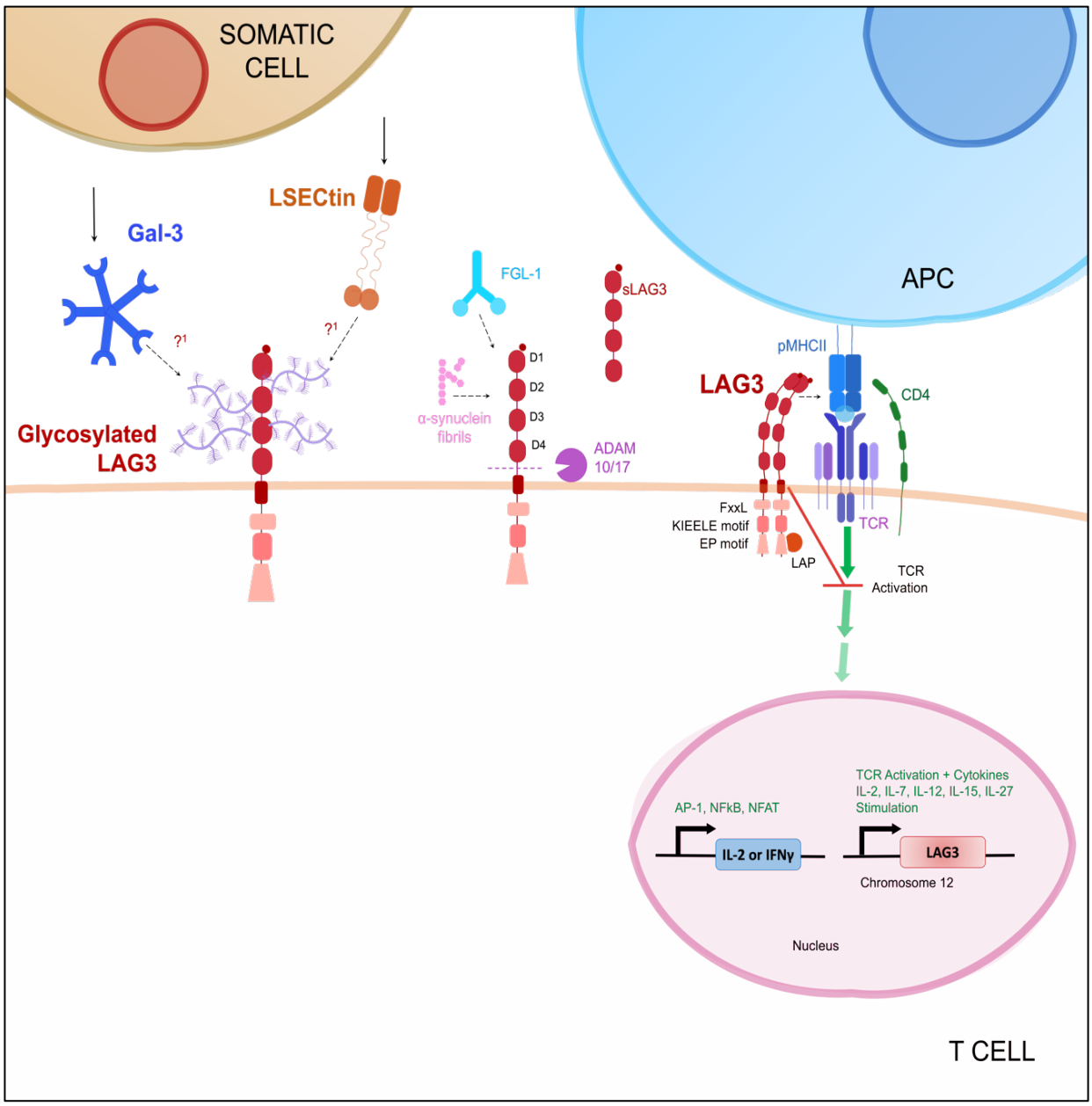


Figure 1. Schematic of LAG3 Interactions and Function. This diagram illustrates the diverse LAG3-ligand interactions during LAG3-mediated T cell inhibition. Activation via T cell Receptor (TCR) or specific cytokine signalling upregulates LAG3 transcription for its surface expression. [1]At the surface, infection or tumor-expressed lectins such as Galectin-3 and LSECtin may engage LAG3 by binding its N-glycosylated residues, although the necessity of N-glycosylation for LAG3-lectin ligation requires elucidation. Confirmed LAG3 ligands, α-synuclein and FGL1, alongside canonical ligand peptide-MHCII (pMHCII), vary in LAG3 oligomerization requirements for binding. Upon ligand engagement, LAG3 inhibits early steps of the TCR signaling pathway, dependent on LAG3's cytoplasmic domain. This impedes activation of transcription factors NFAT, AP-1 and NFkB, ultimately hindering cell cytokine production and proliferation. Metalloproteases ADAM10/17 further regulate LAG3 surface expression by cleaving it, releasing LAG3 in soluble form (sLAG3). Adapted from [21].

1.4.1 LSECtin

Expressed by the liver, lymph node, bone marrow sinusoidal endothelium and TME, LSECtin is part of the DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) family, containing an extracellular domain, a transmembrane domain and an intracellular domain. In addition to soluble isoforms, some LSECtin isoforms are expressed in *ex vivo*-isolated human peripheral blood, thymic DCs and myeloid cells [55,56]. Its extracellular portion comprises a neck domain, influencing oligomerization, ligand-binding affinity and efficient ligand recognition or specificity, as well as a CRD, which binds mannose, N-acetyl-glucosamine (NAcGlc) and fucose residues during Ag capture and recognition [52].

In addition to being expressed by myeloid cells, LSECtin enables pathogen recognition by interacting with glycoproteins of several viruses, augmenting infection of Ebola, Lassa virus, HCV and LCMV [57–59]. Conversely, LSECtin binds soluble HIV-1 glycoprotein, though it cannot capture HIV-1 particles (i.e. virion associated glycoprotein), rendering it ineffective against HIV-1 infection [55]. Its specific role in HIV and interaction with viral ligands require further investigation. Considering its upregulation on APCs in [severe] COVID-19 patients, LSECtin enhances SARS-CoV infection of hepatocytes [60]. Serving as routes of immune escape, pathogens exploit LSECtin for viral immune evasion. Additionally, LSECtin facilitates cell adhesion for DCs and endothelia across various lymph nodes or tissues, enhancing pathogen spread and persistence. While well-appreciated in pathogen detection, clarifying such pathological mechanisms would inform new therapeutic strategies for intervention [61].

In contrast, the role of LSECtin in immune modulation and blunting effector T cell responses, particularly in chronic viral infections and cancer, is emerging, given their shared immunological

similarities [62]. In fact, LSECtin-expressing B16 melanoma suppresses [tumor-specific] T cell activation and proliferation, inhibiting generation of adequate anti-tumor responses *in vitro* and *in vivo* [54]. Through binding LAG3, as an alternative ligand, this LSECtin-mediated immunosuppression enables tumor immune escape, promoting tumor growth and progression within the TME [63]. While the precise mechanisms and signaling pathways underlying inhibitory LSECtin-mediated T cell regulation remain unclear, blocking LSECtin restored CTL IFNγ secretion, slowing tumor growth, mirroring LSECtin-deficient mice.

LSECtin also suppresses hepatic T cell immunity to limit liver immunopathology observed in HBV. Here, administering recombinant LSECtin alleviates disease by downregulating T cell and liver immune responses in mice, suggesting potential interactions with HBV glycoproteins, as well. Such findings merit further research, particularly in HIV infection, where liver disease and inflammation are prevalent [64–67].

While poorly understood, exploring the precise mechanisms of LSECtin's ability to participate in protein-glycan interactions with LAG3 and its relative contribution in instructing LAG3-mediated T cell inhibition could ultimately inform interventions that mitigate immune dysfunction in viral diseases and beyond [21,54,65].

1.4.2 Galectin-3

Galectin-3 is a multifaceted protein within the chimera-type galectin subfamily, which bind beta-galactosides on glycoproteins and extracellular matrix proteins with their CRDs. Additionally, its oligomerization domain enables Gal-3 to cross-link its binding targets. Remarkably, it is found within various cellular compartments, including the nucleus, cytoplasm,

mitochondrion, cell surface and extracellular space, with involvement in cell adhesion, activation, migration, growth, differentiation, cell cycle, apoptosis and immune regulation.

Expressed by many cell and tissue types, including immune cells, epithelia, endothelia, heart, kidneys, liver and lungs, Gal-3 is involved in angiogenesis, organ fibrosis, inflammation, metabolic disorders, inflammatory bowel diseases cardiovascular diseases, neurological conditions and stroke, highlighting its significance in health and disease. Accordingly, Gal-3 serves as a biomarker in monitoring diagnosis, prognosis and as a therapeutic target for various conditions, including cancer as it upregulated by many tumor types during proliferation, metastasis and immune evasion [68,69].

When interacting with immune cells, particularly in cases of inflammation, Gal-3 possesses immunomodulatory functions shaping endothelial and macrophage activation and polarization, DC activation and maturation, and, most importantly, T cell immunosuppression. This multifaceted inhibitor of both the innate and adaptive arms of the immune system dampens its response against cancer, extending to promoting tumor-driven immunosuppression through several mechanisms in the TME [53]. In engaging with and remodeling components such as ECM glycoproteins, for cell-matrix interactions in cell migration, Gal-3 promotes cancer progression and metastasis. By binding to anti-tumor T cells, Gal-3 exerts a comprehensive inhibitory effect upon their function. This includes inhibiting early activation by TCR downregulation. Gal-3 can dampen cytokine production by inducing T cell anergy, a state reversed by depleting surface Gal-3 and even triggers CD8+ T cell apoptosis, exacerbating the immune escape of tumor cells [53,70–72]. In particular, multivalent molecular interactions between T cell surface N-glycans and Gal-3, disrupt TCR clustering, thereby raising the agonist threshold for TCR signaling [73].

Emerging evidence shows that Gal-3-mediated CTL suppression relies on functional LAG3 expression. A mouse tumor model evaluated Gal-3 in shaping antitumor responses in a tolerogenic setting *in vivo*. By inducing LAG3 activity, Gal-3 curtails these terminally differentiated tumor-reactive CD8+ T cells, actively promoting tumor-driven immunosuppression. This process constrains early TCR signaling events and subsequently limits CTL IFNγ production in mice, while Gal-3 depletion increases CTL proinflammatory signalling activation [53]. Similarly, Gal-3 depletion upregulates TCR expression, restoring IFNγ secretion in CD4+ T cells [74]. Tumor-secreted Gal-3 limits IFNγ permeation and subsequently reduces chemokine content and CTL infiltration to the tumor [75]. Conversely, supernatant treatment from Gal-3-depleted cells or treatment with anti-Gal-3 Ab improves tumor-reactive T cell proliferation and IFNγ secretion [71]. Thus, inhibiting Gal-3 in conjunction with T cell-directed immunotherapies, specifically immune checkpoint blockades, can enhance specific immune responses against cancer and even chronic HIV infection.

Independent of its lectin activity, Gal-3 participates in various aspects of the HIV-1 lifecycle, including viral attachment, budding, and intercellular transfer. HIV-1 infection and HIV-Tat protein up-regulate Gal-3 expression, which also enhances HIV budding and transfer [76,77]. In the case of an HIV-1 subtype B infection, Gal-3 expression is induced, where it can interact with viral envelope gp120, facilitating viral attachment and internalization as a PRR. In conjunction with higher Gal-3 plasma concentrations in HIV-1-positive patients, these findings indicate that Gal-3 can improve the infectivity and replication of specific HIV-1 strains [78]. Gal-3 also promotes HIV-1 particle assembly and viral budding or release in T cell lines and primary CD4+ T cells. Co-transfection of cells with Gal-3 and HIV-1 plasmids leads to increased HIV-1 budding, while suppressing Gal-3 expression by RNAi reduces viral budding. By interacting with the proline-rich

region of ALIX via protein-protein interactions, Gal-3 aids ALIX and HIV-Gag p6 association for viral budding, making it an important factor in HIV-1 pathogenesis [77]. Lastly, Gal-3 alters the composition of CD4+ T cell membrane lipid rafts, essential for virological synapse integrity. These supramolecular structures may facilitate the stability and efficiency of cell-to-cell HIV-1 transmission, leading to increased HIV spread within the host [79]. Understanding the interactions between Gal-3 and HIV can provide insights into the development of potential therapeutic strategies targeting Gal-3 to disrupt viral replication and transmission.

Most importantly, uncovering additional mechanisms underlying engagement of LAG3 activation and function can shed light on immune checkpoint biology and T cell exhaustion. Given the complex interplay within tumor microenvironments and chronic infection, Gal-3 could interact with LAG3, potentially hinging on LAG3's extensive N-glycosylation, holds the promise of illuminating mechanisms driving disease progression during immune exhaustion and informing clinical strategies effectively.

1.5 LAG3 in Chronic HIV Infection

In chronic HIV infection, escalating viral load and immune activation drive a complex immunopathological interplay. Continuous HIV replication propels ongoing viral presence. This recurrent antigen exposure intensifies activation of target immune cells, exacerbating chronic inflammation. The resultant depletion of HIV-infected or bystander CD4+ T cells, compromises anti-viral immunity, perpetuating a continuum of disease progression, chronic immune activation and prolonged harmful inflammation [19,20,80].

Accordingly, immune cells invoke expression of ICs, to curb excessive activation and associated immunopathology. Like other ICs, LAG3's main role in chronic infection is likely to prevent

unrestricted immune activity, modulating responses on various immune cells. While achieving the right equilibrium between immunosuppressive and immunostimulatory signals is vital during infection, the immunomodulatory effects of LAG3-mediated immune exhaustion appears to be a double-edge sword when addressing chronic infections. On one hand, having less LAG3 would favour anti-HIV immune responses, alleviating viral burden for the price of immune-mediated pathology. However, through induction of immune cell exhaustion, having more LAG3 controls this immune hyperactivity, albeit at the expense of higher viral load persistence, detrimental when viral clearance is desired [19,20,80].

Indeed, the Fowke lab has shown LAG3 expression is elevated on bulk T cells, central memory T cells, NK cells and iNKT cells in peripheral blood among HIV-positive Kenyan female sex workers compared to healthy controls, even after ART treatment [81–84]. Specifically, iNKT cells harboring higher LAG3 levels is linked to impaired cytokine production upon lipid stimulation in chronic HIV-1, which remains unimproved after ART [81].

Moreover, this upregulation is unfavorably associated with severity of T cell exhaustion, declined CD4 T cell help, higher plasma viral load, disease progression and faster viral rebound upon ART interruption in HIV-positive individuals declined CD4+ T cell help in HIV-positive individuals [13,84,85]. Its [over]expression on both CD4+ and CD8+ T cells exhausts their functionality, reducing their ability to respond to stimuli in HIV-1 infected subjects [84,86]. Eventually, this progressive loss of function is accompanied by physical deletion of cells, in a chronically activated immune system, in addition to HIV-killing of various target and bystander cells [84,86]. In addition to its soluble expression by multiple cell types during inflammation, productive HIV infection also increases Gal-3 production, which can inhibit CTL activation and

killing capacity. In interacting with LAG3, Gal-3 may *further* enhance this CTL dysfunction and, likely, exhaustion on other immune cell subsets expressing LAG3[19].

Possessing innate and adaptive immunological functions, evidence suggests that endothelial cells are more activated and dysfunctional in the milieu of chronic HIV inflammation, as reported in HIV-positive patients [87]. Hence, more LSECtin, expressed by activated liver, lymph node, and bone marrow sinusoidal endothelia, could engage LAG3 and blunt [HIV-specific] effector T cell responses and overactive immune responses in a chronic inflammation, as shown in the liver [61,64–67]. Noting oncological studies referenced earlier, increased LAG3 engagement by lectins can exercise immunosuppression, enabling HIV progression, given its immunological similarities to cancer in inhibiting T cell function [21]. Probing these mechanisms provides insights that address LAG3-mediated T cell suppression and immune dysfunction in HIV.

Compared to non-progressors, LAG3 transcription levels show a positive correspondence with higher viral load set points within rapid progressors. Accordingly, lower LAG3 expression coincides with increased CD4+ T cell counts, indicating its role in T cell depletion and injury in peripheral blood during chronic HIV. Relating to this, LAG3 expression is lower on NK cells of HIV-exposed seronegative populations and elite controllers. As well, compared to control groups, elite controllers and viremic non-progressors display less LAG3 on CD4+ T cells [84,88–90]. Although data on LAG3 blockade in HIV is limited, one study reveals that *ex vivo* LAG3 blockade significantly augments HIV-specific CD4+ and CD8+ T cell responses. Hence, it is possible that LAG3 blockade could not only restore HIV-specific T cell function and CD4 count recovery, originally refractory to current ART, but also slow disease progression and improve therapeutic responses to viral eradication strategies among HIV-infected individuals [84].

Alongside LAG3, other immune checkpoints PD-1, CTLA-4, TIM-3 and TIGIT are also upregulated on exhausted T cells, propelling their dysfunction [91,92]. Specifically, co-expression of LAG3, PD-1 and TIGIT identify CD4+ T cells enriched for HIV DNA, marking a preferential niche for establishing a stable HIV reservoir [93–95]. In conjunction with LAG3-mediated T cell suppression, combined inhibitory signalling may deprive HIV of host factors for its productive infection, thus fostering latency and preferentially HIV persistence during ART, as demonstrated *in vitro* [96].

1.5.1 LAG3 in Therapeutics

Immunotherapeutic approaches targeting inhibitory immune checkpoints has revolutionized cancer treatment paradigms. Indeed, exhausted tumor-infiltrating lymphocytes (TILs), consisting of $CD4^+$ T cells, $CD8^+$ T cells, Tregs, NK cells, B cells, macrophages and DCs, are marked by heighted immune checkpoint expression, correlating with diminished anti-tumor responses and poor prognoses. Disrupting these inhibitory pathways in T cells has enhanced prolonged anti-tumor immunity and clinical responses in advanced malignancies, that were otherwise refractory to traditional treatment. In early clinical trials, monoclonal antibodies targeting both CTLA-4 (ipilimumab) and PD-1 (nivolumab) demonstrated significant anti-tumor effects than using either alone. However, many patients develop resistance and experience low-response rates alongside immune-related side effects. Thus, efforts have pivoted towards targeting alternative immune checkpoint/ligand interactions, like LAG3, to improve clinical outcomes [97].

Upregulated across many tumors and TILs, LAG3 correlates with aggressive tumor progression, poor prognosis and unfavourable outcomes in melanoma, renal cell carcinoma, head and neck squamous cell carcinoma, hepatocellular carcinoma colorectal cancer, non-small cell lung cancer, follicular lymphoma, diffuse large B cell lymphoma and breast cancer and more [98,99].

As ICs act through different mechanisms, targeting multiple ICs simultaneously offers synergistic effects in overcoming exhaustion, and associated resistance, from multiple avenues

and restoring immunity [97]. Due to LAG3's synergistic potential with PD-1, LAG3-targeted therapies are often combined with anti-PD-1 therapies. Compared to 10-fold response monotherapy, dually blocking LAG3 (MK-4280) and PD1 (pembrolizumab) yields a synergistic 50-fold increase in response *in vitro* [98]*.* Likewise, combining LAG3 blockade with anti-PD1, synergistically augments tumor-infiltrating T cell cytokine release and proliferation, ultimately delaying progression of several cancers *in vitro* and *in vivo* [100,101].

Clinically, LAG3 blockade, in combination with PD-1, remarkably exhibits a minimal increase in adverse side effects than PD-1 blockade alone in the RELATIVITY-047 (NCT03470922) trial. In this completed phase III trial, adding anti-LAG3 (relatlimab; first LAG3 mAb) to anti-PD1 (nivolumab) doubles progression-free survival (PFS) to 10.1 months for 714 patients with untreated and unresectable or metastatic melanoma [102,103]. In addition to increasing objective response rates for melanoma, renal cell carcinoma (NCT00351949) patients receiving a higher IMP321 (first LAG3 fusion protein) dose reduced tumor growth and prolonged PFS [97].

As the third FDA-approved IC, LAG3 expands the repertoire of combinatorial blockades for metastatic melanoma, with over 80 trials currently evaluating its clinical success in other cancers. Considering the immunological parallels, FDA-approved LAG3 inhibitors, in addition to lectin inhibitors, could be repurposed to reduce HIV disease severity when combined with pre-existing IC blockades [97].

1.5.2 LAG3 Immunotherapy in HIV

As highlighted, through a dual mechanism, ICs foster HIV persistence. With elevated expression on latently-infected cells alongside other ICs, LAG3 promotes reservoir formation and persistence by suppressing host cellular factors for HIV replication. Secondly, LAG3 represses effector [cytotoxic] function of HIV-specific T cells. This double role in enhancing viral reservoir while simultaneously inhibiting immune responses illustrates the intricate interplay between

ICs and HIV persistence [93]. As part of the "shock and kill" strategy, a LAG3 blockade may exert two distinct effects when given to PLWH on ART. It could "shock" or re-awaken HIV expression preferentially from latently infected CD4+ T cells, exposing them to immune recognition for immune clearance and/or viral-induced apoptosis. Next, LAG3 blockade may reinvigorate functionality and recovery of HIV-specific exhausted T cells, especially CTLs, to "kill" HIV-infected cells [6,18]. Drawing from its success in cancer immunotherapy, high interest exists if this treatment approach can viably reverse latency and recover HIV-specific T cells towards a long-term HIV cure or remission.

As cancer clinical trials frequently exclude PLWH, clinical data examining the use of IC blockades in HIV are limited [18]. However, PD-1 blockade in SIV-infected macaques improves SIV-specific CD4+ and CD8+ T cell responses, proliferation and viremic control, decreasing viral RNA levels and HIV-reservoir size after ART cessation [104,105]. Moreover, combinatorial PD-1 and CTLA-4 blockade enhances effector memory T cells expansion and functionality, inducing greater latency reversal compared to anti-PD-1 alone in ART-treated SIV-infected [106]. Similarly in ART-treated cancer patients with HIV, PD-1 blockade augments proliferation and response of HIV-specific CD4+ and CD8+ T cells while decreasing cell-associated HIV RNA expression. Concurrent with diminished CD4+ T cell-associated HIV DNA, PD-1 blockade induces HIV replication, indicative of latency reversal [95,107,108]. The potency of HIV-reservoir reduction was significantly higher following combinatorial IC blockade [109,110]. Mirroring *in vivo* and *ex vivo* PD-1 and combinatorial blockades, *ex vivo* LAG3 blockade also significantly enhances HIV-specific $CD4^+$ and $CD8^+$ T cell IFNγ responses, suggesting targeting LAG3 may aid HIV reservoir depletion by reversing latency and restoring immune function [84,95,110–112].

Carrying minimal risk of HIV resistance coupled with fewer side effects, understanding LAG3 in HIV infection can better inform LAG3-targeted interventions, potentially reducing disease

severity within patients. Fully unleashing the arsenal of IC therapy, combining LAG3 blockades with other ICs can bolster HIV treatment efficacy for a durable HIV functional cure [19].

1.6 Rationale, Hypothesis and Objectives

Rationale

LAG3 in Immunosuppression: LAG3, an emerging inhibitory immune checkpoint, acts as a negative regulator on immune cells to prevent unrestrained immune activation. However, upon chronic Ag exposure, as in cancer or chronic HIV, the inhibitory effects on T cells are co-opted to evade immune control, leading to immunosuppressive responses linked to immune dysfunction. Suppressing T cell function in chronic disease, this T cell exhaustion is provoked by the upregulation of LAG3, resulting in impaired effector cytokine responses and proliferation[19]. Hence, IC blockades that target LAG3, are an attractive and emerging strategy in immunotherapy to boost both anti-viral and anti-tumor immune responses.

Glycosylation in Immune Regulation: Despite clinical relevance and efficacy of therapeutics targeting LAG3, our current understanding of LAG3 mechanism of action, and its involvement in immunosuppression, is far from comprehensive. Research has identified that LAG3 is extensively glycosylated with multiple N-glycosylation sites. As recognition molecules for glycan-binding proteins or lectins, cell or glycoprotein-expressed N-glycans are integral in immune interactions and signalling, instructing immune responses and IC function upon ligand-engagement [30].

Gaps in Knowledge: However, a significant knowledge gap concerns the precise mechanism of how the proposed lectin-ligands, Galectin-3 and LSECtin, bind LAG3 and the necessity of glycosylation in this interaction. With Galectin-3 and LSECtin expressing CRDs, glycans on LAG3

likely contribute to lectin binding. Hence, elucidating the interaction and contribution of these immunologically-relevant ligands would uncover additional mechanisms of LAG3 engagement and function, broadening the scope of LAG3 biology and T cell exhaustion.

To address these gaps in knowledge, this thesis will determine whether glycosylation of LAG3 is required for Galectin-3 and LSECtin binding and aims to resolve the regulatory effects and functional significance of LAG3-lectin interaction in T cell suppression which remains unknown.

By elucidating these gaps in understanding of LAG3-mediated immunosuppressive processes, this research can inform the interplay between immune exhaustion and disease progression during cancer or chronic infection. This knowledge is vital for optimizing clinical therapies that block lectin interaction or LAG3 function to mitigate immune dysfunction and reinvigorate host immunity, where needed. As such, this study seeks to clarify the role of lectins, LSECtin and Galectin-3, in regulating LAG3-mediated T cell suppression.

Hypothesis: I hypothesize that lectins, LSECtin and Galectin-3, exhibit binding specificity for glycosylated residues on LAG3. Furthermore, upon binding, these lectins will trigger LAG3-mediated T cell suppression, resulting in dose-dependent decline of cytokine production.

Objectives (2):

1. Design a lectin-binding assay to determine whether LAG3 glycosylation is necessary for lectin binding
2. To determine if LSECtin/Galectin-3 binding induces LAG3 function and subsequent T cell inhibition

2 Dissertation Contributors and Contributions

2.1 Contributors to Dissertation Work

I would like to extend my sincere gratitude for the collaborative efforts and invaluable expertise of the esteemed colleagues whose contributions greatly enriched the generated research. Data presented within Chapter 4: Results Section will be eventually submitted for manuscript publication in a scientific journal with the following authors: Shifa F. Mohideen, Colin G. Graydon, Julie Lajoie, and Keith R. Fowke. Contributions of Shifa F. Mohideen (SFM) and fellow colleagues related to Dissertation work are outlined, herein.

Dr. Keith R. Fowke secured study funding from CanCURE 2.0 grant 2019-2023, with Dr. Fowke approving experimental design and overseeing dissertation editing. Natasha Hollett collected blood from healthy adult donors while SFM processed samples for PBMC isolation to conduct PBMC characterization experiments.

Assisting with flow cytometry panel development, Drs. Julie Lajoie, Monika Kowatsch and Keith R. Fowke provided troubleshooting guidance during optimization of the *ex vivo* panel for PBMC and Jurkat characterization (Table 1 and Section 3.5.1). With reference to Table 4, which specifies surface and intracellular panel used in Lectin-mediated suppression experiments, Drs. Monika Kowatsch and Christine Zhang advised marker-fluorochrome channel assignment and controls during initial panel design and optimization. For outlined Tables 1-4 and *in vitro* assays, SFM principally conceptualized panels, executing flow cytometry optimization, sample preparation, staining, acquisition, data analysis and interpretation.

Additionally, I extend my appreciation towards Drs. Thomas Murooka and Colin G. Graydon for their scientific oversight during assay development, optimization and readout of lectin-

mediated LAG3 activity *in vitro*. Approving all designs, their contributions in ensuring feasibility of *in vitro* procedures expedited the attainment of proficient results, following initial conception and development by SFM. For *in vitro* assays and procedures described in Sections 3.6 and 3.7, SFM was responsible for experimental conception, design, optimization and conduction, flow cytometric acquisition and ELISAs, data analysis, interpretation and statistics, and manuscript writing for obtained data presented in this dissertation. I thank the Lentiviral Core at UManitoba for successful production of "LAG3-based lentiviral vectors". Their stringent quality control enabled construction of reliable LAG3+ expressing Jurkat cell lines in our studies.

Lastly, I deeply value the diligent efforts and dedication of Dr. Colin Graydon who principally constructed and established the LAG3+ and LAG3- Jurkat T cell lines, for use in our research projects. His precision and technical proficiency in cell culture and molecular biology ensured the successful creation of reliable cellular models for our experiments, contributing to the robustness and credibility of data. Outlined in Section 3.4, SFM assisted Dr. Colin Graydon in cell line development, including preparation and transformation of competent *E. coli* DH5α, isolation of LAG3-expressing plasmid DNA, transduction of LAG3 lentiviral expression vector into Jurkat T cells and subsequent puromycin selection. After creation, SFM cultured and validated individual Jurkat cell line flasks for characterization and experiments in this thesis.

2.2 Publications and Outputs of Dissertation Work

2.2.1 Peer-reviewed articles

1. Graydon CG, Mohideen S, Fowke KR. LAG3's enigmatic mechanism of action. Frontiers in immunology. 2021:3444.

2. Mohideen S, Murooka TT, Yao XJ, Fowke KR. Intruding the Mind: Unraveling HIV's Invasion of the Neurovascular Unit. Frontiers in Immunology. *In progress*.

2.2.2 Poster Presentations and Abstracts

1. Investigating the Role of LAG3 in HIV-1 Susceptibility (primary research project). Shifa Mohideen (Primary author), Colin Graydon (Co-author), Thomas Murooka (Co-author), Keith R. Fowke (Advisor)

- Poster session presented at:
  - East African Regional Advisory Group Meeting (Nov 4, 2019, Winnipeg, Canada)
  - CanCURE 2.0 Annual meeting (Nov 7-8, 2019, Montreal, Canada and Oct 29-30, 2020, Online)
  - CanCURE 2.0 Committee Advisory Board Meeting and Science Buddy Program (Nov 7-8, 2019, Montreal, Canada and Oct 29-30, 2020, Online)
  - CanCURE 2.0 Basic Sciences – Knowledge Translation trainee workshop (Oct 30, 2020, Online)
  - Canadian Student Health Research Forum (Aug 24-28, 2020, Online)

2. Investigating the Role of Lectins in LAG3 Function and T-cell activity (primary research project). Shifa Mohideen (Primary author), Colin Graydon (Co-author), Keith R. Fowke (Advisor)

- Poster session presented at:
  - CanCURE 2.0 Annual meeting (Oct 27-28, 2021, Online and Oct 20-21, 2022, Online)
  - CanCURE 2.0 Basic Sciences – Knowledge Translation trainee workshop (Oct 28, 2021, Online)
  - Canadian Student Health Research Forum (June 11-15, 2021, Online, June 13-17, 2022, Winnipeg, Canada, and June 12-14, 2023, Winnipeg, Canada)
  - Virtual Microbiology Graduate Symposium (Apr 2022, Online)
  - ComSciCon Canada National Meeting (Aug 11-13, 2022, Toronto, Canada)
  - The Canadian Association for HIV Research (Apr 27-30, 2023, Quebec City, Canada)

3 Materials and Methods

3.1 General Reagents

3.1.1 Media and Buffers

- Phosphate buffered saline (PBS): Gibco PBS pH 7.4 (Fisher Scientific, Ottawa)
- FACS Wash: PBS + 2% heat inactivated Fetal Bovine Serum (FBS; Hyclone, Logan, USA)
- R10 cell culture media: Roswell Park Memorial Institute (RPMI)-1640 (Hyclone) + 10% FBS + 1% Penicillin/Streptomycin (Thermo Fisher Scientific)
- R30 cell culture media: RPMI-1640 (Hyclone) + 30% FBS + 1% Penicillin/Streptomycin (Thermo Fisher Scientific, Waltham, USA), for thawing cells
- Experimental media: ImmunoCult™-XF T Cell Expansion Medium (Cat#10981, Stem Cell Technologies, Vancouver), serum-free media (lacks FBS) for experimental assays
- Freezing media: FBS + 10% dimethyl sulfide (DMSO, tissue culture grade, Sigma, USA)

3.1.2 Staining Antibodies and Controls

Table 1. Surface staining panel used for ex vivo characterization of PBMCs and Jurkats

Antibody Specificity	Clone	Fluorophore	Catalog No.	Vendor	Volume used or dilution
LiveDead	-	AquaVivid	L34966	ThermoFisher	2.5 µL (1:200)
Mouse anti-Human CD3	UCHT1	AF700	557943	BD Pharmingen (San Diego, USA)	1.25 µL
Mouse anti-Human CD4	RPA-T4	BV605	300556	BD Biosciences (San Diego, USA)	2.5 µL
Mouse anti-Human CCR5	2D7	PECy7	557752	BD Pharmingen	2.5 µL
Mouse anti-Human LAG3	3DS223H	PE	12-2239-42	Invitrogen (Carlsbad, USA)	1.25 µL
Mouse anti-Human CD2	RPA-2.10	BUV395	563820	BD Biosciences	1.25 µL
Mouse anti-Human CD28	CD28.2	APC/Cy7	302965	BioLegend (San Diego, USA)	2.5 µL

Table 2. Surface staining panel for N-glycosidase validation

Antibody Specificity	Clone	Fluorophore	Catalog No.	Vendor	Volume used or dilution
LiveDead	-	AquaVivid	L34966	ThermoFisher	2.5 µL (1:200)
Lectin PHA-L (Phaseolus vulgari)	-	AF488	L11270	ThermoFisher	4 µL (1:160)

Table 3. Surface staining panel for LAG3 glycosylation and lactose titration experiments

Antibody Specificity	Clone	Fluorophore	Catalog No.	Vendor	Volume used or dilution
LiveDead	-	AquaVivid	L34966	ThermoFisher	2.5 µL (1:200)
Mouse anti-Human CD3	UCHT1	AF700	557943	BD Pharmingen	1.25 µL
Mouse anti-Human CD4	RPA-T4	BV605	300556	BD Biosciences	2.5 µL
Mouse anti-Human CCR5	2D7	PECy7	557752	BD Pharmingen	2.5 µL
Mouse anti-Human LAG3	3DS223H	PE	12-2239-42	Invitrogen	1.25 µL
Rat anti-Human Galectin-3	M3/38	APC	125420	BioLegend	2 µL
Rat IgG2a, κ Isotype Control	-	APC	400511	BioLegend	2 µL
Mouse anti-Human IgG2A LSECtin	845404	APC	FAB2947A	R&D Systems (Minneapolis, USA)	2.5 µL
Mouse IgG2A LSEC isotype control	-	APC	IC003A	R&D Systems	2 µL
Streptavidin	-	APC	405207	BioLegend	2.5 µL

Table 4. Surface and intracellular panel for Lectin-mediated suppression experiments

Antibody Specificity	Clone	Fluorophore	Catalog No.	Vendor	Volume used or dilution
LiveDead	-	AquaVivid	L34966	ThermoFisher	1.25 µL (1:400)
Mouse anti-Human CD3	UCHT1	AF700	557943	BD Pharmingen	1.25 µL
Mouse anti-Human CD4	RPA-T4	BB515	564419	BD Biosciences	2.5 µL
Mouse anti-Human LAG3	3DS223H	PE	12-2239-42	Invitrogen	1.25 µL
Mouse anti-Human HLA-DR	G46-6	BUV395	564040	BD Biosciences	2.5 µL
Rat anti-Human Galectin-3	M3/38	APC	125420	BioLegend	2 µL
Rat IgG2a, κ Isotype Control	-	APC	400511	BioLegend	2 µL
Mouse anti-Human IgG2A LSECtin	845404	APC	FAB2947A	R&D Systems	2.5 µL
Mouse IgG2A LSEC isotype control	-	APC	IC003A	R&D Systems	2 µL
Streptavidin	-	APC	405207	BioLegend	2.5 µL
Mouse anti-Human IFNγ	2B7	PECy7	506518	BioLegend	2.5 µL
Rat anti-human IL-2	MQ1-17H12	BV605	500332	BioLegend	**4** µL

3.1.3 Stimulants

TCR-dependent stimulation in T-cell activation assays was achieved using ImmunoCult™ Human CD3/CD28 T Cell Activator (Stem Cell Technologies), which provides an *in vivo*-like primary and co-stimulatory signal for CD3+ T cells. As a positive control for cytokine production, a TCR-independent stimulation was employed alongside in assays. Since phorbol 12-myristate 13-acetate (PMA; Sigma) possesses structural similarity to diacylglycerol (DAG), it activates PKCθ and subsequently NF-κB. Moreover, combining PMA with Ionomycin (Io; Sigma), which induces Ca2+ influx from intracellular Ca2+ stores and activates NFAT, enables robust activation of T cells within cellular activation assays, described herein.

3.1.4 Ligands

Table 5. Ligands used in lectin binding assays

Ligand	Fluorophore	Catalog No.	Vendor
Biotinylated Human FGL1 Protein	-	FG1-H82Ey	AcroBiosystems (Newark, USA)
Human Galectin-3	-	599706	BioLegend
Human LSECtin	-	CLGH5250	AcroBiosystems
Lectin PHA-L From Phaseolus vulgari	Alexa Fluor 488	L11270	ThermoFisher
Mouse anti-Human LAG3	-	12-2239-42	Invitrogen

3.2 Source of Biological Samples

3.2.3 Source of human peripheral blood samples

Human peripheral blood samples were obtained from local healthy blood donors at the University of Manitoba with their informed consent for the studies were approved by the University of Manitoba's ethics review committee.

3.2.4 Processing of human peripheral blood

Peripheral blood samples were collected in heparin vacutainers and processed for peripheral blood mononuclear cell (PBMC) isolation by density gradient centrifugation. After centrifuging

whole blood samples at 600xg for 5 min, the upper layer of plasma was separated and disposed. Following a dilution in FACS buffer(PBS + 2% FBS), the remaining whole blood was carefully layered over Ficoll (Lymphoprep; MJS BioLynx Inc., Ontario) and centrifuged at 400xg for 25 min with no brake. The resulting PBMC layer was collected and washed in FACS buffer at 600xg for 7 min, resuspended and then washed with R10 media at 400xg for 10 min. Following resuspension in R10 media, cell counting was performed with trypan blue (HyClone) dye exclusion. Finally, PBMCs were allowed to rest overnight in R10 media prior to use in assays.

3.2.5 Jurkat T-Cell lines

After the initial creation and transduction, as described in section 3.4, Jurkat cells were cultured for two weeks under selection R10 media + 2.5 µg/mL Plasmocin™ (InvivoGen, San Diego, USA) before being frozen as working stocks in liquid nitrogen (-180˚C). Cryopreserved working stocks were thawed when needed, and utilized in experiments for a maximum period of four months before being discarded. Jurkat E6-1 cell lines (ATCC, Manassas, USA) and their created derivative cell lines were passaged in T-75 culture flasks every 2-3 days at a cell density of 1.0-5.0 x 10^5 cells/mL in R10 media (after a two-week plasmocin selection) at 37°C and 5% CO_2.

3.3 General Methods

3.3.1 Flow Cytometry

3.3.1.1 Extracellular Surface Staining

PBMCs or Jurkats were washed in FACS buffer and transferred to 96-well V-bottom plates (Sarstedt, Newton, USA) for staining. Between 5x10^5 and 1x10^6 cells were stained in 50µL per well. Cells were pelleted by centrifuging for 3 min at 500xg with no brake, and then washed in FACS (3 min, 500xg, 4˚C, no brake). After discarding supernatant, 50µL of surface staining

antibody master mix was added to appropriate wells for a 30 min incubation at 4˚C in the dark. Following incubation, cells were subject to a FACS wash (3 min, 500xg, 4˚C, no brake) before resuspension in 200µL of 1% paraformaldehyde fixation buffer (Cytofix, BD Biosciences) or permeabilization for intracellular [cytokine] staining, as per section 3.3.1.2). After fixation, 100µL of FACS buffer was added to achieve a final volume of 300µL before transferring to 5mL FACS tubes for acquisition on the BD-Fortessa flow cytometer (BD Biosciences).

3.3.1.2 Intracellular Cytokine Staining

Surface-stained cells were washed with FACS buffer (3 min, 500xg, 4˚C, no brake), resuspended in CytoFix/CytoPerm buffer (BD Biosciences) that fixes and permeabilizes cells and incubated in the dark for 20 min at 20˚C. After centrifugation (3 min, 500xg, 4˚C, no brake), 50µL of blocking solution comprised of 2.5 mg/mL mouse IgG (ThermoFisher), 10% FBS in a 1X solution of PermWash (BD Biosciences) was added to each well for 15mins at 20˚C to occupy surface Fc receptors for better intracellular staining with intracellular antibodies. Cells were washed (3 min, 500xg, 4˚C, no brake) in 1X PermWash buffer and then resuspended in 100µL intracellular cytokine staining antibody mastermix prepared in 1X PermWash, cells allowing a 1 hour incubation at 20˚C in the dark. Cells were washed in 1X PermWash (3 min, 500xg, 4˚C, no brake), resuspended in FACS buffer to a final volume of 300µL before transferring to 5mL FACS tubes for flow cytometric acquisition on the BD-Fortessa (BD Biosciences).

3.3.1.3 Flow Cytometry Optimization

Before a full stain of cells containing all the antibodies in the panel is run, flow cytometry panels must be optimized to ensure reliable detection of parameters in the experiment. This begins with setting the optimum voltages which maximizes detection of fluorescence signals while

minimizing unwanted background noise of the instrument. To achieve the best signal-to-noise ratio for the specific fluorochromes used in the panel, a voltage titration is performed using unstained cells (either Jurkats or PBMCs). Voltages for each channel are adjusted until its robust standard deviation (rSD) of the fluorescence is 3x the rSD of the electronic noise.

Next, an antibody titration was conducted to identify the optimal dilution for each antibody used to stain its specific marker on the cells. The optimal dilution is determined by maximizing the staining index, without losing the positive population, as per the formula: Staining Index = (median fluorescence of positive population – median fluorescence of the negative population) / (2x rSD of the negative population). Thus, a higher staining index indicates better separation, more reliable identification of positive cells while minimizing background noise and variability in the negative population.

Lastly, voltage titration for each channel was executed by adjusting the instrument's voltages on single-stained cells to optimize the staining index (as per the above formula) without compromising the detection of the positive population. This final step further enhances resolution and separation of the desired signals from background noise, by minimizing spread of the negative population.

3.3.1.4 Compensation

When multiple fluorochromes are used simultaneously within flow cytometry, this can result in some fluorochromes being detected by more than one detector due to their overlapping emission spectra. This spillover can lead to inaccurate measurements and misinterpretation of data.

To remove spillover from analysis, a process called compensation minimizes the signal detected in a particular channel of interest that may arise from a secondary channel. An algorithm within the BD FACS Diva software compensates for this overlap by calculating the signals from single-stained compensation controls for each fluorochrome used. Thus, the emission signal from that specific fluorochrome is captured by its corresponding detector, enabling correct identification and quantification of cell populations.

These controls are prepared by singly staining CompBeads (BD Biosciences) that bind either rat or mouse antibodies for the surface and intracellular markers in experiments. In 400µL of FACS buffer, 1 drop of each rat/mouse CompBeads and negative control Compbeads were added. For each marker in the panel, 1µL of a single antibody was added to 100µL of this aliquoted bead mix and incubated at 20˚C for 10min in the dark before diluting to a final volume of 300µL with FACS buffer.

Likewise, compensation controls for the Live/Dead viability dye were created using ArC Amine Beads (Invitrogen). This involved mixing 1 drop of ArC amine beads with 1µL Live/Dead stain, followed by a 5 min incubation at 20˚C in the dark. For the negative control, 1 drop of ArC negative beads was added to 200µL FACS buffer prior to acquisition on the BD-Fortessa.

3.3.1.5 Data Acquisition and Analysis

Sample acquisition was performed on the BD-Fortessa (BD Biosciences) flow cytometer using BD FACS Diva software (BD Biosciences). The acquired data was exported and analyzed using FlowJo software (v10.8.1; Tree Star Inc, La Jolla, USA).

To ensure reliable data interpretation, a minimum of 100,000 events within the final endpoint gate were collected. This sample size enables adequate analysis and statistics evaluation of two

key outputs of flow cytometry data: proportion (the percentage of cells positive for a specific marker) and median fluorescence intensity (MFI; indicating the number of fluorescent antibody bound per cell).

To accurately define gating regions for each fluorochrome, fluorescence minus one (FMO) controls were used as a reference. These controls involve staining samples with all fluorochromes present in the panel, excluding the antibody of interest. This allows the gate to distinguish true positive signals from autofluorescence, background noise and spillover fluorescence associated with each respective channel.

The hierarchal gating strategy enables the identification and refines analysis of distinct cell populations by sequentially gating on specific markers or subsets of cells. Firstly, a time gate is applied to exclude events with inconsistent flow rates. Next a singlet gate is used to exclude doublet cells and debris, ensuring only single, intact cells are analyzed. After the singlet gate, a lymphocyte gate is drawn, followed by a gate for live cells. Subsequently, specific markers of interest are phenotyped as depicted in each experiment's gating strategy. Finally, parameters such as proportion or MFI can be quantified and analyzed within the defined gates.

3.4 Creation of LAG3+ and LAG3- Jurkat Cell Lines

3.4.1 Preparation of competent E. coli DH5α

The calcium chloride method was used to prepare competent E. coli DH5α cells. Briefly, overnight DH5α culture (LB+10μg/mL + tetracycline) was inoculated in 250 mL SOB medium with shaking at 20°C for growth until an OD600nm of 0.6. Following a 10 min incubation on ice, the pelleted bacterial culture was resuspended in TB buffer with an additional 10 min ice-incubation. DH5α cells were subsequently resuspended in cold TB supplemented with 7% (v/v)

DMSO. Aliquots of 200 µL of competent bacteria were rapidly frozen with liquid nitrogen and stored at –80°C.

3.4.2 Transformation of competent E. coli DH5α

To transform competent E. coli DH5α, 100 – 500 ng of plasmid DNA was used. Aliquots of thawed competent DH5α bacteria were incubated with plasmid DNA for 10 min on ice, followed by a 90 sec heat shock at 42°C and a final 60 sec incubation on ice. Each sample was grown in LB for 60 min at 37 °C with vigorous shaking. To isolate successful ligation reactions, bacteria were plated on LB with the appropriate antibiotic selection.

3.4.3 Plasmid DNA Isolation

To isolate plasmid DNA from successfully cloned colonies, the Qiaprep Spin MiniPrep Kit (Qiagen, Germantown, USA) was used following manufacturer's protocol. LB medium was inoculated with single putative colonies and grown 37˚C overnight with shaking. The following day, pelleted bacteria were resuspended in 150µL P1 buffer (25 mM Tris pH 8, 10 mM EDTA, 100 µg/mL RNAse A). Lysis was achieved by adding 150 µL P2 buffer (0.2 M NaOH, 1% SDS (w/v)). To neutralize lysates, 150 µL cold buffer P3 (11.5 % (v/v) acetic acid, 5 M potassium acetate) was added, precipitating SDS-protein and high molecular weight DNA and RNA complexes, while retaining circular plasmid in solution. Supernatants from lysates (centrifuged 10 min, 14000 rpm), were mixed with 1mL cold ethanol (-20˚C) to precipitate DNA which was pelleted (10 min, 14000 rpm), washed in cold (-20˚C) 70% ethanol and finally, air-dried to remove wash buffer. To elute and collect purified DNA, 50 µL EB Buffer (10 mM Tris·Cl, pH 8.5) was added to the isolated DNA and transferred to a QIAprep spin column in a fresh 1.5 mL eppendorf tube for centrifugation (1 min, 14000 rpm). Adequate plasmid DNA concentration

and purity (A260nm/A280nm ratio) was verified by a NanoDrop Spectrophotometer (ThermoFisher Scientific) before use in lentiviral production and transduction.

3.4.4 Construction of LAG3+ and LAG3- expression Plasmids

LAG3+ and LAG3- expression vectors, used in lentiviral packaging and subsequent transduction of Jurkat clone E6-1 cell line, were built using a third-generation lentiviral gene expression vector, pLenti-P2A-Puro (cat# PS100109; Origene). This lentiviral packaging cassette houses a P2A-Puro marker, enabling puromycin selection in transduced cells.

The full-length LAG3 open reading frame (ORF) (cat# RC220269, Origene, Rockville USA) was inserted into the pLenti-P2A-Puro lentiviral gene expression vector between the SgfI and MluI sites to create the LAG3+ expression vector (cat# CW303487) and subsequently, LAG3+ Jurkats. This was achieved through a straightforward cut-and-ligation procedure employing the appropriate restriction endonucleases.

In contrast, the LAG3- expressing vector comprises the pLenti-P2A-Puro lentiviral vector alone, lacking any insert, to serve as both the negative *and* vector-control in the experiments, described herein. Detailed sequences of the full-length LAG3 insert are accessible in the appendix section.

3.4.5 Lentiviral Transfection and Production

Lentiviral vectors are used for delivery of nucleic acids into a target cell's genome, allowing stable and heritable expression of the gene construct in a population of cells. Using a second-generation packaging system, VSV-G pseudotyped HIV-1 based lentiviral vectors lacking replication competency were created by the Lentiviral Core Facility at the University of Manitoba.

The packaging cells, HEK-293T cells (ATCC), were co-transfected with the "transfer" lentiviral plasmid encoding either the LAG3+ expression vector (full-length LAG3) or the LAG3- expression vector (lacking LAG3 insert), described in section 3.4.4 alongside envelope and packaging plasmids using calcium phosphate-mediated transfection methods. The latter envelope and packaging plasmids encode viral enzymatic, structural and packaging proteins/components required for generation of the lentiviral particles.

For virus collection, ultracentrifugation of supernatants concentrated the resulting lentiviral particles and was frozen at -20°C until use in lentiviral transduction of [target] Jurkat E6-1 cell lines, as detailed in section 3.4.6.

3.4.6 Lentiviral Transduction

As actively dividing cells integrate virus more efficiently for lentiviral transduction, 1x10^{5} Jurkat cells were centrifuged for 7 mins at 400xg in a 15mL falcon tube after confirmation of log-phase growth. Supernatant was discarded and pelleted Jurkat cells were resuspended in 250µL of lentiviral mixture, comprised of 1x10^{5} replication-deficient viral particles and 4µg/ml of polybrene. The cationic polymer, polybrene, neutralizes charge repulsion between virions and the cell surface to enhance transduction efficiency. Herein, Jurkat T-cells that underwent transduction with the lentiviral vector encoding the full-length LAG3 gene were designated as LAG3+ Jurkat cells, while those transduced with the lentiviral vector alone (i.e. lacking LAG3 sequence) were identified as LAG3- Jurkat cells.

After a 2hr incubation at 37˚C and an R10 wash, cells were plated in 500µL R10 in 24 well-plates under 0.4µg/mL puromycin for 72hrs (Sigma) to select for transduced cells. Following the 72 hour incubation, all cells in the untransduced condition (lacks plasmid-borne puromycin-

resistance marker) were deemed dead, as assessed by trypan-blue counting. The surviving fraction of these LAG3+ and LAG3- transduced Jurkats, were recoverable with puromycin selection and further propagated in R10 media for use in studies outlined in this thesis. For a duration of one week, untransduced cells were also retained in R10 + puromycin to verify the absence of any growth before disposal.

3.5 Characterization of LAG3+ and LAG3- Jurkat Cell Lines

3.5.1 Flow Cytometry Panel Optimization

To ensure the comparability of the constructed LAG3+ and LAG3- Jurkat cell lines in experiments,Isought to characterize these cells for various markers via flow cytometry. The similar expression profiles of lineage (CD3, CD4, CCR5) and co-stimulatory (CD2 and CD28) markers were assessed to verify that transduction and puromycin selection procedures did not introduce any undesired variability in the cell lines (Table 1). Moreover, in confirming LAG3 as the sole differing variable between the two cell lines, the specific impact of LAG3 on the outlined study endpoints can be evaluated.

While Jurkat cells are all CD3+CD4+, the fluorescence signal for these key lineage markers appears as a continuous elongated distribution, or smear, on the dot plot. This occurs when the marker of interest is expressed at varying levels within the cell population, either due to inherent biological heterogeneity or technical issues such as non-specific/incomplete Ab binding, as well as improper compensation or voltage settings.

Lacking distinct positive and negative populations to define gating boundaries, the smear expression of CD3 and CD4 by Jurkats precludes the identification of a well-defined CD3+CD4+ parent gate. Hence with sequential lineage gating, the accurate delineation of subsequent

progeny gates based on the expression markers LAG3, CCR5, CD28 and CD2, is consequently affected.

To circumvent ambiguities in defining a positive and negative gate, initial panel development was conducted on PBMCs as they offer distinct positive and negative populations for CD3 and CD4. Thus, PBMCs are preferred for validating panels as this resolution is crucial for optimizing panel performance, sensitivity and specificity, antibody titration and diagnosis of issues related to voltage or compensation with greater clarity and accuracy.

Optimization on isolated PBMCs commenced by performing optimum voltages for the channels included in the panel (Table 1). Then, PBMCs were full stained for the markers of interest, using regular compensation or PLUS beads where appropriate. Here, compensation issues for each colour in the panel were examined by comparing histogram overlays of markers acquired from full stained cells and compensation controls (single-stained beads) specific to the corresponding fluorophores. Inaccurate calculations of the compensation matrix result if the cells fluoresce brighter than their respective controls. Due to erroneous correction of spectral overlap amongst the different fluorochromes, the compensated data may distort true levels of marker expression and diminish resolution sensitivity, causing the observed smearing pattern in experimental samples. To maintain the brightness of compensation signals above that of fully stained cells, PLUS beads were employed for markers exhibiting greater fluorescence intensity than standard anti-mouse compensation beads.

Following revision of compensation, an antibody titration was conducted on resting PBMCs for CD3-AF700, CD2-BUV395, CD28-APCCy7 as the remainder of colours in the panel had undergone titration previously (Table 1). With the inclusion of unstained PBMCs, antibodies

were titrated by single-staining cells at 5µL (manufacturer's concentration), 2.5 µL and 1.25 µL for each of the listed fluorophores.

Then, cells were full stained with appropriate FMO controls, ensuring adequate separation between their positive populations for each marker and their respective compensation controls. To address potential voltage issues, single-stained cells were subject to voltage titration. Following acquisition, the optimal voltage-titrated stain index on PBMCs was calculated for each colour in the panel. After verifying sufficient resolution with CD3+/CD3- and CD4+/CD4- populations, alongside other cellular markers, on full stained PBMCs, panel validation was then shifted to the Jurkats. Based on its optimum voltages, the single-stained constructed cell lines were voltage titrated to better resolve positive and negative populations for the markers of interest. In final characterization of the LAG3+ and LAG3- Jurkat cells, a CD3 FMO and a full-stained PBMC tube were employed for comparison, achieving optimal clarity and separation to conclude panel validation.

3.6 Assessment of LAG3 Glycosylation on Lectin Binding

3.6.1 N-glycosidase Treatment

The enzymatic action of Peptide:N-glycosidase F (PNGaseF) (NewEngland Biolabs, Ipswich, USA) enables the removal of N-linked glycans from glycoproteins. This feature makes it a valuable tool to investigate glycosylation in various cellular processes, such as receptor-ligand interactions, glycoprotein structure and function, signaling pathways or immune responses.

To efficiently remove N-glycans from Jurkat cells, a time-titration at 0hr, 1hr, 2hr, 4hr and 6hr using uniform concentrations of PNGaseF was first performed to empirically determine an optimal duration of enzyme treatment. Thereafter, the extent of de-glycosylation was validated

by staining PNGaseF-treated cells with 4 µL of diluted PHA-L (ThermoFisher) alongside LiveDead, as per Table 2 and Section 3.3.1.1. PHA-L, also known as *Phaseolus vulgaris* leucoagglutinin, is a lectin that binds tri- or tetra-antennary (terminal) complex N-glycans. By conjugating PHA-L with AlexaFluor488 dye, the binding of PHA-L to cell-surface N-glycans can be detected via flow cytometry. Consequently, a duration of 6hr for enzyme treatment was optimal in yielding minimal PHA-L binding. This minimal PHA-L binding would indicate lower expression of surface N-glycans, verifying the activity of glycosidase enzyme.

As such for glycosidase treatment, 1 x 10^6 LAG3+ and LAG3- Jurkat cells were treated with or without 2000U/well of PNGaseF in 200µL of supplied GlycoBuffer 2 (50 mM sodium acetate) in 96-well V-bottom plates. After a 6hr incubation at 37˚C, cells were thoroughly washed for subsequent analysis of L-PHA binding or downstream experiments with lectin treatment, described herein.

3.6.2 Lectin Binding Assay

As part of Objective 1, to investigate the requirement of LAG3 glycosylation for lectin-binding, N-glycosidase (PNGaseF) was used to remove glycan residues on both LAG3+ and LAG3- Jurkats, as described above. Following glycan removal of glycans, LSECtin (Acrobiosystems) and Galectin-3 (BioLegend) were then added to both cells to evaluate lectin-binding efficiency with or without (+/-) N-glycosidase treatment. A dose-response curve was generated by incubating 1 x 10^6 LAG3+ and LAG3- cells with gradually increasing concentrations (0µg/mL (i.e. untreated; media only), 25µg/mL, 50µg/mL, 100µg/mL or 200µg/mL) of Galectin-3 or LSECtin. Cells were treated with lectins for 2hr in serum-free Immunocult media (StemCell Technologies) at 37˚C to allow binding to occur between the lectins and the glycosylated residues of LAG3. For PNGaseF-

treated cells, lectin concentrations yielding saturation (200µg/mL) were used to examine the effect of deglycosylation on lectin-binding. As a positive control for ligand-binding, cells were treated with 50µg/mL of FGL1, which binds non-glycosylated regions of LAG3 [48]. Prior to extracellular staining with the antibody panel, described in Table 3, cells were washed in PBS to remove any unbound lectin and minimize any background fluorescence. Finally, flow cytometry was used to compare the percentage (%) of LAG3+ and LAG3- cells expressing either LSECtin or Galectin-3 to quantify the efficiency of lectin-binding. Bar graphs consist of n=3 independent iterations. A $p<0.05$ was considered significant using Mann Whitney U-test: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.6.3 Lactose Competitive Inhibition Assay

To verify that lectin and LAG3 interaction (i.e. CRD-glycan interaction) occurs through glycosylated regions of LAG3, β-lactose was used as a competitive inhibitor to selectively disrupt CRD-glycan interactions. In principle, this disaccharide's structural similarity with target glycans on surface glycoproteins enables it to outcompete glycosylated structures by effectively occupying the CRD binding site of lectins. Thus, lactose's competitive binding to lectins, LSECtin and Galectin-3, would displace lectins from the cells.

Briefly, 1 x 10^6 LAG3+ and LAG3- Jurkats were treated with 100µg/mL of either Galectin-3 or LSECtin for 2hrs, as above. To assess and confirm the extent of competitive inhibition, a lactose gradient was conducted by incubating lectin-treated cells with 2-fold increasing concentrations of β-lactose (Sigma). Cells received a final concentration of 0mM (i.e. untreated; media only) 25mM, 50mM, 100mM, 200mM, 400mM β-lactose in 200µL of serum-free ImmunoCult media (StemCell Technologies) to displace cell-bound lectins. After the 1.5hr lactose incubation at

37˚C, cells were washed and spun down in a 96-v bottom plate. To detect the reduction of Galectin-3 or LSECtin binding at higher concentrations of lactose, cells were surface-stained with the antibody panel described in Table 3 for flow cytometry. Bar graphs consist of n=3 independent iterations. A $p<0.05$ was considered significant using Mann Whitney U-test: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.7 Lectin Engagement of LAG3 activity

3.7.1 Cellular Activation and Cytokine Detection

Following glycosidase and lectin treatment, LAG3+ and LAG3- cells were stimulated to obtain baseline T-cell activation levels and ultimately assess the impact of lectin binding on suppressing T-cell activation. Stimulation via ligation of anti-CD3 (primary signal) and anti-CD28 (co-stimulation) antibodies mimics the natural TCR signalling that occurs upon T cells and antigen-presenting cell (APCs) contact during an immune response. Moreover, co-stimulation ensures a robust and optimal immune response by preventing T cell anergy or tolerance. Altogether, this process triggers specific signaling cascades, resulting in T cell activation, survival, proliferation and most importantly, cytokine production.

For the assessment of lectin-mediated LAG3 activity, a concentration and time-dose curve for T-cell activation stimulus was first conducted to elucidate the optimal concentration and duration for activating cells with anti-CD3 and anti-CD28, as per Objective 2.

This dose-response curve was generated by supplementing 1 x 10^6 LAG3+ and LAG3- cells with increasing amounts of (0 µL (i.e. unstimulated; media only), 6 µL, 12 µL, 25 µL, 50 µL and 100 µL) of cocktail ImmunoCult Human CD3/CD28 T Cell Activator (StemCell Technologies) in 500 µL of serum-free ImmunoCult media in 96 well v-bottom plates at 37˚C. This cocktail enables

robust activation and expansion of T cells. For detection of cytokine production (via Flow Cytometry), 0.66 µL/mL GolgiStop (containing momensin; BD Biosciences) and 1 µL/mL GolgiPlug (containing brefeldin A; BD Biosciences) were added to wells immediately prior to simulation, to prevent transport of cytokines though the Golgi and enable intracellular cytokine staining, as outlined in section 3.3.1.2. Hence, in samples lacking GolgiStop and GolgiPlug, supernatants were harvested at the end of the assay to quantify cytokines by Enzyme-linked immunosorbent assay (ELISA), as detailed in 3.7.1.1. At the timepoints of 0h, 1h, 2h, 4h, 6h, 8h and 12h, each condition was stained for endpoints of IL-2 and IFNγ, a readout of T-cell activation, followed by analysis by flow cytometry. From this titration assay, the optimal stimulus, of 25µL of the anti-CD3/anti-CD28 cocktail, was selected to activate 1 x 10^6 LAG3+ or LAG3- cells for 4h.

To examine the induction of LAG3 activity by LSECtin and Galectin-3 and their subsequent differential inhibitory strengths on T cell activation, a gradient of lectin concentrations from 0-400 µg/mL were applied to 1 x 10^6 glycosidase-treated (or untreated) LAG3+ and LAG3- Jurkat cells, as described in sections 3.6.1 and 3.6.2. In parallel to the indicated anti-CD3/CD28 stimulation, cells were also stimulated with 0.02 µg/mL PMA and 1 µg/mL Ionomycin for 4h. This strong stimulus serves as a positive control for cytokine detection in experiments. After the indicated stimulation time, cells were stained intracellularly (sections 3.3.1.1 and 3.3.1.2) for the endpoints of IL-2 and IFNγ, as per Table 4 before acquisition on BD-Fortessa flow cytometer (BD Biosciences).

Flow cytometry compared the differential strength of inhibition for ligands by quantifying the percentage % of treated LAG3+ and LAG3- cells expressing IL-2 and IFNγ to assess dose-

dependent cytokine responses and evaluate lectin-mediated T-cell suppression. Bar graphs consist of n=3 independent iterations. A $p<0.05$ was considered significant using Mann Whitney U-test: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.7.1.1 ELISA Detection of IL-2 and IFNγ

ELISAs for IL-2 (Human IL-2 ELISA MAX™ Deluxe set; 431804, BioLegend) and IFNγ (Human IFNγ ELISA MAX™ Standard set; 430101, BioLegend) were performed on supernatants generated in T-cell activation assays, according to the manufacturer's protocol. ELISA assays were terminated with the Stop Solution for TMB Substrate (BioLegend), which stabilizes colour development for acquisition of absorbances. Run alongside standards and samples were blank samples, composed of solely buffer that lacks protein, 1X Assay Diluent A. Blank wells were used to subtract any background absorbance from standard and sample data, for accurate absorbance readings.

All standards and samples were plated in duplicate, thus final data is result of the averaged values. Plates were read at 450nm using a Gen 5 3.11 BioTek Synergy microplate reader (Agilent Technologies, Santa Clara, USA) instrument, with correction at 570nm as per manufacturer's instructions. The lower limit of detection for both IL-2 and IFNγ kits is 4 pg/mL.

After correcting for dilution factors, IL-2 and IFNγ concentrations were quantified from the linear range of their respective standard curves by plotting absorbance readings against standard concentrations. A standard curve was generated with an R of 0.9929 (IL-2) and R=0.9968 (IFNγ). Final concentrations were graphed and analyzed using Prism9 (ver 9.5.1, GraphPad, San Diego, USA).

3.8 Statistics and Analysis

As mentioned in Section 3.3.1.6**,** flow data was analyzed using FlowJo Software (v10.8.1; Tree Star Inc). Statistical analysis for ELISA and Flow Cytometry data was conducted in GraphPad Prism9 (ver 9.5.1, GraphPad Software). Given the low number of independent technical replicates, the Mann Whitney U-test (a non-parametric, unpaired test for continuous variables) was used to compare differences in surface marker and cytokine expression across treatments between 2 independent samples (i.e. LAG3+ Jurkats vs. LAG3- Jurkats). ELISA and Flow Cytometry data are visually represented in bar graphs as median ± SD, whereby a p-value of < 0.05 was deemed statistically significant when assessing differences between the cell lines.

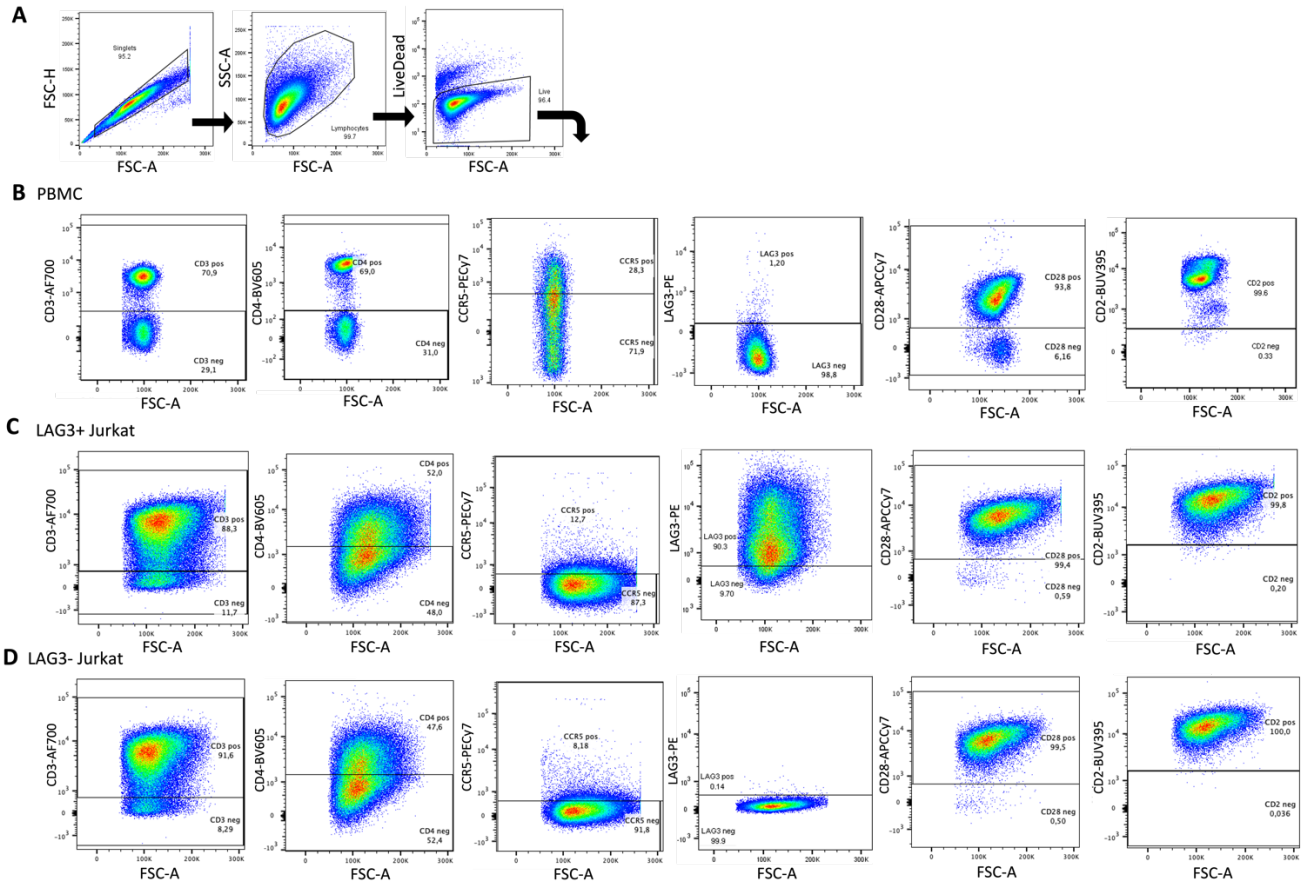
4 Results

4.1 Validation of Jurkat Characterization Flow Cytometry Panel

To validate the constructed LAG3+ and LAG3- Jurkat T cell lines,Iaimed to develop a flow cytometry panel to assess the expression of various lineage and activation markers (outlined in Table 1). Though uniformly CD3+CD4+, the smear expression of CD3 and CD4 by Jurkats precludes identifying distinct positive and negative populations for a defined CD3+CD4+ gate [113,114]. Stemming from biological heterogeneity or antibody, compensation or voltage configuration issues, these gating ambiguities subsequently impact delineating accurate progeny gates for LAG3, CCR5, CD28 and CD2 expression [115]. For ease in diagnosing issues and optimizing panel performance, healthy donor PBMCs were chosen for validating the Jurkat Characterization panel as they exhibit clear CD3 and CD4 populations, as in Figure 2.
As observed, meticulous titration of antibodies and voltages on single-stained PBMCs, minimized the spread of the negative population while maximizing detection of the dim positive population for each fluorophore [115]. As confirmed by flow plots, this enhanced resolution aids gating precision of the descendant markers CCR5, LAG3, CD28 and CD2 on full-stained PBMCs.

Moreover, ensuring that compensation beads fluoresce brighter than fully-stained PBMCs for each fluorophore, mitigates compensation and technical smearing issues [116]. This reinforces our confidence that any observed marker smearing primarily reflects inherent biological variability within PBMC-derived primary T cells, and, by extension, the Jurkat T cell lines.

Figure 2. Validation of Characterization Panel on Resting PBMCs and Jurkat T cell lines



(A) Arrows indicate the gating sequence. FSC-height (FSC-H) versus FSC-area (FSC-A) first identifies singlets, followed by gating for lymphocytes based on Side Scatter-area (SSC-A) versus FSC-A. Next, live cells, identified as Live/Dead AquaVivid negative, are then compared for staining profiles of CD3, CD4, CCR5, CD2, CD28 and LAG3 between (B) PBMCs and (C) LAG3+ and (D) LAG3- Jurkat T cell lines for panel validation. Specifically, from CD3+ cells, CD4+ cells were identified then further segregated into progeny gates of CCR5, CD2, CD28 and LAG3.

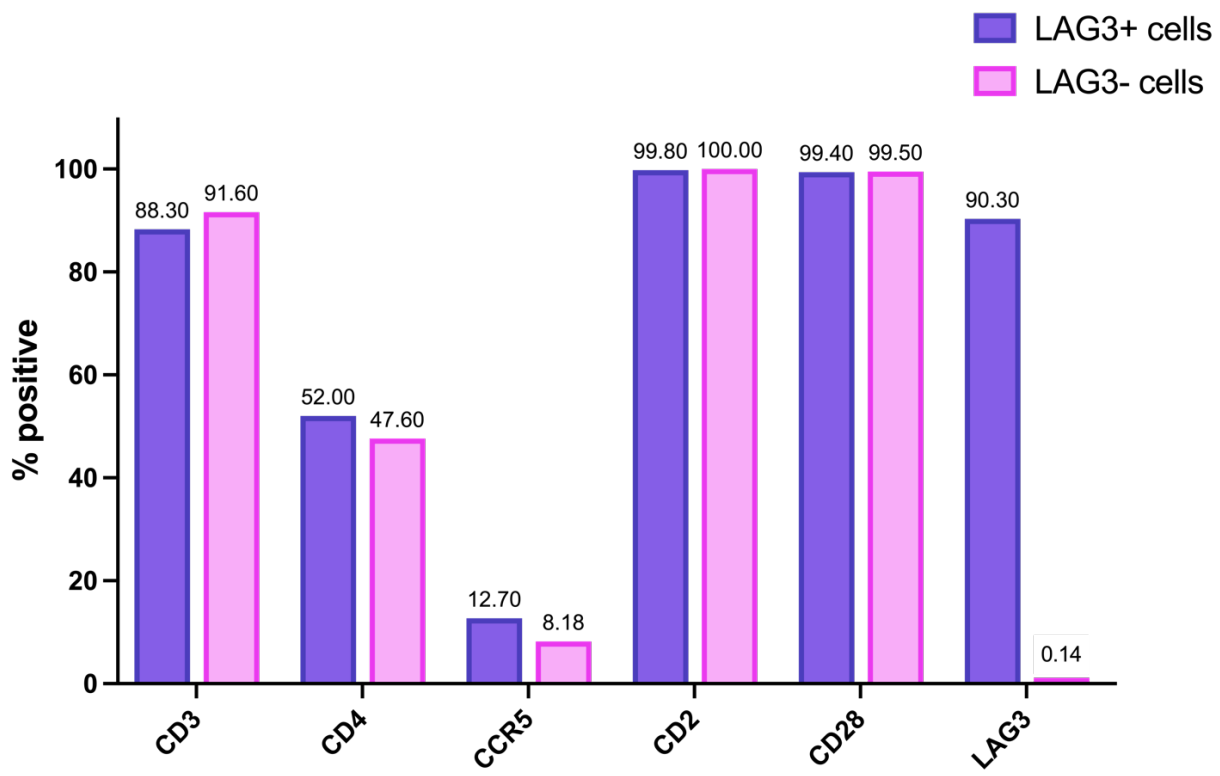
Additionally, the constructed cell lines were voltage titrated to better resolve positive and negative populations for the selected markers. Assessing the validated panel on full-stained Jurkats, the CD28 and CD2 positive populations on both constructed lines appear distinct in contrast to the other evaluated markers, in agreement with existing literature. Employing FMO controls for all markers during characterization provided additional assurance that smear pattern of expression, particularly for CD3, CD4, CCR5 and LAG3, on both cell lines in Figure 2 mirrors its biology [113,114]. These steps collectively strengthened our certainty to conclude panel validation for precise LAG3+ and LAG3- Jurkat characterization.

4.2 Characterization of LAG3 cell line model

The limited availability of LAG3 cell line models has impeded extensive research concerning LAG3's mechanism of inhibitory function [21,34,45]. Considering this, our LAG3 model was generated using the Jurkat CD4 T cell lines, given their extensive use in delineating our current knowledge of the TCR signalling pathway [10]. After transducing Jurkats with lentiviral vectors with LAG3 ORF (LAG3+ cells) or without insert (LAG3- cells) with subsequent puromycin selection, the aim was to characterize the constructed model via flow cytometry. This characterization ensures the comparability of the constructed LAG3+ and LAG3- cell lines, before examining LAG3's inhibitory effect on T cell activation in experiments.

Assessing the similar expression profiles of lineage (CD3, CD4 and CCR5) and co-stimulatory (CD2 and CD28) markers demonstrates that the resting LAG3+ and LAG3- cell lines are comparable (Figure 3). In this regard, lentiviral transduction, puromycin selection or even culturing methods did not introduce any undesirable variability between the two cell lines, with very minimal differences in levels of percent positive expression for each marker.

Figure 3. *Ex vivo* Characterization of LAG3+ and LAG3- Jurkat cell lines



Jurkat cells were transduced by lentivirus vector with containing full-length LAG3 ORF (LAG3+ cells) or without LAG3 insert (LAG3- cells; vector control) and subjected to puromycin selection. Summary bar graphs represent characterized resting Jurkats cells, measuring expression of CD3, CD4, CCR5, CD2, CD28 and LAG3 from Figure 2. LAG3+ and LAG3- cells exhibit comparable profiles for the evaluated lineage and functional markers. Thus, differences in cell behaviour can be attributed to LAG3 expression, the major distinguishing factor.

Moreover, in confirming LAG3 expression as the main detectable differing variable between the two resting cell lines, the specific impact of LAG3 on the outlined study endpoints can be evaluated. In our flow cytometry analysis, Jurkats transduced with the vector housing the full LAG3 ORF (LAG3+) displayed high LAG3 expression levels of 90.30%. Conversely, I observed that LAG3 expression on cells transduced with the vector alone (LAG3-) was negligible, measuring only 0.14%, as anticipated (Figure 3). By design, any differences in cell behaviour with respect to activation or cytokine production are a direct consequence of the differing LAG3 expression, and in turn, its activity between these constructed cell lines. Such a design highlights its suitability for various experimental contexts to specifically investigate our outlined research objectives.

4.3 Validation of N-glycosidase Activity

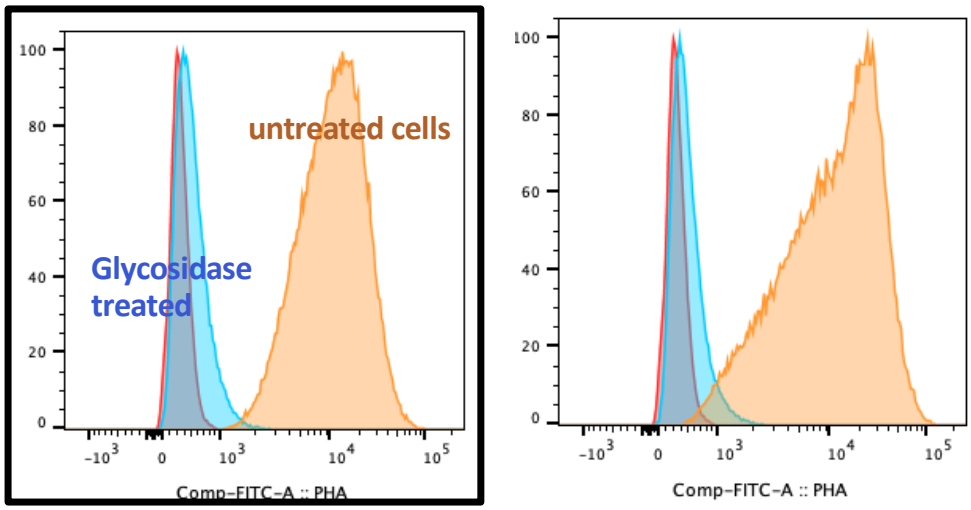
To resolve Objective 1 concerning the necessity of LAG3 glycosylation for lectin binding, PNGaseF N-glycosidase activity upon the constructed LAG3+ and LAG3- Jurkat cells was first validated to address this knowledge gap. Specifically, live LAG3+ and LAG3- cells were subject to PNGaseF glycosidase treatment, which cleaves N-glycans from glycoproteins, deglycosylating the cells [117]. With global N-glycan removal on both cell lines, this can clarify the importance and impact of glycosylation, regarding LAG3, on lectin binding of Galectin-3 and LSECtin for Objective 1 [and Objective 2].

Using flow cytometry, PHA (phytohemagglutinin)-L staining confirmed the efficacy of glycosidase activity on PNGaseF-treated Jurkats (detailed in Table 2 and Section 2.3.1.1). PHA-L is a lectin that specifically binds tri- or tetra-antennary (terminal) complex N-glycans on the cell surface. Conjugating PHA-L with FITC, enables us to detect the binding of PHA-L to cell surface

N-glycans to evaluate the extent of deglycosylation of LAG3 and the glycosylation repertoire of the glycosidase-treated Jurkat cells [118–120]. In contrast to untreated cells, optimal PNGaseF deglycosylation would yield reduced PHA-L binding by lower PHA-L fluorescence intensity, indicating minimal surface N-glycan expression. Such outcomes confirm effectiveness of N-glycosidase in N-glycan removal for use in subsequent assays [29,117,120,121].

Indeed, flow cytometric analysis illustrates that a substantial portion of LAG3+ Jurkat cells in the untreated condition exhibit a higher fluorescence intensity for PHA-L-FITC binding (orange), signifying intact N-glycans (Figure 4). However, PNGaseF treatment effectively removes N-glycans from live cells, portrayed by the diminished fluorescence intensity and, in turn, cell surface binding of PHA-L (blue), compared to untreated LAG3+ cells (orange). This decline in PHA-L staining depicted by the leftward shift of the curve, from untreated (orange peak) to glycosidase-treated (blue peak) LAG3+ cells, confirms PNGaseF's success in cell surface N-glycan removal, approaching the level seen in the unstained condition (red peak), which lacks PHA-L FITC conjugate altogether. A similar trend of reduction in fluorescence intensity from altered glycosylation status was observed with LAG3- Jurkat cells, also revealing a leftward shift in flow cytometry histograms amongst treated and untreated conditions (Figure 4).

Figure 4. Flow Cytometric Validation of N-glycosidase Activity on LAG3+ and LAG3- Jurkat cells



LAG3+ Jurkat **LAG3- Jurkat**

Live Jurkat cells were either treated with PNGaseF (blue peak) or left untreated (PBS only; orange peak) for verification N-glycosidase activity via Flow Cytometry. Histogram overlays illustrate both LAG3+ and LAG3- Jurkat cells stained with PHA-L-conjugated FITC, a lectin binding terminal complex N-glycans on the cell surface, or left unstained (red peak). PNGaseF treatment (blue) effectively removes N-glycans from live cells, reducing cell surface PHA-L binding (leftward shift) compared to PBS untreated control (orange), verifying N-glycosidase effectiveness in N-glycan removal from the cell surface. The y-axis indicates relative number of cells at different levels of PHA-L staining intensity (x-axis), measured in arbitrary units.

In distinguishing between intact N-glycans on cells and those removed by glycosidase, our findings verify the effectiveness PNGaseF glycosidase, providing conclusive evidence of N-glycan removal and modifying glycosylation profiles of these cells [29,117,120,121]. Conducting this validation was crucial, paving the way for conducting downstream experiments that concern lectin binding assessments, as described in our outlined study objectives. These results mark a fundamental step in our research, enabling deeper exploration of glycosylation and its significance in LAG3-mediated processes and interactions, with potential implications for T cell biology.

4.4 Lectins Demonstrate Specificity for LAG3 binding

4.4.1 Glycosylation of LAG3 is necessary for LSECtin and Galectin-3 binding

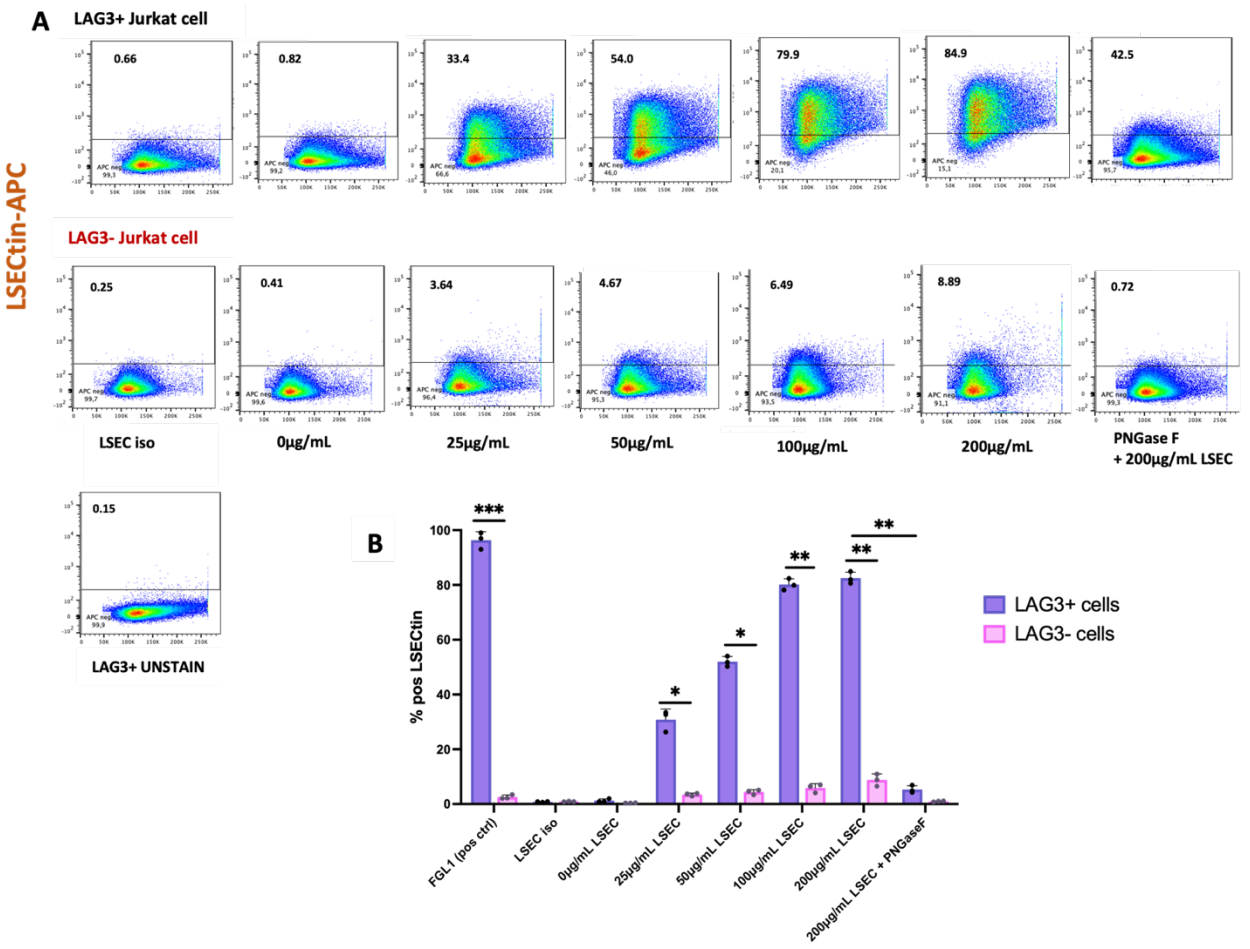
Whether soluble or membrane-bound, lectins contain carbohydrate-recognition domains (CRDs) enabling them to bind glycans on cell-surfaces or glycoproteins [29,30,51]. Despite the recognized importance of these interactions during immune recognition and response, the specific requirements and mechanisms of LAG3 glycosylation for lectin binding and its consequences upon T cell suppression remain elusive [29,30,51]. Given LAG3's extensive N-glycosylation, Objective 1 of this dissertation seeks to clarify the necessity and specificity of N-glycosylation on LAG3 in mediating binding of lectin-ligands, LSECtin and Galectin-3.

Having validated PNGaseF N-glycosidase efficacy on both LAG3+ and LAG3- Jurkat cells for global N-glycan removal, LAG3's glycan profiles can be manipulated to empirically characterize the role and impact of N-glycosylation, or lack thereof, for LAG3-lectin engagement [29,73,117,118,120,121]. Thereafter, resting LAG3+ and LAG3- Jurkat cells were exposed to a two-fold increasing concentration gradient (0-200µg/mL) of LSECtin (LSEC) or Galectin-3 (Gal-3) with or

without PNGaseF-treatment. Targeting non-glycosylated regions of LAG3, treating cells with 50μg/mL FGL-1 serves as a positive control for ligand binding, as it is known to bind the non-glycosylated regions of LAG3's D1 and D2 domain [48]. From the lectin dose curve in Figures 5 and 6, flow cytometry compared the percent (%) positive of LAG3+ and LAG3- cells expressing either LSEC or Gal-3 to quantify extent of LAG3-lectin binding.

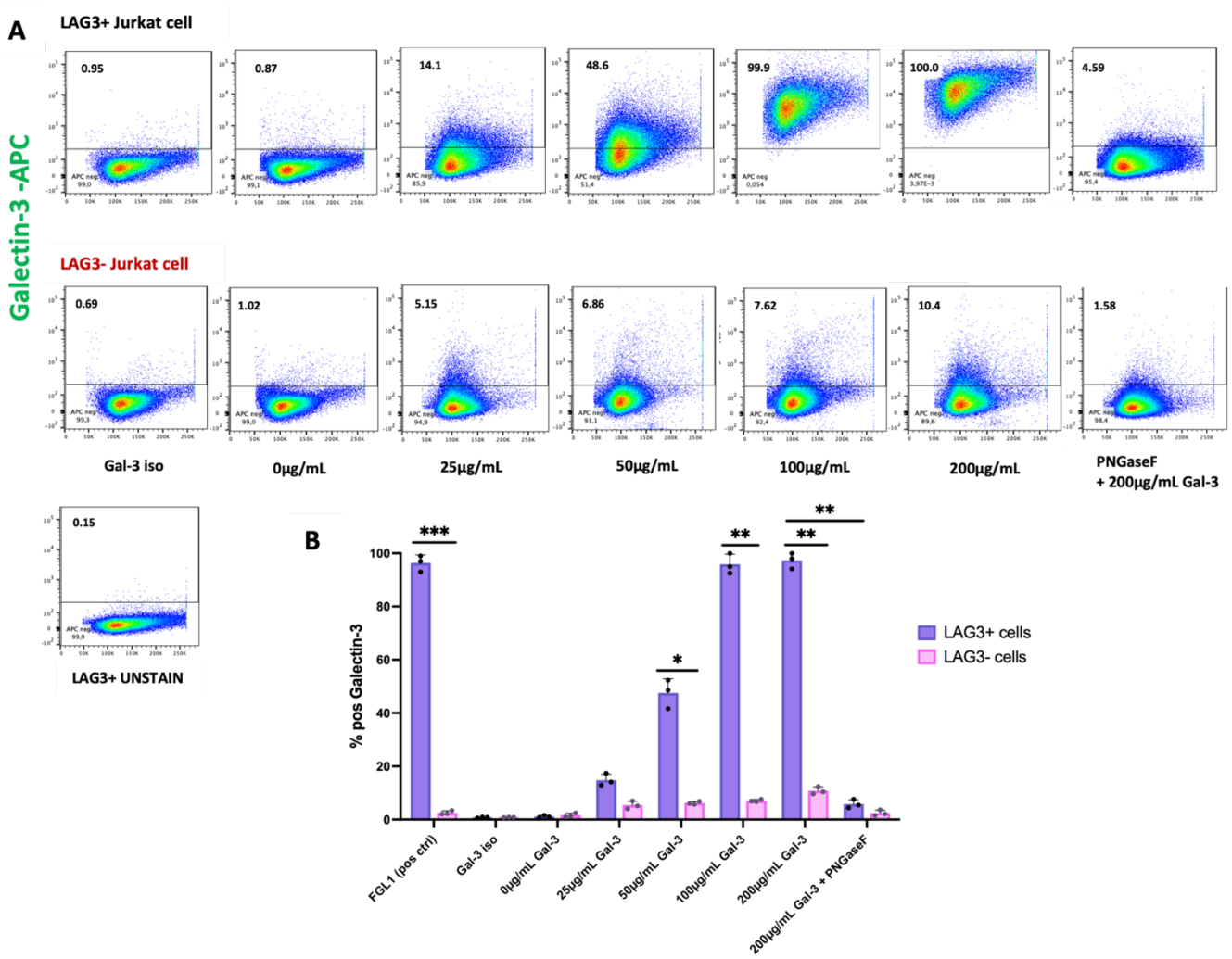
Regarding Figure 5, concurrently with the rise of exogenous LSEC concentration, increasing median percent positive LSEC expression is exemplified on both untreated (with N-glycans intact) LAG3+ and LAG3- Jurkats, demonstrating a dose-dependent trend of binding. Contrasting with marginal changes in exogenous LSEC expression observed for LAG3- cells, its specificity for LAG3 is evident with higher and more pronounced changes in LSEC-binding on LAG3+ cells. This difference is statistically significant at 25μg/mL, 50μg/mL, 100μg/mL and 200μg/mL of LSECtin ($p=0.0486$; $p=0.0375$; $p=0.0092$ and $p=0.0089$, respectively). Similarly, in Figure 6, as Gal-3 concentration increases, it mirrors this dose-dependent pattern with a statistically significant specificity for binding LAG3 at 50μg/mL, 100μg/mL and 200μg/mL of Galectin-3 ($p=0.0269$, $p=0.0082$ and $p=0.0069$, respectively).

Figure 5. Assessment of LAG3 Glycosylation for LSECtin Binding



LAG3-LSECtin (LSEC) binding efficiency was evaluated by exposing LAG3+ and LAG3- Jurkat cells to an LSEC concentration gradient (0-200µg/mL). At 200µg/mL LSEC concentration that yielded saturated binding, both cell lines were deglycosylated using N-glycosidase (PNGaseF) to confirm necessity of LAG3 glycosylation for LSECtin-binding. LSECtin concentrations (µg/mL) are indicated directly below. The upper left corners of flow plots (A) and corresponding bar graphs (B) represent n=3 independent experiments for the percentage of LAG3+ or LAG3- cells staining positive for LSEC to quantify dose-dependent lectin-binding. The y-axis of flow plots depicts the median fluorescence intensity, measured in arbitrary units, for cells staining at varying levels of LSECtin-APC intensity, plotted against the FSC-area (FSC-A) of cells. Targeting non-glycosylated regions of LAG3, FGL-1 is a positive control for ligand binding. LSEC iso; isotype antibody control for non-specific background binding of anti-LSECtin-APC staining antibody. Statistical significance was determined using the Mann Whitney U-test: *p<0.05, **p<0.01, ***p<0.001.

Figure 6. Evaluation of LAG3 Glycosylation for Galectin-3 Binding



LAG3-Galectin-3 (Gal-3) binding efficiency was measured by exposing LAG3+ and LAG3- Jurkat cells to a Gal-3 concentration gradient (0-200µg/mL). At a Gal-3 concentration of 200µg/mL that yielded saturated binding, both cells were deglycosylated using N-glycosidase (PNGaseF) to confirm necessity of LAG3 glycosylation for Galectin-3-binding. Galectin-3 concentrations (µg/mL) are indicated directly below. The upper left corners of flow plots (A) and corresponding bar graphs (B) represent n=3 independent experiments for the percentage of LAG3+ or LAG3- cells staining positive for Gal-3 to quantify dose-dependent lectin-binding. The y-axis of flow plots depicts the median fluorescence intensity, measured in arbitrary units, for cells staining at varying levels of Galectin-3-APC intensity, plotted against the FSC-area (FSC-A) of cells. Targeting non-glycosylated regions of LAG3, FGL-1 is a positive control for ligand binding. Gal-3 iso; isotype antibody control for non-specific background binding of anti-Galectin-3-APC staining antibody. Statistical significance was determined using the Mann Whitney U-test: *p<0.05, **p<0.01, ***p<0.001.

With respect to Figures 5 and 6, in engaging N-glycans, the increase in LSECtin and Galectin-3 binding on LAG3+ cells from 25-100µg/mL of ligand follows a relatively linear progression, both reaching their saturation point at 100µg/mL of ligand. Notably, the maximal binding is higher for Gal-3 at 99.9% LAG3+ cells positive for Gal-3 expression, as opposed to LSEC at 79.9% (Figures 6a and 5a, respectively). Compared to 14.1% Gal-3+ expression at 25µg/mL of Gal-3, LSECtin initially binds LAG3+ Jurkat cells higher at 33.4% at the same concentration. Accordingly, this enhanced binding is reflected in the gradual and shallower inclines of LSEC-binding, opposing the stark elevation in Gal-3 binding observed between the 25-50 µg/mL and 50-100 µg/mL conditions on LAG3+ Jurkats. While steeper inclines for Galectin-3 indicate swifter and stronger LAG3-binding than LSECtin at higher Gal-3 concentrations, the initially high LSEC-binding implies a higher affinity between LAG3 and LSECtin.

Providing a measure of lectin-binding affinity to LAG3, the EC50 (Half-maximal Effective Concentration) value represents the concentration of LSEC or Gal-3 at which 50% of the maximal binding to LAG3 occurs on LAG3+ Jurkat cells. Amongst the independent triplicates (n=3), the lower EC50 value for LSECtin (38.4286 µg/mL) compared to Galectin-3 (50.5024 µg/mL) indicates LSECtin has higher binding affinity to LAG3, interacting stronger than Galectin-3. These binding curves inform the specificity and relative strength of lectin-LAG3 binding within this linear concentration range, better characterizing the dynamics of these molecular interactions.

For PNGaseF-treated cells, lectin concentrations yielding saturated binding (200µg/mL) were used to assess how PNGaseF-mediated N-glycosylation removal impacts lectin-binding to both LAG3+ and LAG3- cells [29,117,120,121]. This approach aimed to confirm the requirement of LAG3 N-

glycosylation for effective binding of both LSECtin and Galectin-3, addressing Objective 1. Compared to the untreated 200µg/mL LSEC condition which yields maximal binding on LAG3+ Jurkats, glycosidase-treatment in the 200µg/mL LSEC + PNGaseF condition significantly diminishes percent positive LSEC-binding on LAG3+ Jurkats ($p= 0.0086$; Figure 5). Similarly, this statistically significant reduction Gal-3 binding is observed upon PNGaseF treatment in the 200µg/mL Gal-3 + PNGaseF condition ($p= 0.0079$; Figure 6). Notably, PNGaseF deglycosylation curtails binding of both lectins on LAG3+ cells to levels akin to untreated LAG3- cells which bind exogenous lectins minimally, even with their N-glycans intact [29,117,120,121].

While these results impart molecular insights into the nature of this interactions, they do not fully capture its nuances. Proving that the carbohydrate-recognition domain (CRD) of lectins and glycans of LAG3 facilitate these engagements, rather than a protein-protein interaction, would further clarify these intricacies.

Altogether, these observations illustrate the dose-dependent specificity of LSECtin and Galectin-3 show for binding LAG3, as additional ligands for LAG3. And upon altering the glycosylation profiles of cells, these findings confirm the necessity of N-glycosylation in mediating LAG3's interactions with Galectin-3 and LSECtin, resolving a significant knowledge gap of Objective 1. Beyond LAG3-lectin binding assessments, these results facilitate subsequent research concerning N-glycosylation in LAG3-mediated processes and its implications for modulating T cell activity, as per Objective 2.

4.4.2 CRD-glycan Interaction Validation with Competitive Inhibitor β-lactose

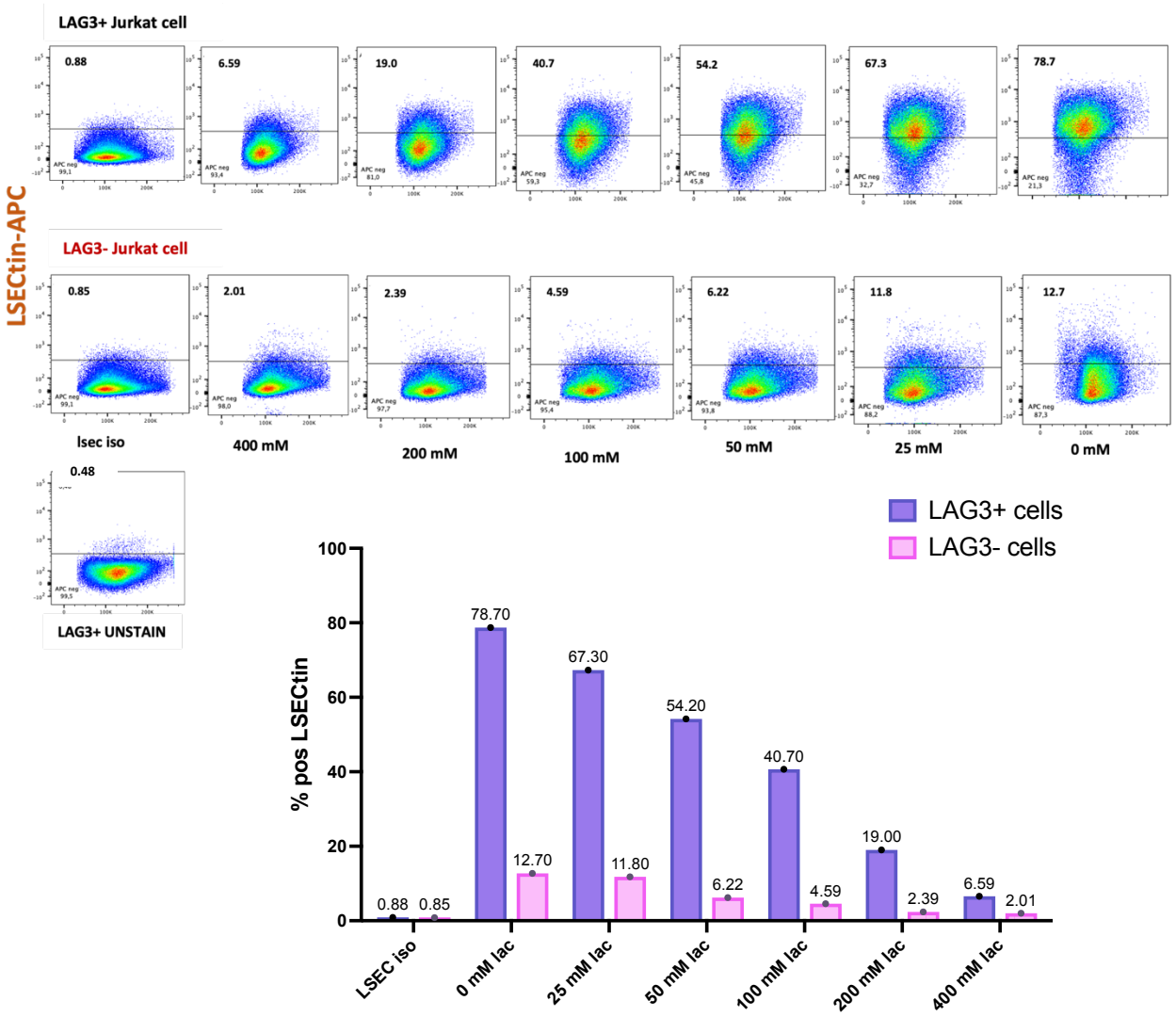
In efforts to verify that lectin-LAG3 engagement occurs through the carbohydrate-recognition domain (CRD) of the lectin and the N-glycan of LAG3, a competitive inhibition assay with β-

lactose was conducted. Leveraging its structural similarity to target glycans of LAG3, β-lactose outcompetes these N-glycans by competitively occupying the CRD binding pocket of lectins, displacing lectins from the cells [78,122–126]. With selective CRD-glycan disruption, the competitive inhibitor β-lactose aims to substantiate Objective 1 whereby LSECtin and Galectin-3 binding transpires via glycosylated residues of LAG3 through a CRD-glycan interaction, rather than a protein-protein interaction.

Specifically, this CRD-glycan disruption was executed by pre-treating LAG3+ and LAG3- Jurkat cells with either 100μg/mL LSECtin or Galectin-3, then subjecting pre-treated cells to a β-lactose gradient (0mM - 400mM). Thereafter, degree of lactose competitive inhibition was evaluated with flow cytometry by assessing changes in cell-surface binding of the respective lectins on both LAG3+ and LAG3- cells.

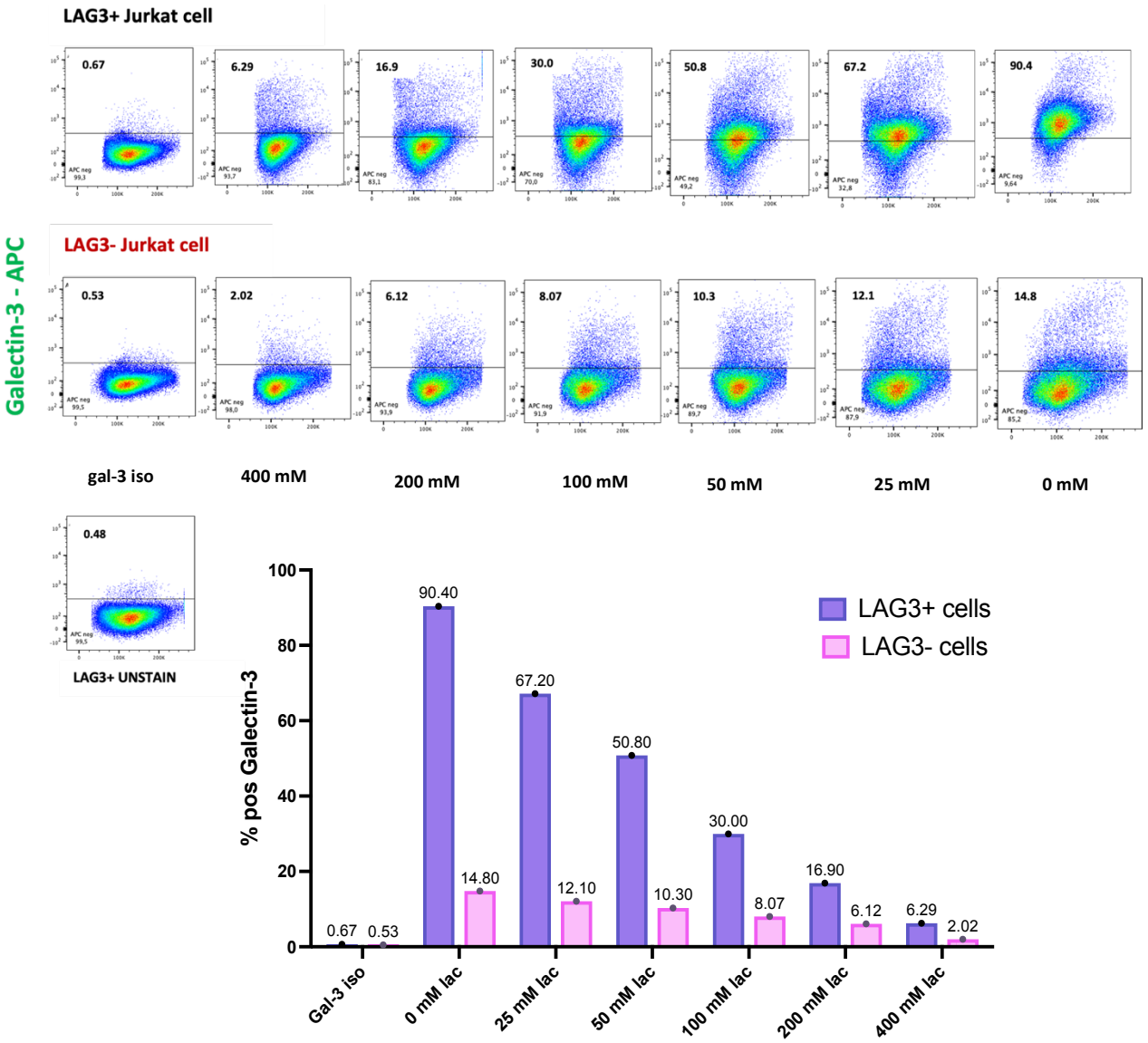
In addition to PNGaseF, which deglycosylates cells, lectin-LAG3 binding was disrupted by administering β-lactose. Indeed, the increase in lactose concentrations revealed a clear reduction of percent positive cell-surface binding for both LSECtin and Galectin-3 in Figures 7 and 8, respectively. Even with lectin concentration consistent, this observation demonstrates the escalating dose-dependent competitive inhibition by lactose. When comparing binding patterns between LAG3+ and LAG3- Jurkats, initial LSECtin and Gal-3 binding and its reductions upon lactose addition are more pronounced for LAG3+ cells, contrasting with minimal change observed for LAG3- cells. In line with the previous results for Objective 1, this discrepancy reaffirms that LSECtin and Galectin-3 exhibit specificity for LAG3. Hence, the presence of LAG3, or lack thereof evidently shapes lectin binding dynamics and ensuing competitive inhibition.

Figure 7. Validation of CRD-glycan Interaction: β-lactose Competitive Inhibition of LSECtin



CRD-glycan interaction during LSECtin-LAG3 binding was confirmed by evaluating extent of competitive inhibition with β-lactose. Structurally, like glycans on LAG3, lactose outcompetes CRD-binding site of LSECtin. LAG3+ and LAG3- Jurkat cells pre-treated with 100µg/mL LSECtin, were exposed to a β-lactose gradient (0mM - 400mM), to disrupt CRD-glycan interactions, displacing cell-bound LSECtin. Upper left corners of flow cytometry plots and corresponding bar graphs show the percentage of LAG3+ or LAG3- cells staining positive for LSECtin, quantifying reduction in LSECtin cell-surface binding and extent of lactose competitive inhibition. The y-axis of flow plots depicts the median fluorescence intensity, measured in arbitrary units, for cells staining at varying levels of LSECtin-APC intensity, plotted against the FSC-area (FSC-A) of cells. Lactose concentrations (mM) are indicated directly below flow plots and bar graphs. Both graphs depict results of a single independent iteration.

Figure 8. Validation of CRD-glycan Interaction: β-lactose Competitive Inhibition of Galectin-3



CRD-glycan interaction during Galectin-3-LAG3 binding was confirmed by evaluating extent of competitive inhibition with β-lactose. Structurally, like glycans on LAG3, lactose outcompetes CRD-binding site of Galectin-3. LAG3+ and LAG3- Jurkat cells pre-treated with 100µg/mL Galectin-3, were exposed to a β-lactose gradient (0mM - 400mM), to disrupt CRD-glycan interactions, displacing cell-bound Galectin-3. Upper left corners of flow cytometry plots and corresponding bar graphs show the percentage of LAG3+ or LAG3- cells staining positive for Galectin-3, quantifying reduction in Galectin-3 cell-surface binding and extent of lactose competitive inhibition. The y-axis of flow plots depicts the median fluorescence intensity, measured in arbitrary units, for cells staining at varying levels of Galectin-3-APC intensity, plotted against the FSC-area (FSC-A) of cells. Lactose concentrations (mM) are indicated directly below flow plots and bar graphs. Both graphs depict results of a single independent iteration.

In occupying the CRD sites on lectins, the decrease in LSECtin binding on LAG3+ cells follows a relatively linear pattern at each lactose concentration (Figure 7). In contrast, a stark reduction in Galectin-3 binding is observed between the 0mM and 25mM condition of β-lactose on LAG3+ Jurkat cells (Figure 8). As opposed to gradual or shallower declines, steeper declines observed for Galectin-3, reflect a swifter and more potent disruption of lectin-binding than LSECtin, particularly at lower lactose concentrations. In this regard, lactose was marginally better at initially displacing Galectin-3 from LAG3+ cells than LSECtin. However, the inverse appears true when comparing LAG3- Jurkats in both conditions. Although $IC50_{lactose}$ values seem comparable for both lectins in this single iteration, conducting additional assay replicates would better quantify each lectin's affinity for lactose, improving precision of relative binding affinities and strengths of interaction of each lectin towards LAG3. For instance, a lower $IC50_{lactose}$ value for Galectin-3 reflects a potentially higher affinity between lactose and Galectin-3, coinciding with a lower affinity between LAG3 and Galectin-3 within this lactose concentration range.

Examining the linear range of the curves presented herein, the efficacy of lactose-mediated inhibition begins to plateau from 200mM onwards. Testing concentrations beyond 400mM lactose would confirm the threshold lactose concentration to reach saturation, indicating the limit of lactose's competitive binding. Nevertheless, 50mM to 100mM lactose is typically employed for assessing CRD-glycan interactions, as documented in literature [78,122–126].

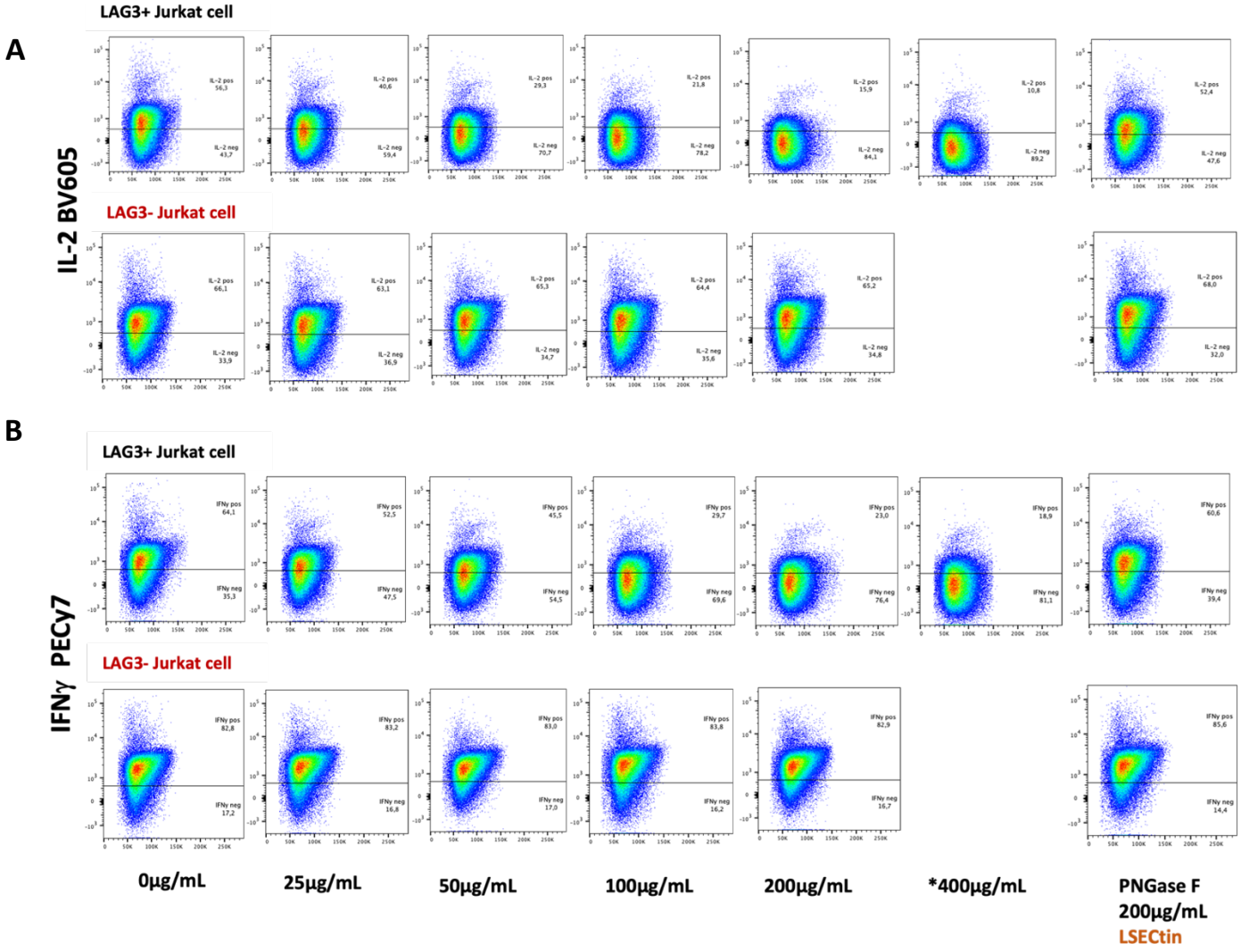
Through β-lactose disruption of CRD-glycan interactions, LSECtin and Galectin-3 specifically bind LAG3 in a carbohydrate or glycan-dependent manner, requiring glycosylation of LAG3. Altogether, these findings substantiate earlier results obtained for Objective 1.

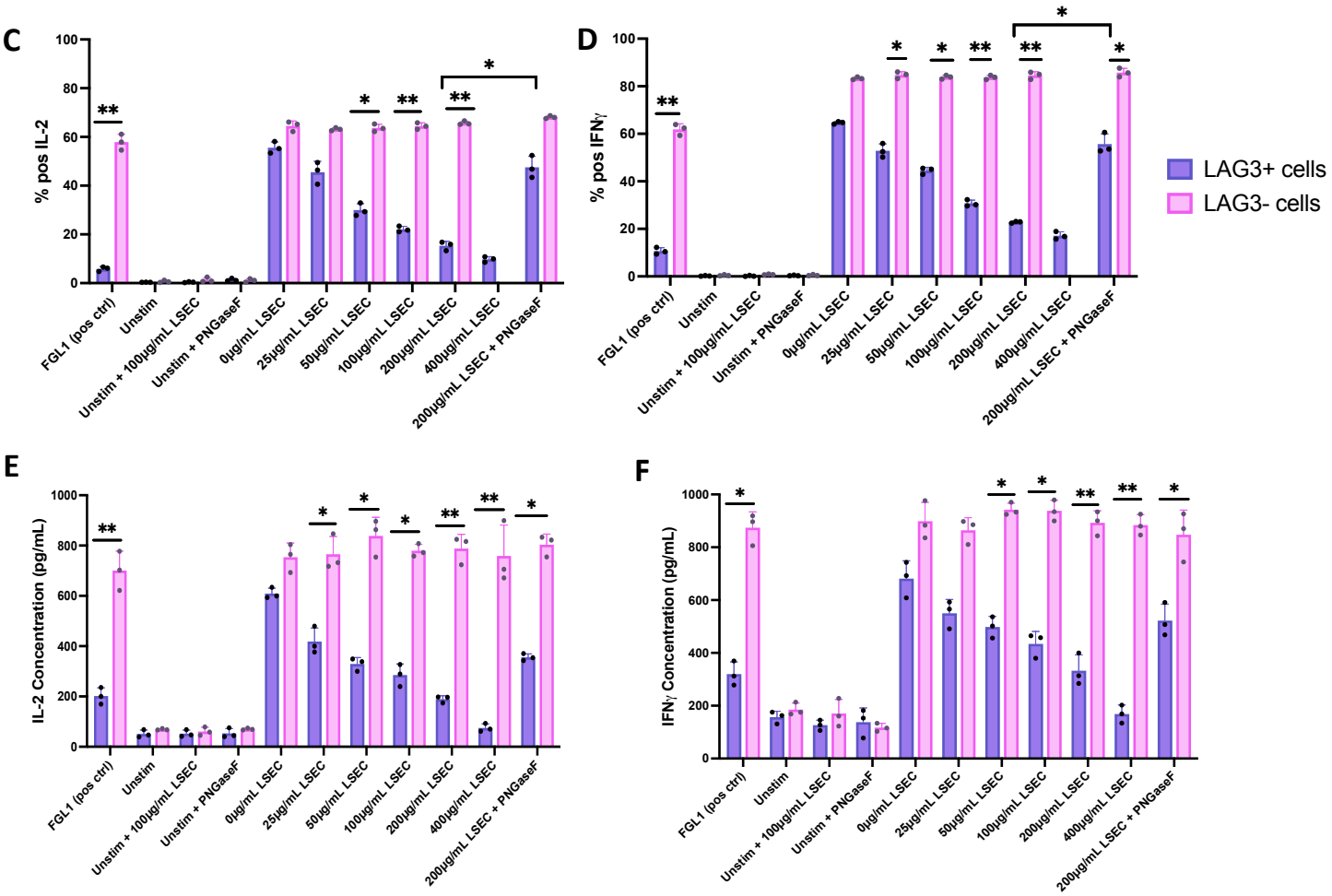
4.5 Lectin Binding Engages and Augments LAG3-mediated T cell suppression

While lectin binding of LAG3 through N-glycosylated residues were demonstrated, the functional consequences and immunological significance of this interaction on LAG3 function remain unexplored. Understanding how lectins modulate LAG3 biology to instruct T cell exhaustion and immune responses could better inform interventions that counter immune dysfunction. In line with Objective 2, this study sought to investigate whether, following binding, lectins can engage LAG3 inhibitory activity and subsequently inhibit T cell responses.

For assessment of lectin-mediated LAG3 activity, a concentration gradient (0-400 µg/mL) of Galectin-3 or LSECtin was applied to LAG3+ and LAG3- cells, treated with or without PNGaseF glycosidase. These cells were then strongly stimulated with anti-CD3 and anti-CD28 for robust T cell activation, expansion and cytokine production. To examine the dose-dependent reduction in cytokine responses, flow cytometry compared the percentage (%) of LAG3+ and LAG3- cells expressing intracellular IL-2 and IFNγ, endpoints of T cell activation. This comparison provides a measure of the differential inhibitory strengths of LSECtin and Galectin-3 that augment LAG3-driven T-cell suppression, as depicted in Figures 9 and 10, respectively.

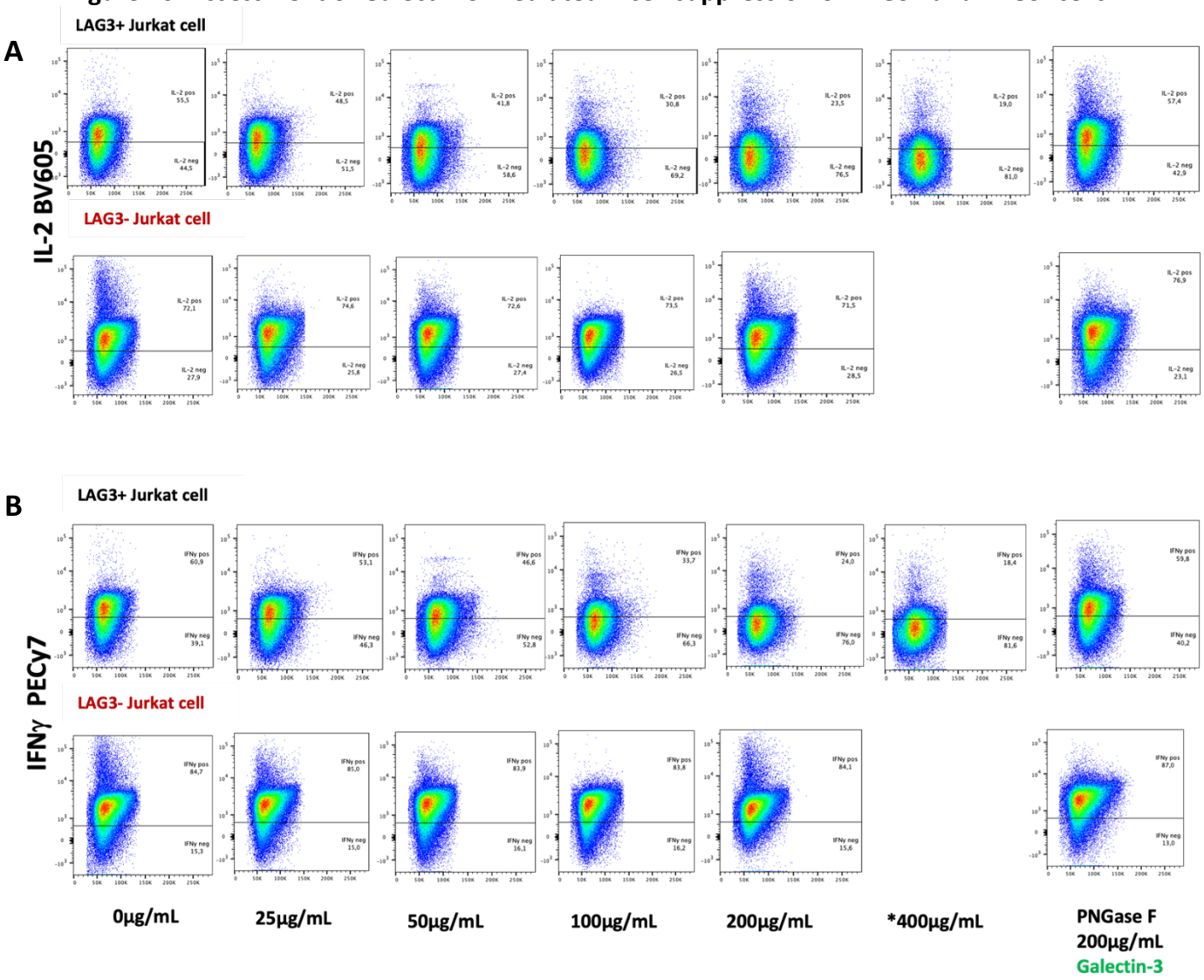
Figure 9. Assessment of LSECtin-mediated T-cell suppression of LAG3+ and LAG3- Jurkat cells

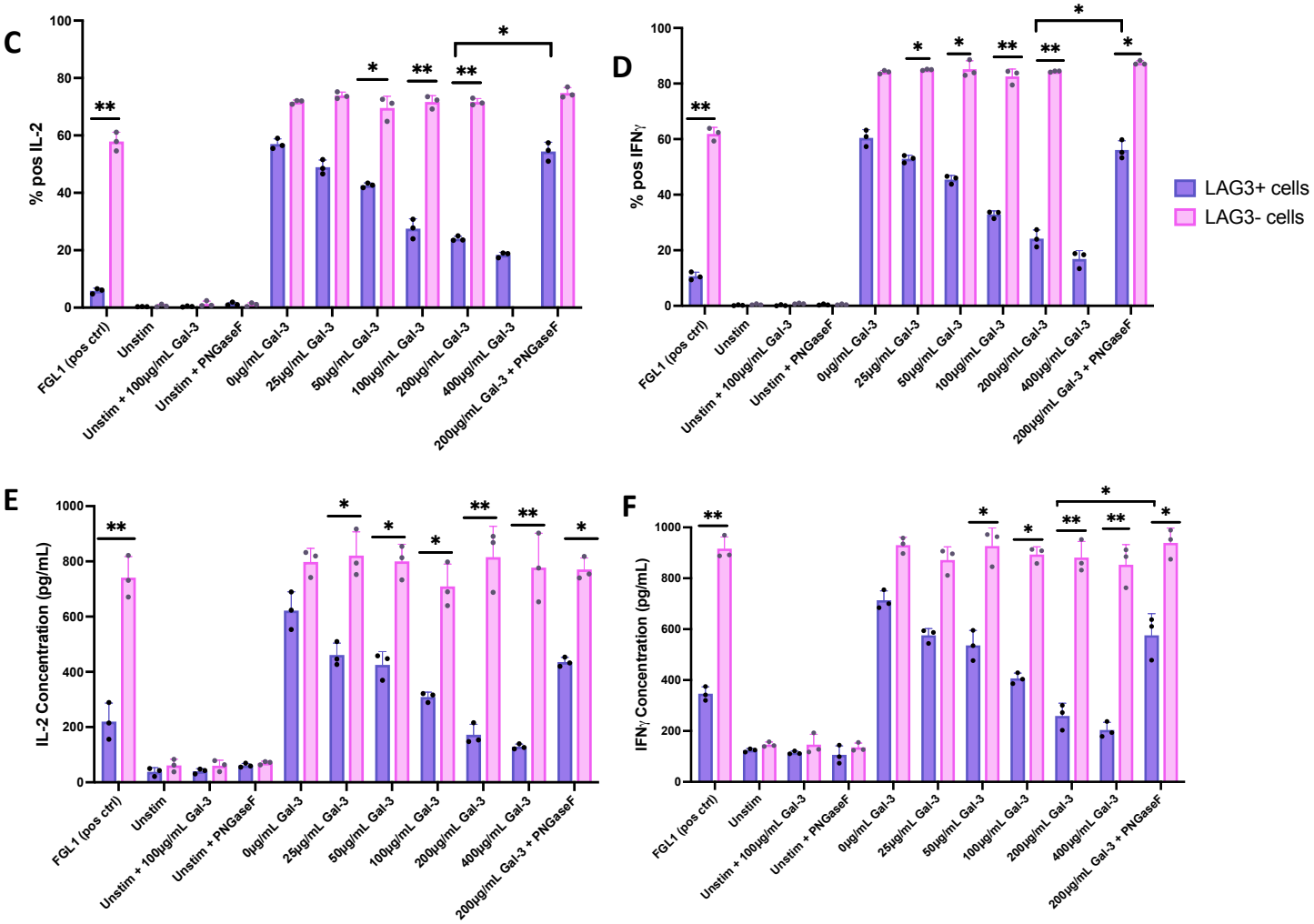




As indicated below each graph, LSECtin (LSEC) concentration gradients (0-400µg/mL) on anti-CD3 and anti-CD28 stimulated LAG3+ and LAG3- cells were used to assess whether LSECtin engages LAG3 activity, and its dose-dependent impact on T cell inhibition (IL-2 and IFNγ production). At the saturating concentration of 200µg/mL LSECtin, PNGaseF-treatment was used to reduce LSEC binding on Jurkats to investigate restoration of intracellular cytokine levels. Flow dot plots and corresponding bar graphs illustrate percentage of LAG3+ or LAG3- Jurkat cells staining positive for intracellular IL-2 (A and C) or IFNγ (B and D), quantifying lectin engagement of LAG3 and subsequent T cell suppression. Targeting non-glycosylated regions of LAG3, FGL-1 is a positive control for ligand binding and T cell inhibition. Considering expenses associated with flow cytometry, *400µg/mL LSEC treatment of LAG3- Jurkats was deemed unnecessary and redundant, as saturated cytokine responses were observed at preceding concentrations. Simultaneously, ELISAs were conducted to measure soluble extracellular IL-2 (E) and IFNγ (F) responses, as an additional readout for the specified aims. Based on n=3 independent experiments, statistical significance was determined using the Mann Whitney U-test: *p<0.05, **p<0.01, ***p<0.001.

Figure 10. Assessment of Galectin-3-mediated T-cell suppression of LAG3+ and LAG3- cells





As indicated below each graph, Galectin-3 (Gal-3) concentration gradients (0-400µg/mL) on anti-CD3 and anti-CD28 stimulated LAG3+ and LAG3- cells explored whether Galectin-3 engages LAG3 activity, and its dose-dependent impact on T cell inhibition (IL-2 and IFNγ production). At the saturating concentration of 200µg/mL Galectin-3, PNGaseF-treatment was used to reduce Gal-3 binding on Jurkats to investigate restoration of intracellular cytokine levels. Flow dot plots and corresponding bar graphs illustrate percentage of LAG3+ or LAG3- cells staining positive for intracellular IL-2 (A and C) or IFNγ (B and D), quantifying lectin engagement of LAG3 and subsequent T cell suppression. Targeting non-glycosylated regions of LAG3, FGL-1 is a positive control for ligand binding and T cell inhibition. Considering expenses associated with flow cytometry, *400µg/mL Gal-3 treatment of LAG3- Jurkats was deemed unnecessary and redundant, as saturated cytokine responses were observed at preceding concentrations. Simultaneously, ELISAs were conducted to measure soluble extracellular IL-2 (E) and IFNγ (F) responses, as an additional readout for the specified aims. With n=3 independent experiments, statistical significance was determined using Mann Whitney U-test: *p<0.05, **p<0.01, ***p<0.001.

In Figures 9 and Figure 10, increasing concentrations of LSECtin and Galectin-3 are accompanied with progressively diminished intracellular IL-2 and IFNγ responses within LAG3+ Jurkat cells, while LAG3- Jurkat cells exhibit uniform cytokine levels regardless of lectin dose. At 50µg/mL, 100µg/mL and 200µg/mL of exogenous LSECtin, this difference in median percent positive IL-2 levels is statistically significant ($p=0.0431$, $p=0.0094$ and $p=0.0096$, respectively; Figures 9A and 9C). Comparing intracellular IFNγ expression, a statistically notable distinction emerges between LAG3+ and LAG3- Jurkat cells for LSECtin concentrations of 25µg/mL, 50µg/mL, 100µg/mL and 200µg/mL ($p=0.0496$, $p=0.0493$, $p=0.0092$ and $p=0.0081$, respectively; Figures 9B and 9D). Contrary to unvarying cytokine levels in LAG3- Jurkat cells, increasing concentrations of Galectin-3 results in a dose-dependent reduction of cytokine expression within LAG3+ cells, mirroring patterns observed with LSECtin-treated LAG3+ Jurkat cells (Figure 10). For IL-2, this statistically significant divergence is apparent at Gal-3 concentrations of 50µg/mL, 100µg/mL and 200µg/mL ($p=0.0407$, $p=0.0088$ and $p=0.0082$, respectively; Figures 10A and 10C). Likewise, 25µg/mL, 50µg/mL, 100µg/mL and 200µg/mL of Galectin-3 yield statistically significant differences in IFNγ expression ($p=0.0370$, $p=0.0331$, $p=0.0065$ and $p=0.0054$, respectively; Figures 10B and 10D).

In parallel conditions to the above lectin-cytokine gradients, supernatants from both cell lines were harvested to quantify extracellular, or secreted, IL-2 and IFNγ production by ELISA as an additional readout of T cell activation status. In line with results obtained from flow cytometry, these statistically significant dose-dependent declines in cytokine production within LAG3+ Jurkat cells by LSECtin and Galectin-3 remain consistent and even more pronounced in the conducted ELISAs. This is evident through the greater number of statistically significant

differences in cytokine production present across the various lectin concentrations tested (Figures 9E, 9F, 10E and 10F).

As evidenced by results from conducted ELISAs and flow cytometry, the reproducibility of these prominent dose-dependent trends of lectin-mediated T cell suppression within LAG3-expressing Jurkat cells, confirm the specificity of LSECtin and Galectin-3 for triggering LAG3 activity. Most importantly, compared to conditions without LSECtin or Gal-3 (0µg/mL), the presence of lectin, even at 25µg/mL, can lower cytokine production in a LAG3-dependent manner (Figures 9 and 10). Thus, while the presence of LAG3 crucially influences initial cytokine levels, upon lectin-binding, both Galectin-3 and LSECtin exhibit the ability to engage LAG3 function and augment its inhibitory activity, provoking lower cytokine responses and subsequent T-cell suppression.

Across flow cytometry and ELISA experiments and treatments illustrated in Figures 9 and 10, IFNγ production appears to be greater for both LAG3+ and LAG3- cells, when compared to levels of IL-2. As expected, this distinction becomes apparent when assessing maximal expression of IFNγ and IL-2 on LAG3- cells across conditions. LAG3- cells, lacking inhibitory LAG3, will demonstrate maximal cytokine expression, providing a basis for comparison across treatments. Despite not reaching their maximal cytokine repression saturation point within the tested interval of 0-400µg/mL of lectin, lectin-mediated suppression begins to plateau by 200 µg/mL of either lectin ligand on LAG3+ cells (Figures 9A-D and 10A-D).

As inhibitory LAG3 activity is engaged with increasing LSECtin and Galectin-3 concentrations, LAG3-expressing cells follow a relatively linear and progressive reduction in median percent positive IFNγ and IL-2 expression. When comparing the ranges that demarcate the maximum to

minimum value for percent positive cytokine expression, these values amongst the two investigated cytokines are similar between Gal-3 and LSECtin. Notably, this results in a comparable linear decline in cytokine response for Gal-3 and LSECtin (Figures 9A-D and 10A-D).

In each of the four lectin-dose curves obtained through flow cytometry, this linear diminution is punctuated by a region of more pronounced cytokine reduction. In the case of IFNγ, this occurs between 50-100µg/mL for both Gal-3 and LSECtin. For IL-2, visible downregulations arise at 25-50µg/mL of LSECtin and 50-100µg/mL of Gal-3 (Figures 9C, 9D, 10C and 10D). At these highlighted intervals of steeper declines, their corresponding lectin concentrations could exert, in a LAG3-dependent manner, a stronger inhibitory effect on cytokine production. Concerning IL-2 reductions, LSECtin shows an earlier concentration interval compared to Gal-3 (Figures 9A and 10A). This suggests that the strength of cytokine inhibition by LSECtin may be more potent than that of Gal-3 at the same corresponding concentration.

Accordingly, measuring the LSECtin or Gal-3 concentration needed to inhibit 50% of cytokine production within LAG3+ Jurkats, the IC50 (half-maximal inhibitory concentration) value quantifies the potency or inhibitory strength of either lectin in suppressing cytokine response. In the dose curves, LSECtin showed a lower IC50 value for IL-2 (49.226 µg/mL) and IFNγ (65.489 µg/mL) compared to Gal-3 ($IC50_{IL-2}$: 62.4189 µg/mL and $IC50_{IFNγ}$: 85.9899 µg/mL, respectively). Coinciding with lower IL-2 and IFNγ levels across LSECtin-treated conditions, the lower LSECtin IC50 values suggest its greater potency in curtailing production of both these cytokines by augmenting LAG3 inhibitory activity relative to Gal-3. Further analyzing the calculated IC50 values indicate that Gal-3 is more effective at inhibiting IL-2 (62.4189 µg/mL) compared to IFNγ (85.9899 µg/mL), based on higher IFNγ expression observed across various Gal-3

concentrations. Consistent with the globally higher IFNγ levels for both lectins, this patten is reflected in LSECtin, which consequently exhibits stronger suppression of IL-2 production (49.226 μg/mL) throughout the tested concentrations relative to IFNγ (65.489 μg/mL).

Altogether, these lectin dose curves illustrate the specificity and differential inhibitory strengths of LSECtin and Gal-3 in suppressing cytokine responses within LAG3-expressing Jurkats. These findings better inform the dynamics of these interactions in modulating T cell responses, emphasizing the nuanced regulatory roles of these lectins in guiding immune responses [30].

As a sub-aim related to Objective 1, this thesis sought to elucidate how N-glycosylation affects lectin-mediated T cell suppression and its implications for restoring intracellular cytokine responses within LAG3+ and LAG3- cells. To address this subsidiary objective, lectin concentrations yielding saturated binding (200μg/mL) were utilized to assess the consequences of PNGaseF N-glycan removal[29,117,120,121]. This approach once again served to confirm the necessity of LAG3 N-glycosylation for lectin-binding, as well.

Compared to 200μg/mL LSEC ligand-alone conditions that maximally suppress IL-2 and IFNγ within LAG3+ Jurkats, glycosidase-treatment in the 200μg/mL LSEC + PNGaseF conditions significantly restores percent positive IL-2 and IFNγ expression on LAG3+ Jurkats (p=0.0476 and p=0.0498, respectively; Figures 9C and 9D). Similarly, statistically significant IL-2 and IFNγ restoration occurs in PNGaseF-treated LAG3+ cells in the 200μg/mL Gal-3 + PNGaseF conditions (p=0.0484 and p=0.0490; Figure 10C and 10D). Of note, this restoration in cytokine expression is almost to the level of untreated (no PNGaseF) LAG3+ cells incubated with 0μg/mL ligand conditions for LSECtin and Gal-3 (Figures 9C, 9D, 10C and 10D). Furthermore, these outcomes are replicated in ELISAs conducted on supernatants from this assay.

In summary, PNGaseF deglycosidation disrupted the dose-dependent cytokine reduction observed in Gal-3 and LSECtin-incubated LAG3+ cells, enabling higher IL-2 and IFNγ responses in PNGase-F treated cells and LAG3- cells compared to untreated LAG3+ cells. Fulfilling the research gap of Objective 2, in altering N-glycosylation profiles, lectin-binding on LAG3+ Jurkats is diminished, alleviating lectin-mediated T cell suppression, even in the presence of ligand. Thus, LSECtin and Galectin-3 binding induce LAG3 activity, exhibiting differential inhibitory strengths. This reinforces importance of N-glycosylation in lectin-LAG3 interactions, and its pivotal role in modulating LAG3-mediated processes. As regulators of the immune response, uncovering these additional mechanisms that engage LAG3 function and fine-tune immune regulation expands our knowledge of IC biology and T cell exhaustion.

5 Discussion

5.1 Validation of Jurkat Characterization Panel

Comprehensively validating our characterization panel, outlined in Table 1, confirmed its efficacy on full-stained primary resting PBMC-derived T cells. Then, I assessed this validated panel to ultimately describe the expression profiles of various lineage and functional markers on the constructed Jurkat T cell lines. Even with already distinct resolution for positive and negative populations of CD3 and CD4 on PBMCs, the inclusion of their respective FMOs ensured unambiguous gating precision and population discrimination [115,127].

Including all FMO controls to precisely delineate the smear patterns of CD3, CD4, CCR5, and LAG3 on both constructed cell lines parallels their underlying biology, documented in prior research [113,114]. This supports the biological relevance and credibility of these findings and panel validity, improving downstream data quality by effectively capturing their expected biology. Consistent precision of the validated panel, when assessed on PBMCs and subsequently Jurkat T cells, enabled accurate detection of target marker expression profiles, demonstrates its reproducibility. This affirms its suitability for precise characterization of LAG3+ and LAG3- Jurkat cells for use in subsequent experiments and reinforces the reliability of associated results.

5.2 Characterization of LAG3+ and LAG3- Jurkat Cell Lines

The unique, yet enigmatic, inhibitory mechanism of LAG3 can inform immune exhaustion, but remains a challenge to decrypt with the scarcity of reliable LAG3 cell line models, prior to this study [20,21]. Bridging this critical research gap, this dissertation overcame this significant challenge in LAG3 research, by generating a robust and versatile LAG3 cell line model to address the limited availability of such models. Extensively utilized in broadening knowledge of

TCR signaling which is implicated in the LAG3 mechanism, leveraging Jurkat CD4 T cell lines is an appropriate platform for developing the LAG3 model to assess its inhibitory function[10,36].

Creating the LAG3+ and LAG3- Jurkat cell lines through lentiviral transduction, flow cytometry-based characterization was performed to ensure the two cell lines were comparable. Both resting LAG3+ and LAG3- cell lines demonstrated highly similar expression profiles of evaluated lineage (CD3, CD4, CCR5) and co-stimulatory (CD2 and CD28) markers (Figure 3). Besides LAG3 expression, the close uniformity in percent positive expression levels for each marker signify that transduction, puromycin selection and culturing procedures do not create bias nor unfavorable variation amongst both our cell lines.

To characterize how LAG3 suppresses the TCR pathway within the constructed Jurkat T cells, the study design ensured that LAG3 expression was the sole variable that differentiates the two cell lines. Measurement of LAG3 expression on each resting cell line strongly confirms this. As expected, Jurkat cells transduced with the full LAG3 ORF (LAG3+) exhibited high LAG3 expression levels of 90.30%, while those transduced with the vector alone (LAG3-) displayed negligible LAG3 expression at 0.14% (Figure 3). Providing a favourably elevated signal-to-noise ratio with the high LAG3 expression, this clear distinction in LAG3 expression marks a fundamental aspect of our model's design [36]. Offering reproducibility and flexibility for LAG3 mechanistic studies, this Jurkat model mitigates limitations of primary T cells, including biological variability, swift LAG3 downregulation following activation and low resting LAG3 expression [20,21]. Such constraints diminish signal clarity and subsequent pronounced effects in PBMC-derived T cells with the fewer LAG3-expressing cells, making them less suitable for evaluating LAG3 function on T cell activation [36].

Notably, the LAG3+ Jurkat T cell lines exhibit a range of expression for LAG3, rather than a defined population. This smear LAG3 expression pattern was fundamentally verified through comprehensive flow cytometric optimization of compensation, antibody and volt titration, in addition to cytometry-based characterization with PBMCs. This validation ensures the reliability and accuracy of the observed LAG3 expression profile in the LAG3+ Jurkat cell line, reflecting the inherent biological variability. Using the lentiviral system to create the LAG3+ Jurkat cells herein, transducing cells with lentivirus encoding the full-length LAG3 ORF generates this substantial variation in LAG3 levels [128].

Beyond copy number variation, integration of the lentiviral LAG3 expression vector at different genomic loci within each Jurkat cell gives rise to the diverse LAG3 levels among the LAG3-expressing population, as in Figure 2. Moreover, integration near regulatory enhancer and repressor elements, or epigenetic modifications, influences LAG3-expression vector accessibility to transcriptional machinery. Such factors further modulate gene expression patterns and contribute to the observed surface LAG3 expression diversity among individual Jurkat cells [128].

Due to the differences in cellular context and activation states, the high level of LAG3 expression on Jurkat T cells, an immortalized leukemic CD4 T cell line, does accurately represent nor reflect the lower physiological LAG3 levels on primary CD4 T cells after antigen stimulation. As stated, primary T cells rapidly downregulate LAG3 following activation[20,21,36]. Accordingly, compared to the Jurkat cell lines in Figure 2, LAG3 expression is considerably lower on resting primary T cells from healthy-donor PBMCs, mirroring low baseline LAG3 levels on primary T cells in literature. In contrast to Jurkats, such factors lower the number of LAG3-expressing

primary cells or a weaker LAG3 signal, inconducive for assessing LAG3 activity in primary T cells [20,21,36].

Encompassing various subsets, primary CD4 T cells also exhibit a range of LAG3 expression levels rather than a uniform response post-antigen stimulation. As alluded to, the duration and intensity of antigenic stimulation correlates with extent of T cell exhaustion [12–16]. Hence, while levels of LAG3 on primary CD4 T cells can vary considerably with degree of exhaustion, a substantially narrower range of LAG3 expression is observed in physiological settings than that of the LAG3+ Jurkat cells [20,21,36,97]. As an exhaustion marker for CD4 and CD8 T cells following repetitive antigen stimulation, increased LAG3 expression is linked to elevated viral or tumor loads, underscoring its significance in infectious diseases and cancer, respectively. Notably, the highest LAG3 expressors are frequently observed among terminally-exhausted CD4+ and CD8+ T cells, as documented in chronic HIV infection. However, future research should clarify LAG3 expression and activity on across other immune cell types from disease patients, particularly chronic HIV, which remain poorly defined [20,21,36,84,97,111].

While Jurkat T cells may not fully recapitulate the biological diversity and diverse responses of various primary T subsets or other immune cell types, the LAG3-expressing Jurkat cells offer heterogeneity, contrasting a clonal or defined cell population. This variability in surface LAG3 expression among individual Jurkat cells may partially model natural variation in activation status, regulation, functional states or responses to stimuli during physiological immune responses. In this regard, differential outcomes may emerge among various T cell subsets expressing LAG3, albeit at a low levels. Indeed, LAG3 expression varies among different types of immune cells and conditions, highlighting its diverse immunoregulatory roles [20,21,36,97].

Translating this to primary immune cells, this Jurkat system lends reproducibility and flexibility for studying LAG3, bridging insights into how LAG3 expression heterogeneity impacts ligand interactions and immune cell functionality. This is vital for refining immunotherapy targeting LAG3 across various immune cell subsets, highlighting the complexity of LAG3 regulation and its relevance to T cell responses and immune modulation.

Although no perfect model exists for decrypting LAG3 activity, employing Jurkats enables confident assessment of LAG3's specific impact on various experimental endpoints, aligned with the proposed research objectives in this dissertation. In addition to bridging a notable research gap, the devised model has validity and versatility in drawing accurate inferences and conclusions into LAG3 inhibitory function, broadening its scope in T cell biology and immune exhaustion.

5.3 PNGaseF N-glycosidase Activity Validation

As detection molecules in immune recognition and response, N-glycosylation on surface of cells and glycoproteins are indispensable in virtually all signaling and cell-cell interactions within immunity. In determining T cell interactions with other soluble or cell-surface bound glycan-binding proteins (GBP), these glycans regulate pivotal aspects of T cell homeostasis and activation, amongst others, during lectin binding. Having multiple binding partners, these N-glycan-bearing glycoproteins may induce pleiotropic effects, contingent on the specific GBP it may bind [30,51].

While multiple N-glycosylation sites have been identified for LAG3, the precise effects of LAG3 glycosylation for lectin binding and its outcomes for LAG3-mediated T cell suppression remain unclear [53,54,63,67,72] Hence, probing the multifaceted and specific contributions of N-glycans in

LAG3-ligand interactions will resolve the complex, yet obscure, interplays governing T cell modulation and immunosuppression. Moreover, to avert attributing a single function to a particular glycan type, manipulating N-glycosylation profiles to empirically identify glycan functions in their specific contexts of T cell exhaustion is necessary [30,51].

As the most effective enzymatic method allowing N-linked glycan removal from glycoproteins, the catalytic action of PNGaseF N-glycosidase serves as a valuable biochemical tool. It has been extensively employed in workflows for identifying and characterizing N-linked glycosylation in glycoprotein activity and function in such contexts [29,117,120,121]. As such, this thesis sought to validate PNGaseF N-glycosidase activity to evaluate its efficacy in deglycosylating both LAG3+ and LAG3- Jurkat cells before its use in addressing our experimental aims.

Additionally, in PNGaseF activity assessment, PHA-L, a lectin with specific affinity for complex N-glycans, is frequently used in research to discern the presence or absence of N-glycans on cell-surface glycoproteins, like LAG3. Furthermore, this approach facilitates the evaluation of the extent of cell surface deglycosylation, thereby confirming effectiveness of PNGaseF activity following enzymatic treatment [118–120]. As evidenced by our flow cytometric validation, untreated LAG3+ and LAG3- Jurkat cells demonstrated elevated fluorescence intensity for PHA-L binding depicted in orange in histogram overlays, indicating the presence of intact N-glycans on the cell surface (Figure 4).

Shown by the blue peak, PNGaseF effectively removes N-glycans from Jurkats, diminishing PHA-L cell surface binding compared to untreated controls (orange). Comparison of treated samples to its respective controls in both cell lines helps establish the specific effects of N-glycosidase activity. Specifically, this leftward shift of the blue histogram from glycosidase-treated

conditions verifies successful N-glycan removal by PNGaseF, effectively deglycosylating both cell lines [29,117,120,121]. Nearing the red peak, which represents the unstained condition lacking PHA-L FITC, PNGaseF demonstrates its ability to substantially alter the N-glycosylation status in both LAG3-expressing and non-expressing Jurkats (Figure 4). This stark contrast in modulating glycosylation patterns allows us to discern between intact N-glycans and those removed by PNGaseF. These findings provide additional validation, strongly supporting PNGaseF effectiveness and highlight the extent to which glycosylation profiles can be modified through enzymatic deglycosylation, as also demonstrated by others [29,117,120,121].

Despite widespread use of this tool in workflows for characterizing N-linked glycan structures or protein glycosylation sites, our exploration is limited to flow cytometric validation. As in proteomic analysis and studies, further validations of deglycosylation, of especially LAG3, could examine changes in molecular weight, shifts in electrophoretic mobility or alterations in glycoprotein structure following treatment. Beyond mass spectrometry, other studies affirmed N-glycosidase's targeted removal of specific glycan structures through western blot analysis [29,41,117,120,121]. In fact, analyzing N-glycosidase treatment via western blot played a crucial in verifying the precise N-glycosylation sites on LAG3 glycoprotein as illustrated in a landmark LAG3 study by Baixeras et al [41]**.** Such confirmation would yield additional assurance for our presented findings.

Nevertheless, our flow cytometric validation of N-glycosidase activity and the corresponding changes in glycosylation status in both LAG3+ and LAG3- Jurkat cells, substantiates the validity, consistency and reliability of our enzymatic deglycosylation approach. This provides a sound platform to disrupt these N-glycans, enabling deeper exploration of glycosylation in LAG3-

mediated processes and interactions for subsequent investigations of lectin binding and its impact on T cell activity.

5.4 Analysis of LAG3 and Lectin binding

5.4.1 Relative Strength and Specificity of Lectin-LAG3 interactions

In the intricate landscape of immune interactions, lectins assume pivotal roles in immune modulation and signaling, dynamically finely tuning cellular immune responses. Containing carbohydrate recognition domains (CRDs), these lectins recognize many glycosylated structures on cell-surface glycoproteins such as immune checkpoint molecules, to execute a diverse array of functions. Given the pleiotropic effects of these glycan-binding proteins, their mechanisms of binding their glycoprotein counterparts, and the consequences thereof, must be empirically characterized [29,30,51]. With Lymphocyte Activation Gene 3's (LAG3) extensive glycosylation, the lectins, Galectin-3 and LSECtin have been proposed to bind glycans on LAG3, potentially engaging LAG3 function and suppressing T cell responses [19–21,53,54,72]. Resolving this knowledge gap, Objective 1 of this dissertation sought to clarify the necessity and specificity of N-glycosylation on LAG3 in mediating binding of lectin-ligands, LSECtin and Galectin-3.

After manipulating cell surface N-glycosylation profiles, percent (%) positive of LAG3+ and LAG3- cells, the cell-bound LSECtin (LSEC) and Galectin-3 (Gal-3), were compared to quantify lectin-LAG3 engagement under increasing lectin concentrations for Objective 1. The generated lectin dose curves for LAG3+ and LAG3- cells not subjected to PNGaseF, both exemplify a dose-dependent pattern for binding LAG3 as exogenous LSEC and Gal-3 concentrations rise in Figures 5 and 6, respectively. With respect to prominent exogenous lectin expression on LAG3+ cells, statistically different to LAG3- cells, indicates LSECtin and Galectin-3's specificity for binding

LAG3. When comparing binding patterns between LAG3+ and LAG3- Jurkat cells from β-lactose competitive assays in Figures 7 and 8, dose-dependent reductions in LSECtin and Gal-3 binding upon lactose addition are more pronounced for LAG3+ cells, contrasting the minimal change for LAG3- cells. This discrepancy, consistent with previous results for Objective 1, reaffirms the specificity of LSECtin and Galectin-3 for LAG3. Hence, presence of LAG3, or lack thereof evidently shapes lectin binding dynamics and ensuing competitive inhibition.

While dose curves for lectin-LAG3 engagement have not been extensively characterized in literature, Xu et al. and Kouo et al. used co-immunoprecipitation assays to verify both soluble LSECtin and Galectin-3 physically interact with soluble LAG3 protein, respectively [53,54]. This provides valuable insights and physical confirmation of the molecular interactions of LAG3 with lectins underlying immune modulation and for therapeutic targeting. This concentration-dependent pattern of binding in this thesis aligns with published findings investigating a different linear range of concentrations for Gal-3 (0-150µg/mL) and LSECtin (0-1µg/mL) [53,54]. By testing a more expansive range of concentrations, this dissertation refines these molecular binding kinetics and affinity between lectins and N-glycosylated LAG3 that may occur during immune recognition and modulation.

While no glycan analysis was conducted in the cited studies, both Galectin-3 and LSECtin bound LAG3 in a dose-dependent way, providing additional support for their specificity towards LAG3 [21,53,54,72]. Thus, degree of LSECtin and Galectin-3 binding is evidently influenced by presence or absence of LAG3. By capitalizing on the strengths of a high signal-to-noise ratio regarding LAG3 expression on LAG3-expressing Jurkat cells, such experimental setup overcomes the limitations of low LAG3 expression on primary T cells during steady state [21,34]. In this regard, differential

LSECtin and Galectin-3 binding may emerge among various T cell subsets expressing LAG3, albeit at a low level. Appreciating the functional significance and complexity of such lectin-LAG3 interactions in immune regulation is vital for tailoring interventions to specific immune cell populations. Such insights aid targeted approaches that address the distinct roles of cell subsets in immune responses.

When comparing Figures 5 and 6, the steeper inclines for Gal-3 binding that interrupt the linearity of the dose curve, suggesting a swifter and stronger association with LAG3 than LSECtin at higher Gal-3 concentrations. These stark changes in lectin binding prompts speculation about certain concentrations intervals that likely act as thresholds for lectin multimerization. Considering the capacity of both these lectins to oligomerize, this multimerization tendency is more pronounced in soluble forms compared to monomeric, membrane-bound cell-surface ligands [56,129]. Additionally, the extensive glycosylation of LAG3 at its N-residues, characterized by large and heavy glycan branches provides multiple binding sites and may better facilitate the transition from monomeric to oligomeric forms, in addition to cooperative ligand binding [130]. Certainly, more replicates beyond n=3 can be conducted to better characterize the linearity of dose-dependent binding. Understanding the dynamics of these molecular engagements remains a significant unknown and stands as an area for future research to elucidate of lectin binding to LAG3 is monomeric or multimeric.

Indeed, the lower LSEC EC50 value than that of Gal-3 indicates the higher binding affinity of LSECtin for LAG3, highlighting the relative strength and specificity of lectin-LAG3 interactions. This observation coincides with higher IC50 values obtained for LSEC than Gal-3 from β-lactose competitive inhibition assays. With LSEC more resistant to lactose disruption and displacement

from LAG3 given its higher affinity for LAG3, this reinforces the consistency and reliability of the findings across different metrics. Acknowledging the limitation of a single iteration for lactose competition assays, determining IC50 values with subsequent replicates would better clarify the nature of lectin binding and dynamics and subsequent competitive inhibition.

Deciphering how monomeric or multimeric versions informs the affinity to LAG3 involves considering the contribution of avidity, which represents the overall strength of multivalent interactions. Alternative approaches such as SPR or bio-layer interferometry (BLI) to accurately measure binding affinity (K_a) between the two lectins and LAG3. In the prolonged inflammatory environments of chronic infection or the TME where LSECtin and Galectin-3 are upregulated, insights into ligand-receptor behaviour can inform their roles in augmenting disease pathogenesis [54,63,72,73,131]. Thus, despite LSECtin's higher affinity, the abundance of Galectin-3 and potentially better multimerization capacity could outcompete LSECtin for binding LAG3 if both are present in the inflammatory milieu of question, influencing associated functional consequences. Given diversity of LAG3 ligands, their hierarchy in importance and function warrants resolution. This knowledge contributes to the broader comprehension of immune regulation for further exploration of lectin-mediated immune responses and immune checkpoint modulation.

5.4.2 N-Glycosylation of LAG3 is Necessary for LSECtin and Galectin-3 Binding

After establishing the specificity of LAG3-lectin binding, Objective 1 aimed to elucidate the structural requirement of N-glycosylation in governing these interactions. Here, at saturating concentrations of lectin, which yield maximal binding, PNGaseF was used to assess how

removal of cell-surface N-glycosylation affects lectin-binding to both LAG3+ and LAG3- cells [29,73,117,118,120,121].

Notably, PNGaseF-mediated deglycosylation significantly reduced LSECtin and Galectin-3 binding to LAG3+ Jurkat cells, even at saturating ligand concentrations in Figures 5 and 6, respectively. This emphasizes the consistent impact of PNGaseF treatment across different lectins. Such comparative analyses underscore the specificity of the observed effects on lectin binding. Addressing a significant and previously unresolved knowledge gap in literature, in the context of LAG3, these pivotal findings indicate that N-glycan moieties are a fundamental molecular determinant for LSECtin and Galectin-3 binding [21,53,54,72].

To verify that lectin-LAG3 ligation occurs through CRD of lectins binding to N-glycosylated regions of LAG3, β-lactose was administered to selectively disrupt CRD-glycan interface. Mimicking target glycans on surface glycoproteins, this competitive inhibitor is commonly used in research for modulating galectin-glycan interactions in glycan-binding assays. In outcompeting glycosylated structures and occupying the CRD binding site of lectins, β-lactose displaces lectins from cells, verifying that a CRD-glycan interaction occurs, and not a protein-protein interaction [52,67,122,123,131,132]. In Figures 7 and 8, the ability of lactose to diminish lectin binding on LAG3+ cells, underscores its effectiveness in displacing lectins from glycosylated regions of LAG3. Validating the specificity of CRD-glycan interactions over protein-protein interactions, LSECtin and Galectin-3 engage with LAG3 in a glycan-dependent manner, specific to N-glycan structures. This lends support and fulfills the research gap of Objective 1, affirming the necessity of N-glycosylation of LAG3 for lectin-binding.

The use of both PNGaseF and β-lactose as independent methods to disrupt lectin-LAG3 binding, particularly at the CRD-glycan interface, strengthens this study's design and robustness of findings, implicating N-glycans as a key molecular determinant. In advancing understanding of the molecular binding characteristics, the approaches employed herein still do not fully capture the complexity and nuances of this engagement. While studies exploring lectin binding within the context of LAG3 glycosylation are nonexistent, these observations regarding Galectin-3 and LSECtin recognition of N-linked glycans align with findings documented in prior literature [52,67,122,123,131,132].

Using live cell proximity labeling, Joeh et al. mapped N-glycan-mediated Galectin-3 interactions, demonstrating that Gal-3 engages with β-galactoside containing glycoconjugates. This interaction, facilitated by the CRD domain of Galectin-3, distinctly recognizes and binds to N-acetyllactosamine (LacNAc), a β-galactoside on complex N-linked glycans [124]. Another study highlighted that Gal-3 binding protein (LGALS3BP), a hyperglycosylated protein with multiple N-linked glycosylation sites could interact with Gal-3 to mediate cell-cell adhesion in the TME, relying on LacNAc [72,73,133,134].

For LSECtin, a study probing the overall glycome of T cells assessed the contribution of N-linked glycosylation in facilitating LSECtin adhesion. These activated T cells were cultured in the presence of tunicamycin which impeded formation of N-glycosidic linkages and subsequent adhesion of LSECtin to both primary and Jurkat T cells. This study also utilized a series of LSECtin-CRD point mutants, completely abolishing the binding of soluble LSECtin-Fc to T cells, which is mediated through a protein-glycan mechanism [65]. A previous study regarding LSECtin, binds D-mannose, N-GlcNAc (N-acetyl glucosamine), and even, to a lesser extent, L-fucose in a

Ca2+-dependent manner [52,65]. Through NMR and glycan-binding arrays, LSECtin exhibits substantial affinity for highly branched N-linked glycans terminating in GlcNAcβ1-2Man, in addition to recognition of LacNAc [125,126]. Altogether, previous research collectively reveals the binding of N-glycans by LSECtin and Galectin-3, reinforcing the molecular insights elucidated in this dissertation. Clarifying the molecular basis of lectin-LAG3 binding makes this the first study to verify the occurrence of LAG3-lectin interaction through N-glycosylated residues of LAG3.

As a recognition motif or interface for cognate ligands, the demonstrated requirement of LAG3 N-glycosylation highlights the significance of post-translational modifications in glycan-mediated immune interactions, recognition and response. In directing cell-cell and cell-matrix interactions, post-translational regulation of N-glycosylation shape the functional landscape of immune checkpoint regulation, holding implications for modulating immunosuppression during health and disease [29,30,120,131,133].

For instance, aberrant N-glycosylation towards more branched N-glycans typifies tumor-infiltrating T lymphocytes and cancer cells during tumor progression and metastasis. Frequently observed in chronic inflammation and cancer, these glycome alterations, coupled with its increased expression, impact biological outcomes of the tumor-immune interface [29,30,53,54,71,120,131,133]. In such contexts where LSECtin and Galectin-3 are extruded in surplus, LAG3 may develop increased N-glycan branching, potentially enhancing the valency, binding affinity and avidity of interactions with these TME-secreted lectins, as glycan-protein interactions are often intrinsically weak. As an adapter to cross-link its binding partners, tuning N-glycosylation density can control cell-surface or ECM lattice formation with Galectin-3 or LSECtin, regulating T cell homeostasis and signal transduction [52,54,55,63,72,74,124,130]. In cases of

chronic inflammation during infection or cancer, the functional role of lectin-binding may serve to facilitate LAG3 crosslinking and colocalization at the immune synapse to orchestrate its inhibitory function and provoke T cell suppression [21,45,46]. The pleiotropic roles of lectins highlight the need to manipulate N-glycosylation profiles to empirically characterize Galectin-3 and LSECtin function and hierarchy of importance in the context of LAG3-mediated T cell exhaustion [29,30,51].

In addition to a more nuanced clarification of these molecular events, such resolution would illuminate how the N-glycan repertoire influences immune and T cell modulation. By selectively influencing lectin engagement with LAG3, this knowledge may inspire novel immunotherapeutics to fine-tune immune responses in various disease contexts [19,21,34,99].

Altogether, results presented herein illustrate the specificity of LSECtin and Galectin-3 for binding LAG3, verifying the necessity of N-glycosylation in mediating these interactions. Resolving a significant knowledge gap, this study clarifies the molecular determinants and dynamics of these engagements, in confirming LSECtin and Galectin-3 as additional ligands for LAG3. These findings expand the ligand repertoire of LAG3, enrich our appreciation of LAG3 biology in immune interactions, providing a foundation for further investigations in LAG3-mediated process and T cell exhaustion.

5.5 Lectin Impact on LAG3 Function

5.5.1 Lectin-induction Enhances LAG3 activity and T cell Suppression

Establishing the necessity of N-glycosylation for LAG3-lectin binding, its functional consequences and immunological significance in LAG3-related mechanisms and T cell suppression warrant

inquiry. Exploring how lectins modulate LAG3 to instruct immune exhaustion could refine interventions to mitigate immunological impairment, where needed [29,30,51].

Aligned with Objective 2, this thesis aimed to examine the capacity of LSECtin and Galectin-3 to induce LAG3 suppressive function and subsequently impede T cell responses. A Galectin-3 and LSECtin concentration gradient, with or without PNGaseF, was applied to both cell lines, followed by anti-CD3/CD28 stimulation for robust T cell activation. The differential inhibitory potencies of LSEC and Gal-3 in augmenting LAG3-mediated T cell suppression was evaluated by comparing the dose-dependent cytokine reduction of IL-2 and IFNγ within both LAG3+ and LAG3- cells.

In Figures 9 and 10, mirroring trends in LSECtin-treated cells, increasing exogenous Gal-3 concentrations causes dose-dependent cytokine reduction in LAG3+ cells, contrary to uniform cytokine levels in LAG3- cells irrespective of lectin dose. The maximal cytokine expression in LAG3- cells, lacking suppressive LAG3, establishes a reference point for comparison, justifying its use across conditions. The statistically significant distinction in IL-2 and IFNγ expression between LAG3+ and LAG3- cells, underscores the specific impact of lectin-mediated induction of LAG3 activity. Furthermore, this differentiation emphasizes the impact of lectins on T cell cytokine responses is contingent on the presence or absence of LAG3, illuminating T cell modulation and exhaustion during lectin-LAG3 engagement.

Concurrently for the specified aims, ELISAs measured soluble extracellular IL-2 and IFNγ. This confirmatory assay served as an additional metric for assessing T cell activation, as a proxy for LAG3 activity. ELISA results mirror the statistically significant dose-dependent cytokine declines in flow cytometry, robustly validating lectin-mediated T cell suppression within LAG3-

expressing Jurkat cells (Figures 9E, 9F, 10E and 10F). The reproducibility of these trends across modalities confirms the specificity of LSECtin and Galectin-3 for inducing LAG3 activity and highlight the strength of the study. Although flow cytometry advantageously detects cell-specific intracellular cytokine response, it lacks quantification ability, a limitation that may be concerning for clonally phenotypic cell lines. As a secondary independent assay herein, ELISAs can quantify supernatant cell-free proteins, ideal for cross-study comparisons. Cytokines function by being secreted, acting extracellularly in paracrine or autocrine fashions. By measuring extracellular cytokine response, this renders ELISA data more biologically meaningful and physiologically relevant in understanding impacts on T cell exhaustion [135,136].

As an IC, LAG3 inhibits cytokine production and activation of T cells that express it in various contexts, including chronic viral infection and cancer [21,45,137–140]. Notably, lower cytokine responses from the LAG3+ cells, lacking lectin treatment, compared to LAG3- cells signify that LAG3 inhibitory activity was functional. In the absence of MHCII ligand, Guy *et al*. illustrated ligand-independent inhibitory effects of LAG3, depressing TCR signaling responses more than LAG3- T cells. To assess LAG3 function independently, CD8+ and CD4+ T cells from $LAG3^{+/+}$ and $LAG3^{-/-}$ mice were stimulated with plate-bound CD3ε and CD28 Abs without MHC class II and antigen-presenting cells. In absence of MHCII, $LAG3^{-/-}$ CD8+ and CD4+ T cells exhibited higher proliferation and calcium flux than $LAG3^{+/+}$ counterparts. Confirmed by immunoblot analysis, confocal microscopy demonstrates that $LAG3^{-/-}$ CD4+ and CD8+ T cells express increased proximal (p-ZAP70) and distal (p-ERK, p-AKT) TCR signaling activation than $LAG3^{+/+}$ T cells at the immune synapse. These results collectively indicate that the mere presence of LAG3 functions can independently disrupt TCR signaling, irrespective of MHCII ligand [139,140].

Additionally, this group observed ligand-independent effects of signaling restoration with LAG3 blockade antibodies [139,140]. Thus, LAG3 could inhibit T cell activation through both LAG3 ligand–dependent and –independent mechanisms. Likewise, LAG3 antibodies could promote T cell activation either by blocking LAG3 ligands or in a ligand-independent manner. Most importantly, the presence of lectin, even at 25µg/mL, can lower cytokine production in a LAG3-dependent manner, compared to conditions lacking LSECtin or Gal-3 in Figures 9 and 10. Therefore, the presence of LAG3 significantly influences initial cytokine levels. However, upon binding with lectin, both Galectin-3 and LSECtin demonstrate the capacity to engage LAG3 and enhance its inhibitory function, resulting in even lower cytokine responses and subsequent T cell suppression.

These dose-dependent findings presented herein align with earlier studies examining Galectin-3 or LSECtin inhibitory action in the context of LAG3 or T cell biology, thereby reinforcing the consistency of these observed phenomena [53,54,63,65,130,141–143]. Compared to LAG3-KO CD8+ T cells which consistently produced maximal IFNγ *in vitro,* 0-150 µg/mL exogenous Gal-3 suppressed IFNγ production in LAG3-expressing CTLs. Following anti-CD3 and anti-CD28 stimulation, this study also showed that 0-25 µg/mL of Gal-3 could reduce the frequency of IFNγ-producing CD8+ T cells from LAG3+ but not LAG3-deficient mice [53]. Similarly, Xu et al. showed that LSECtin on melanoma cells could diminish IFNγ production from LAG3-expressing CD4+ and CD8 T cells after anti-CD3 stimulation. This outcome was replicated with 10µg/mL LSECtin which significantly reduced IFNγ secretion in ELISAs. Functionally, mediated by both recombinant and *in viv*o tumor-expressed LSECtin, these inhibitory effects on IFNγ responses were partially alleviated by the LAG3 blocking antibody C9B7W. This partial restoration raises the possibility that glycan-CRD interactions, not hindered by C9B7W, may persist, allowing for

continued LSECtin binding to LAG3 and ensuing T cell inhibition [54]. In dampening tumor-specific or effector T cell responses, previous literature supports the immunosuppressive role of Gal-3 and LSECtin in controlling cytokine production, specifically relying on LAG3 [53,54,63,65,72,74,130,141–143]. This reinforces the credibility of the findings presented in this dissertation.

Being heavily N-glycosylated, LAG3 possesses several glycan binding sites for lectin binding [41]. In connection with a sub-aim of Objective 1, this thesis explored how N-glycosylation impacts lectin-mediated T cell suppression and its functional role on restoring intracellular cytokine responses. With lectin concentrations yielding saturated binding, Objective 2 evaluated the functional consequences of PNGaseF N-glycan removal, in addition to reaffirming necessity of LAG3 N-glycosylation for lectin-binding [29,117,120,121]. Of note, PNGaseF deglycosylation significantly enhances IL-2 and IFNγ expression within Gal-3 and LSECtin-incubated LAG3+ cells, in contrast to 200µg/mL lectin-alone conditions that maximally suppress cytokine within LAG3+ Jurkat cells. This cytokine recovery nearly approaches levels observed in untreated (no PNGaseF) LAG3+ cells, lacking lectin ligand (Figures 9 and 10). Additionally, these results are reproduced in ELISAs. This indicates that, even in the presence of ligand, PNGaseF deglycosylation, which rids extracellular N-glycans, effectively curtails lectin-binding on LAG3+ cells. This restoration of cytokine levels mitigates lectin-driven T cell inhibition, highlighting the significance of N-glycosylation in modulating lectin-LAG3 interactions and T cell activity [120].

N-glycosylation constitutes a recognition interface for LAG3-expressing cells to communicate and engage with binding partners in their environment. As a crucial aspect of LAG3 biology, post-translational glycosylation influences LAG3 function through lectin ligation, fine-tuning T cell signaling and immunomodulatory properties for glycan-mediated immune interactions, recognition and response [29,30]. For example, removal of N-linked glycan expression with

PNGaseF, even impairs the suppressive capabilities of splenic T reg cells [120]. With lectins having pleiotropic effects, PNGaseF utilization advantageously enabled empirical characterization and appreciation of involvement and impact of N-glycosylation and lectin-binding in LAG3 biology, potentially refining LAG3-immunoregulatory circuits, as outlined in the study objectives [29,30,117,120,121].

As stated, LSECtin and Galectin-3 bind highly branched N-glycans on glycoproteins and extracellular matrix proteins with their CRDs. Their oligomerization domains enable them to further cross-link its binding targets [30,52,53,55,74,121,130,143]. In fact, multivalent molecular interactions between T cell surface N-glycans and Gal-3, disrupt TCR clustering, thereby raising the agonist threshold for TCR signaling and regulating T cell activation [72,73,75,118,121]. In engaging with and remodeling components such as ECM glycoproteins, for cell-matrix interactions in cell migration, these lectins promote cancer progression and metastasis [72]. Similarly, LAG3's substantial N-glycosylation may facilitate cell-surface or ECM lattice formation with Galectin-3 or LSECtin [130]. Such a lattice could amplify LAG3 crosslinking and stable colocalization with the TCR at the immune synapse, to restrain T cell activation. As noted in Section 5.4.2, such glycome alterations influence IC and T cell regulation and biology, thereby modulating immune suppression at the disease-immune interface. Further unraveling cellular glycomes could reveal novel immune regulatory circuits, broadening our knowledge of immune regulation. The abundance and diverse roles of lectins in infection or cancer, emphasizes the need to clarify their hierarchy of importance during LAG3-mediated T cell exhaustion to better inform targeted immunotherapeutic strategies [29,30,73,130]. This expands the spectrum of interactions that shape LAG3 function and regulation, illustrating their complexity in LAG3 biology and immune modulation as potential therapeutic targets [21,34,45,46,97].

5.5.2 Relative Lectin-mediated T cell Inhibition

From the generated lectin-cytokine gradients with flow cytometry in Figures 9 and 10, the determination of IC50 values quantifies the relative inhibitory strength of each lectin in suppressing cytokine response. Exhibiting lower IC50 values for IL-2 and IFNγ compared to that of Gal-3, LSECtin demonstrates greater potency in curtailing cytokine production. Coinciding with these results, the lower EC50 values for LSEC compared to Gal-3 signify the higher binding affinity of LSECtin for LAG3, highlighting the relative strength and specificity of lectin-LAG3 interactions in section 5.4.1. Despite the lack of binding site analysis and comparative studies surveying both lectins and their impacts, these dose curves collectively support that LSECtin augments LAG3 inhibitory activity more effectively than Gal-3. This illustrates the specificity and differential potency by which LSECtin and Gal-3 restrict cytokine production in a LAG3-dependent manner.

Interestingly, a recent study failed to detect a specific interaction between LAG3 and LSECtin or Galectin-3 nor for the role of these lectins in LAG3-mediated T cell inhibition [140]. This study used LAG3-expressing Jurkat reporter T cells, whereby membrane-bound anti-CD3 antibody on stimulator K562S cells, from human K562's, activates LAG3+ reporter cells. Analyzing the disparity in findings reveals that LSECtin–expressing K562S cells strongly bound various fusion proteins much stronger than soluble fusion LAG3-Ig in this study. This outcome is likely due to low expression of LSECtin on stimulator K562S cells and low concentration (3-30μg/mL) of LAG3-Ig fusion protein used, causing a low signal-to-noise ratio on both sides that limits pronounced LSECtin-LAG3 binding. This might lead to preferential lectin interaction with the heavily glycosylated Fc portion of other Ig fusion proteins, potentially overshadowing specificity for binding the LAG3-Ig fusion protein. Likewise, LSECtin-expressing K562S cells marginally

increased cell conjugate formation independent of LAG3 presence. Contrasting findings in this thesis, LSECtin-expressing stimulator K562S cells did not elicit an inhibitory signal in LAG3+ reporter cells. This discrepancy could be due to the overpowering activating signal of stimulator cells possibly overriding any LSECtin-induced inhibitory signal, particularly if it is low to moderately expressed on K562S stimulator cells. As well, LAG3+ reporter cells incubated with K562S and soluble Galectin-3 did not bind nor inhibit LAG3+ reporter cell activation. With the experimental setup of few LAG3+ reporter cells and low concentration of soluble Gal-3 used (1 µg/mL), at 4°C, Gal-3 may prefer binding K562S stimulator cells than LAG3+ cells. This contributes to the lack of Gal-3 interaction with LAG3. Thorough assessing data and experimental conditions employed in this study suggests that both Galectin-3 and LSECtin interact with cell-surface glycosylated proteins generically but do not act as specific binding partners for LAG3, regardless of LAG3+reporter cell expression [140].

In the prolonged inflammatory environments of chronic infection or the TME, the surplus extrusion of LSECtin and Galectin-3, along with their distinct impacts on restraining T cell activation, emphasizes the nuanced regulatory roles of these lectins in instructing immunity [30]. In possessing multiple ligands, some of which bind non-redundantly, may enable LAG3 to modulate the immune response in varied ways, in addition to LAG3-independent mechanisms. Along with the immunomodulatory properties of other LAG3 ligands, resolving this hierarchy in lectin-driven immunosuppression during diseases pathogenesis is crucial to discern if some ligands are more important than others for LAG3 functionality [54,72,131]. Evidently, this outcome depends on the relative abundance or concentration of these receptor-ligand binding partners *in situ*, critically influencing effector T cell activity and the functional sequelae of these interactions [28].

Despite LSECtin's higher affinity, the greater abundance and potentially superior multimerization capacity of Galectin-3 might surpass LSECtin for binding LAG3 in the TME or infection milieu, possibly making it a prominent ligand for LAG3 in those settings. The presence of both soluble and membrane-bound ligands introduces an additional layer of complexity and control. Moreover, Maruhashi *et al*. demonstrated FGL1 bound activated T cells from LAG3-deficient mice, implying FGL1 might exert inhibitory effects, independently of LAG3 [45]. This LAG3-independent observation could also apply for LSECtin and Galectin-3. Much work is required in elucidating whether these LAG3 ligands function independently or exhibit additive or synergistic effects in LAG3 inhibitory function for entry into the immune synapse. Additionally, it is important to delineate if certain LAG3–ligand interactions are disease-specific and therapeutically targetable [29,30,73,130]. These questions justify inhibiting lectin-LAG3 engagements, alongside existing immunotherapy, to restore T cell responses, mitigate immune exhaustion and improve patient outcomes, where necessary [97].

Despite clinical successes of LAG3 immunotherapy, numerous aspects of LAG3 function remain enigmatic and unresolved, hampering further studies in addition to the limited data on LAG3-blockade enhancing HIV-specific T cell responses [18,19,84]. As an IC that curtails immunopathology or autoreactivity, LAG3 inhibits cytokine production and activation of [exhausted] T cells that express it in chronic viral infection and cancer [21,45,137–140]. Earlier investigations specifically revealed LAG3 impedes expansion and infiltration of both $CD4^+$ and $CD8^+$ T cells [33]. LAG3 is exploited, like other ICs, by tumors to disrupt antitumor T-cell immune responses, rendering them hypofunctional [97–99].

Indeed, engagement by its immunosuppressive ligands, often expressed by viral-infected cells, tumor cells or the inflammatory milieu, dampens levels T cell function, furthering immune dysregulation and exhaustion. As stated, Gal-3 and LSECtin have both inhibited helper T cell and CTL IFNγ responses in a LAG3-dependent manner *in vitro* and *in vivo*, promoting tumor-driven immunosuppression [38,39,54,74]. Interestingly, tumor-secreted Gal-3 limits IFNγ permeation, subsequently lowering chemokine-dependent CTL tumor infiltration [71,75]. Tumor-derived LSECtin inhibited tumor-specific T cell proliferation and IFNγ secretion, dampening antitumor immunity *in vitro* and *in vivo* [54]. Through binding LAG3, this LSECtin-mediated immunosuppression enables tumor immune escape, promoting tumor growth and progression [63]. Similar to acute viral infection *in vivo*, LSECtin also suppresses hepatic T cell immunity in HBV, significantly decreasing proliferation and secretion of IFNγ and IL-2 in primary T cells [54,64,65]. Hence, as part of LAG3-mediated functions, this imbalanced or decreased IL-2 and IFNγ production, key T cell growth factors, attenuates Th effector responses and cytotoxic activities within cell-mediated immunity [42,53,54].

The limited IL-2 and IFNγ availability aggravates the exhausted T cell phenotype, hindering its capacity to eliminate tumor or viral-infected cells and necessitating heightened signal strength for full T cell activation and effective effector responses [13,14,23,80,144]. The severity of T cell exhaustion correlates with heightened HIV viral load and diminished CD4 T cell help in HIV-positive individuals. In this environment, increased Treg activity along with other regulatory cell subsets and anti-inflammatory cytokines depress Th1-dominant responses, skewing subset-specific responses of Th2 humoral immunity [13,14,144,145]. Relying on proinflammatory cytokines, such as IL-2, IFNγ and TNFα, Th1 responses initiate cell-mediated immunity, activate macrophages, and stimulate local CTL activation, a mainstay for eradicating tumor and HIV-

infected cells. Accordingly, lectin-driven IL-2 and IFNγ deprivation may shift the bias towards Th2 Ab-mediated responses, that counter extracellular parasites, suboptimal for antiviral or antitumor immunity. The potential for more immune exhaustion induced by lectins, which reduce IFNγ and IL-2 availability for cells and Th1 responses, hinders a desired, robust and sustained immune response, which could prolong disease severity within patients [146–148].

Thus, lectins not only instruct LAG3-mediated immunosuppression, but also engender an additional mechanism of viral or tumor immune escape [73,131,142]. By augmenting LAG3 inhibitory activity on T cells, lectin interaction may help shift the balance towards an immunosuppressive microenvironment, broadening LAG3's immune regulatory impacts. Moreover, tumor cells or viral-infected cells can proliferate unfettered by overwhelming and evading hypofunctional immune control, which is incrementally quelled with cancer or AIDS progression [12,54,61]. Revealing further mechanisms underlying engagement of LAG3 function enriches knowledge of immune checkpoint biology and T cell exhaustion in health and disease. These insights support disrupting lectin-LAG3 interactions to alleviate immune exhaustion.

In this way, Galectin-3 and LSECtin could be additional targets for adjuvant immunotherapy, as part of combinatorial immunotherapeutic approaches alongside LAG3 antibody blockades. Targeting multiple inhibitory pathways simultaneously could augment the efficacy of existing LAG3 immunotherapy, fine-tuning aberrant immune responses where needed and improving patient outcomes [20,30,34,53,97].

As confirmed ligands of LAG3, detailed herein, these lectins expand the scope of LAG3 biology and T cell exhaustion, in intricately modulating immune activation and suppression in various contexts. Beyond the specificity and necessity of N-glycosylation for lectin binding to LAG3 in

Objective 1, this thesis also confirms, that upon engagement, Galectin-3 and LSECtin induce LAG3 function and subsequent T cell suppression, albeit at different inhibitory strengths, as per Objective 2. This reinforces the importance of post-translational N-glycosylation in coordinating lectin-LAG3 interactions, as additional layer that regulates LAG3-driven processes. As summarized in Figure 11 below, this dissertation delineates the mechanisms by which N-linked glycans and lectins instruct LAG3-mediated T cell suppression, ultimately contributing to induction of T cell exhaustion during disease and fulfilling a critical research gap in LAG3 literature.

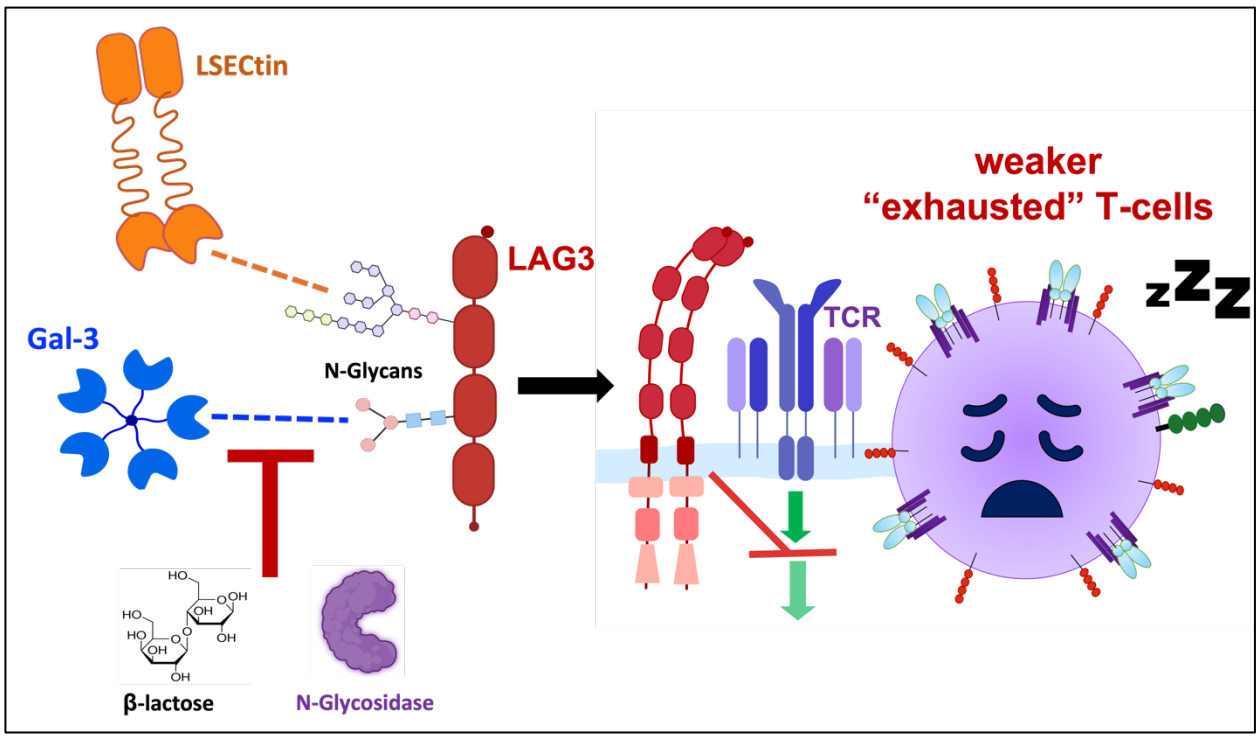


Figure 11. Role of Lectins in LAG3 Function and T cell Activity. Lectins, LSECtin and Galectin-3 specifically bind N-glycosylated residues of LAG3, triggering LAG3 inhibitory function and augment T cell suppression and immune exhaustion.

5.6 Strengths and Limitations

With the employed experimental design and methodologies, this section emphasizes the study's strengths, highlighting its methodological robustness and innovation in its design. Simultaneously it acknowledges and addresses inherent limitations that impact the interpretation of the obtained results, for a comprehensive evaluation.

Jurkat T cell line as LAG3 Model:

Overcoming the scarcity of reliable LAG3 models that hindered LAG3 research, this dissertation generated and utilized a robust LAG3 cell line model. While an ideal model for studying LAG3 inhibitory mechanisms would use primary human cells, their low baseline LAG3 expression, rapid LAG3 downregulation post-activation and substantial biological variability limits their utility. These factors result in fewer LAG3-expressing cells or a lower signal-to-noise ratio, inconducive for detecting and evaluating a pronounced effect by LAG3 in primary T cells. Although imperfect, the ease of activation and extensive use of Jurkat CD4 T cells in elucidating TCR signaling, a process implicated in the LAG3 mechanism, makes them a suitable model system to assess LAG3 inhibitory function on TCR signaling [10]. Lentiviral transduction of Jurkat cells was chosen to stably express LAG3 allowing indefinite use of cell lines, ensuring replicability, minimal experimental variability and a consistent standardized system for studying the LAG3 mechanism. By ensuring LAG3 expression as the sole differing variable between the LAG3+ and LAG3- cell lines, this fundamental aspect of the model's design enables confident assessments of LAG3's specific impact on the outlined study endpoints and subsequent experiments, underscoring its suitability. With the high LAG3 expression, this high signal-to-noise ratio favorably yielded statistically significant differences in dose-dependent trends between LAG3+ and LAG3- cells regarding lectin-binding (Objective 1) and lectin-mediated T cell

suppression (Objective 2), enhancing the biological relevance and credibility of findings. Hence, the presence of LAG3, or lack thereof evidently shapes lectin binding dynamics, competitive inhibition and T cell activity. The developed model is both valid and versatile for investigating and broadening LAG3's scope in T cell biology and immune exhaustion. Despite exhibiting T cell-like characteristics, Jurkat cells, lack the full biological diversity of primary T cell subsets. This simplified *in vitro* system may not fully capture the complexity of physiological conditions and T cell responses *in vivo*, limiting the ability to encapsulate the interplay between immune cells and their tumor or infection microenvironment. Thus, relevance of findings to *in vivo* scenarios and physiological contexts should be cautiously extrapolated.

Sample Size:

A prominent limitation concerns the low number of independent technical replicates for comparing endpoints between LAG3+ and LAG3- cells. While suitable for non-parametric data, this small sample size limits the reliability of the Mann Whitney U-test, compromising robust interpretation of statistical significance for results obtained in Objectives 1 and 2. The acknowledged limitation of a single independent iteration for lactose competition assays further accentuates this constraint. Hence, subsequent replicates beyond n=3 in all assays would better characterize the nature of lectin binding, competitive inhibition and lectin-mediated inhibition, broadening the applicability of study findings towards LAG3 biology.

Method of TCR Stimulation:

As LAG3 acts through the TCR pathway, Jurkat T cells were activated in a TCR-dependent manner before evaluating lectin-mediated LAG3 activity in Objective 2. Using the Raji B cell line with superantigens like SED or SEB, mimics peptide stimulation through MHCII-TCR cross-linking

to robustly activate Jurkat and human T cells. This well-characterized TCR-based method engages relevant co-receptors through respective ligands present on Raji B cells, including LAG3 (MHCII), CD4 (MHCII) and CD28 (CD80/CD86) [19,21,32,36]. However, aligning with the dissertation's objective to explore alternative LAG3 ligands, conducting functional assays in the presence of MHCII, a prominent LAG3 ligand, would confound this objective. To avoid this confounding effect of an MHCII-based stimulant, TCR-based stimulation using soluble anti-CD3 (primary signal) and anti-CD28 (co-stimulation) antibody was chosen instead. By preventing T cell anergy, anti-CD28 stimulus effectively activates T cells but does not engage other co-receptors beyond CD28. Consequently, it may not activate cells as strongly nor use the same signaling pathways as typical antigen stimulation during an immune response [19,21,32,36]. However, this soluble stimulation can a large fraction of T cells simultaneously, inducing sufficient cytokine production to explore inhibitory function of LAG3 and its alternative ligands.

Cytokine Detection by Flow Cytometry and ELISA:

As independent complimentary methods, flow cytometry and ELISAs substantiate each other in verifying lectin-mediated T cell and cytokine suppression, as a proxy for LAG3 inhibitory activity. This cross-verification across modalities highlights the study's strength, reproducibility of findings and potential translatability into therapeutic strategies. Specifically, flow cytometry is attractive for detecting cell-specific intracellular cytokines. The visual representation by flow plots enables observation of general population trends in the sample condition. This enhances the clarity and interpretation of cell-specific results [149]. Besides lacking quantification ability, the expense of flow cytometry contributed to low sample size numbers, and requires larger sample volumes compared to ELISA, presenting a limitation [135,149]. Ideal for cross-study comparisons, ELISAs quantify cell-free soluble extracellular cytokine, allowing monitoring of

cumulative cytokine production throughout the assay. By measuring secreted extracellular cytokine, this renders ELISA data more biologically and physiologically relevant in understanding the impact of cytokine function on T cell suppression [135,149].

N-glycan Interaction with β-lactose and PNGaseF:

In addition to using PNGaseF N-glycosidase, β-lactose competition was employed to disrupt lectin-LAG3 binding, advantageously offering two distinct methods to validate the evidence of N-glycan interaction with the tested lectins. PNGaseF N-glycosidase provides a sound platform for global N-glycan removal from LAG3. By discerning the presence or absence of N-glycans on LAG3 with PHA-L binding, validating the N-glycosidase efficacy was crucial in achieving clear assessments of lectin binding [29,117,120,121]. Considering the pleiotropic effects of lectins, this valuable tool empirically characterized the importance and impact of N-linked glycosylation on lectin-LAG3 interactions and LAG3-mediated processes in T cell immunosuppression as per the study aims [29,30,117,120,121]. The competitive inhibitor β-lactose provides an additional controlled method to modulate lectin-LAG3 ligation, allowing for the dissection of its specific molecular determinants. In resolving relative binding affinities of lectin-LAG3 interaction, β-lactose disruption of CRD-glycan interaction proved LSECtin and Galectin-3 specifically bind LAG3 in a glycan-dependent manner, supporting earlier results. The use of both PNGaseF and β-lactose as independent methods to disrupt lectin-LAG3 binding at the CRD-glycan interface, strengthens this study's design and robustness of findings regarding the requirement of LAG3 glycosylation. Addressing a previously unresolved gap in LAG3 literature, this will be the first study to verify that N-glycans are a key molecular determinant in lectin-LAG3 interactions, subsequently augmenting LAG3 inhibitory activity [20,53,54].

5.7 Prospects for Future Research

From insights and limitations addressed in this study, prospects for future research can probe unexplored avenues, addressing knowledge gaps by refining methodologies and pursuing innovative approaches. This would enhance the precision and scope of investigations further unraveling the intricate dynamics of lectin-LAG3 interactions during T cell immunosuppression. Despite clinical successes of LAG3 immunotherapy, various aspects of LAG3 function remain unresolved, impeding further research into its mechanism and ligand-binding partners [18,19,84].

Alternative Measures of T cell Function: As an IC curtailing immunopathology, LAG3 suppresses responses of [exhausted] $CD4^+$ and $CD8^+$ T cells expressing it during chronic viral infection and cancer [54]. Thus, it is worthwhile to explore alternative endpoints related to T cell functionality such as signaling, activation, expansion and infiltration, both *in vitro* and *in vivo* [33]. In addition to repeating previous *in vitro* experiments with larger sample size to strengthen validity of results, the effect of LAG3 on T cell proliferation can be assessed with dilution of CFSE-stained cells upon each cell division within assays, for instance.

Ligand-specific Signaling Perturbations: Additionally, it is unclear whether different LAG3 ligand affect different signaling pathways via differential engagement of LAG3. Previous findings of LAG3 inhibiting calcium flux, NFAT, Th1 cytokines, especially at proximal TCR signaling events, suggest a broader impact on signal transduction by inhibiting other pathways, beyond TCR [21,36,46]. Employing kinomics provides insight into how LAG3 and its ligands may disrupt different branches of the TCR signaling pathway by assessing kinase activity.

Quantification of Relative LAG3-Lectin Affinities and Binding Dynamics: Despite LSECtin's higher affinity, the abundance of Galectin-3 *in vivo* along with its potentially superior multimerization capacity could outcompete LSECtin for binding LAG3 if both are present

simultaneously, influencing associated functional consequences. Warranting further resolution, future investigations may encompass competitive inhibition assays between LSECtin and Galectin-3 to determine the hierarchy of these ligands in engaging cell-expressed LAG3 *in vitro*. Approaches such as surface plasmon resonance (SPR) or bio-layer interferometry (BLI) could accurately measure binding affinity (K_a) between the two lectins and LAG3 [29,54,130,150]. Considering the contribution of avidity, representing the overall strength of multivalent interactions, these approaches can also quantitatively measure how monomeric or multimeric lectin forms informs the affinity to LAG3. This knowledge broadens comprehension of immune regulation for further exploration of lectin-mediated immune responses and IC modulation.

Western Blot Validation of PNGaseF and Point Mutants: Subsequent work includes verifying PNGaseF activity by subjecting PNGase-F treated soluble LAG3 to western blot analysis. Due to the removal of N-glycan moieties with PNGaseF treatment, deglycosylated LAG3 would exhibit a smaller molecular weight than glycosylated LAG3 with its N-glycans intact. By assessing shifts in molecular weight following deglycosylation, it becomes possible to identify the different deglycosylated LAG3 glycoforms, thereby enhancing confidence in the presented findings [41]. To elucidate and confirm the specific N-glycosylation sites on LAG3, future investigations will involve conducting western blot verification, alongside studies assays presented herein, with constructed LAG3 point mutants at its N-glycosylation sites. These consensus sites are defined by Asn-X-Thr/Ser sequences, where X represents any amino acid except for proline [21,41,117,119,130]. With the hopes of identifying and blocking the CRD-glycan mechanism during lectin-LAG3 ligation and ensuing T cell suppression, LSECtin or Galectin-3 CRD point mutants can identify residues essential for N-glycan recognition [55,65,130].

LAG3 Function Across Primary Immune Cells: With limited biological representation of the various T cell subsets, LAG3-expressing Jurkat cells, a specific CD4+ T cell line, cannot recapitulate the diverse responses of primary T cells, especially when studying specific aspects of immune modulation. As LAG3 is expressed on other immune cells, future work should investigate how LAG3 mediates its inhibitory signal across different immune cell types and its significance in various disease contexts, especially chronic HIV infection, which remain inadequately defined [21,97]. To address this gap, future work may involve characterizing LAG3 expression and activity on primary T cells and other cell types from cancer or chronically-infected patients. This is particularly valuable for LAG3 blockade having greater therapeutic utility, when LAG3 is elevated on T cells in such disease contexts [20,34]. Other pertinent questions that concern LAG3 glycosylation and lectin binding for future research include: What is the extent of and specific glycosylation patterns that exist on LAG3 across different immune cell types and how do they affect LAG3 function? What is the significance of these lectin interactions with glycosylated LAG3 in immune modulation? Beyond conventional CD4+ and CD8+ T cells, differential LSECtin and Galectin-3 binding may emerge among various T cell subsets, expressing LAG3, according to their stages of differentiation [53].

LAG3 *In vivo* Studies: While this thesis demonstrates the differential potency of LSECtin and Gal-3 in instructing LAG3-mediated T cell suppression *in vitro*, it does not fully recapitulate the complexity of *in vivo* conditions. The lack of comparative studies surveying both lectins and their impacts warrant animal studies with transgenic and LAG3-/- mice, frequently used in LAG3 research[151–155]. In exploring how LSECtin and Gal-3 shape LAG3 function and regulation during cancer and HIV infection, it is crucial to clarify their hierarchy of importance in LAG3-mediated immune exhaustion and functional sequelae in the TME or infection milieu *in vivo* [29,54,72,130,131].

Similar to acute viral infection *in vivo*, both lectins have shown LAG3-dependent inhibition of effector responses, proliferation and infiltration of CD4+ and CD8+ T cells *in vitro* and *in vivo*, promoting disease-driven immunosuppression [53,54,63,65,72,74,130,141–143]. Further *in vivo* work should examine if lectin-LAG3 driven cytokine deprivation aggravates T cell exhaustion, skews Th1 responses towards Th2 humoral immunity and alters T cell elimination of tumor or viral-infected cells by engendering viral or tumor immune escape [13,14,144,146–148]. These outcomes may broaden LAG3's immune regulatory impacts, dampening disease-specific immunity [13,54,97].

Incorporating LAG3 blockades and Lectin Inhibitors: With limited data on LAG3-blockade in enhancing HIV-specific T cell responses, one must delineate if certain LAG3–ligand interactions are therapeutically targetable with LAG3 antibody blockades and lectin inhibitors to reinvigorate host immunity [18,19,29,51,72,84,131,150]. The individual clinical successes of lectin inhibitors and LAG3 blockades approved in cancer immunotherapy spurs questions about their application in chronic HIV infection. Altogether, this would better inform combinatorial immunotherapeutic approaches to alleviate immune exhaustion and reduce disease severity for better patient outcomes in cancer and chronic HIV infection [18,97,109,131,146–148].

5.8 Significance

This addressed the intricate mechanisms of T cell suppression mediated by LAG3 and lectin binding, informing critical aspects of immune regulation. Addressing a significant knowledge gap, I informed the mechanism by which lectin-ligands, Galectin-3 and LSECtin, bind LAG3, determining the necessity of N-glycosylation in this interaction. As recognition molecules, this study deciphered that LAG3's N-glycans guide and enable this interaction whereby, LSECtin and Galectin-3 exhibit specificity for binding glycosylated residues on LAG3. Hence, this study

enriches understanding of how such engagements with its lectin partners potentially facilitate immune interactions and signaling, directing IC function within immune responses. Moreover, the validated Jurkat model, used for studying LAG3 activity and its mechanisms herein, provides a foundation for further investigations into molecular dynamics and processes behind T cell inhibition by LAG3.

Ligand-binding by lectins may be a previously underappreciated mechanism that instructs immunosuppressive responses by governing LAG3 activity and T cell inhibition. Upon binding, this study confirms these lectins engage LAG3 activity, subsequently inducing LAG3-mediated T cell suppression, resulting in dose-dependent decline of cytokine production. In elucidating the interaction and contribution of these immunologically-relevant ligands, the study uncovered additional mechanisms of LAG3 engagement and its functional significance, broadening the scope of LAG3 biology and T cell exhaustion. By unveiling these previously underexplored immunosuppressive processes, this research can illuminate the intricate interplay underlying immune exhaustion and disease progression during cancer or chronic infection.

Beyond the academic contexts, the impact of this research extends clinically for designing more targeted and effective interventions. Understanding the role of lectin interaction in regulating LAG3 activity can optimize and inform the efficacy of a lectin or LAG3 blockade in relieving undesired T cell suppression, alongside current immunotherapies in such immune-related diseases. By reversing cellular exhaustion, I can reinvigorate host immunity and amplify both anti-tumor and anti-viral immune responses to better combat disease progression. Deciphering the mechanics of these “LAG3 brakes” and its binding partners, helps us in augmenting current

clinical and cure strategies, and ultimately, alleviate the burden of cancer and HIV/AIDS, globally.

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6 Appendices

6.1 Plasmid Sequences of LAG3 ORF for LAG3+ Jurkat T cells

ATGTGGGAGGCTCAGTTCCTGGGCTTGCTGTTTCTGCAGCCGCTTTGGGTGGCTCCAGTGAAGCCTCTCC
AGCCAGGGGCTGAGGTCCCGGTGGTGTGGGCCCAGGAGGGGGCTCCTGCCCAGCTCCCCTGCAGCCCCAC
AATCCCCCTCCAGGATCTCAGCCTTCTGCGAAGAGCAGGGGTCACTTGGCAGCATCAGCCAGACAGTGGC
CCGCCCGCTGCCGCCCCCGGCCATCCCCTGGCCCCCGGCCCTCACCCGGCGGCGCCCTCCTCCTGGGGGC
CCAGGCCCCGCCGCTACACGGTGCTGAGCGTGGGTCCCGGAGGCCTGCGCAGCGGGAGGCTGCCCCTGCA
GCCCCGCGTCCAGCTGGATGAGCGCGGCCGGCAGCGCGGGGACTTCTCGCTATGGCTGCGCCCAGCCCGG
CGCGCGGACGCCGGCGAGTACCGCGCCGCGGTGCACCTCAGGGACCGCGCCCTCTCCTGCCGCCTCCGTC
TGCGCCTGGGCCAGGCCTCGATGACTGCCAGCCCCCCAGGATCTCTCAGAGCCTCCGACTGGGTCATTTT
GAACTGCTCCTTCAGCCGCCCTGACCGCCCAGCCTCTGTGCATTGGTTCCGGAACCGGGGCCAGGGCCGA
GTCCCTGTCCGGGAGTCCCCCCATCACCACTTAGCGGAAAGCTTCCTCTTCCTGCCCCAAGTCAGCCCCA
TGGACTCTGGGCCCTGGGGCTGCATCCTCACCTACAGAGATGGCTTCAACGTCTCCATCATGTATAACCT
CACTGTTCTGGGTCTGGAGCCCCCAACTCCCTTGACAGTGTACGCTGGAGCAGGTTCCAGGGTGGGGCTG
CCCTGCCGCCTGCCTGCTGGTGTGGGGACCCGGTCTTTCCTCACTGCCAAGTGGACTCCTCCTGGGGGAG
GCCCTGACCTCCTGGTGACTGGAGACAATGGCGACTTTACCCTTCGACTAGAGGATGTGAGCCAGGCCCA
GGCTGGGACCTACACCTGCCATATCCATCTGCAGGAACAGCAGCTCAATGCCACTGTCACATTGGCAATC
ATCACAGTGACTCCCAAATCCTTTGGGTCACCTGGATCCCTGGGGAAGCTGCTTTGTGAGGTGACTCCAG
TATCTGGACAAGAACGCTTTGTGTGGAGCTCTCTGGACACCCCATCCCAGAGGAGTTTCTCAGGACCTTG
GCTGGAGGCACAGGAGGCCCAGCTCCTTTCCCAGCCTTGGCAATGCCAGCTGTACCAGGGGGAGAGGCTT
CTTGGAGCAGCAGTGTACTTCACAGAGCTGTCTAGCCCAGGTGCCCAACGCTCTGGGAGAGCCCCAGGTG
CCCTCCCAGCAGGCCACCTCCTGCTGTTTCTCATCCTTGGTGTCCTTTCTCTGCTCCTTTTGGTGACTGG
AGCCTTTGGCTTTCACCTTTGGAGAAGACAGTGGCGACCAAGACGATTTTCTGCCTTAGAGCAAGGGATT
CACCCTCCGCAGGCTCAGAGCAAGATAGAGGAGCTGGAGCAAGAACCGGAGCCGGAGCCGGAGCCGGAAC
CGGAGCCCGAGCCCGAGCCCGAGCCGGAGCAGCTC