

LAG3 expression profile and the mechanisms regulating
LAG3 expression and function

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Abstract

During persistent activation of immune cells, immune checkpoints are upregulated and inhibit the cell function. While potentially desirable for autoimmunity and excessive immune activation, immune checkpoints are harmful during cancer and potentially harmful during infection. Immune checkpoint inhibitors have revolutionized the treatment of several forms of cancer. Lymphocyte activation gene-3 (LAG3) is among the most promising immune checkpoints for targeting in cancer due to its efficacy and selective expression. However, much about LAG3 remains unknown. The goal of this thesis was to provide a greater understanding of LAG3 expression and mechanism of action. Because LAG3 research is typically focused on T cells, LAG3 is consequently often ignored on other cells. Also, the LAG3 mechanism of action is mostly uncharacterized due in part to the lack of conserved inhibitory motifs and a consistent model of study.

This thesis found that a high frequency of monocytes express LAG3. This expression was confirmed by two separate and frequently used antibodies for LAG3 detection. However, these antibodies detected significantly different fractions of cells expressing LAG3. These antibodies were then tested on LAG3-negative or LAG3-positive Jurkat cells where it was shown that the polyclonal antibody bound non-specifically and with less sensitivity than the monoclonal antibody.

Next, LAG3 expression on T cells was shown to be downregulated when cells were activated in the presence of toll-like receptor (TLR) agonists. Suggesting a mechanism of downregulating LAG3 when an infection is active.

To study the LAG3 mechanism, a model of LAG3 activity was created using transduced Jurkat cells. This model reflected LAG3-mediated inhibition of IL-2 after activation with superantigen. This model was used to identify MAPK pathway as a branch of the TCR signaling pathway influenced by LAG3 expression.

This study sheds light on LAG3 expression and mechanisms of LAG3 expression and function, helping to inform further research into LAG3.

Dedication

I dedicate this thesis to my sons. I am beyond excited to watch you grow up to be the kind, strong men I know you will become. Humanity will know much more by then than now, so this thesis will be out of date. Therefore, I recommend you stop reading this now and use that time instead to call your parents with your augmented reality brain interface. We love you both, unconditionally.

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Table of Contents

Table of Contents

Abstract.....	ii
Dedication.....	iv
Acknowledgements.....	iv
Table of Contents.....	vi
List of Tables	x
List of Figures	xi
List of Acronyms	xii
Overview:.....	1
1 Introduction	2
1.1 Introduction to mammalian immunology	2
1.1.1 Innate immunity	2
1.2 Adaptive immunity.....	4
1.2.1 T cell immunity.....	5
1.2.2 T cell exhaustion.....	10
1.3 Immune checkpoints	12
1.4 LAG3.....	13
1.4.1 LAG3 structure.....	13
1.4.2 LAG3 expression	14
1.4.3 LAG3 function	16
1.4.4 LAG3 mechanism of action	18
1.4.5 LAG3's role during disease	25
1.5 Rationale, hypotheses and objectives	32
1.5.1 Rationale	32
1.5.2 Objectives.....	34
2 Materials and Methods.....	35
2.1 General reagents.....	35
2.1.1 Solutions.....	35
2.1.2 Plasmids.....	35
2.1.3 Antibodies and dyes.....	36
2.1.4 Toxins and stimulants	37
2.2 Biological samples.....	38
2.2.1 Ethics.....	38
2.2.2 Source of peripheral blood	38
2.2.3 Processing of peripheral blood.....	38
2.2.4 Cell lines	39
2.3 General methods.....	39
2.3.1 Flow cytometry.....	39
2.4 Surface expression of LAG3 on cell subsets	42

2.4.1	Flow cytometry optimization	42
2.4.2	Analysis	43
2.5	LAG3 expression kinetics	43
2.5.1	Activation with anti-CD3/anti-CD28 beads	43
2.6	Proliferation and cytokine detection	44
2.6.1	Proliferation measurement	44
2.6.2	Cytokine and LAG3 expression measurement	44
2.7	Creation of LAG3 ⁺ cell lines.....	45
2.7.1	Lentiviral production.....	45
2.7.2	Lentiviral transduction	45
2.8	LAG3 function on cell lines	46
2.8.1	Activation for IL-2 detection.....	46
2.8.2	IL-2 ELISA	46
2.8.3	Activation for kinomics array	47
2.8.4	Kinomics array	47
2.8.5	Phospho-flow cytometry.....	48
2.9	Statistics.....	49
3	Thesis-related publications	50
3.1	Peer reviewed articles	50
3.2	Poster presentations	50
4	<i>Ex vivo</i> surface expression of LAG3.....	54
4.1	Rationale.....	54
4.2	Hypotheses	54
4.3	Objectives	54
4.4	Results	55
4.4.1	LAG3 across cell subsets.....	55
4.4.2	Monoclonal and polyclonal antibody discrepancies	63
4.5	Summary.....	69
4.6	Discussion.....	69
5	Regulation of LAG3 expression on T cells	71
5.1	Rationale.....	71
5.2	Hypotheses	71
5.3	Objectives	71
5.4	Results	72
5.4.1	LAG3 expression kinetics on primary T cells.....	72
5.4.2	Impact of TLR activation on LAG3 expression	75
5.5	Summary.....	77
5.6	Discussion.....	77
6	LAG3 and primary T cell activation.....	79
6.1	Rationale.....	79
6.2	Hypothesis	79

6.3	Objectives	80
6.4	Results	80
	6.4.1 Cytokines	80
	6.4.2 Proliferation.....	80
6.5	Summary.....	88
6.6	Discussion.....	88
7	Impact of LAG3 on T cell receptor signal transduction.....	90
7.1	Rationale.....	90
7.2	Hypothesis	91
7.3	Objectives	91
7.4	Results	92
	7.4.1 Creation of cell line model.....	92
	7.4.2 Characterization of cell line model	94
	7.4.3 Role of cytoplasmic domain on LAG3 expression in Jurkat cell line	94
	7.4.4 IL-2 production after activation with SEE and SED.....	97
	7.4.5 Impact of LAG3 on signal transduction.....	99
7.5	Summary.....	106
7.6	Discussion.....	107
8	Discussion.....	110
8.1	LAG3 expression across cell types.....	112
	8.1.1 LAG3 expression varies, with monocytes exhibiting highest levels	112
	8.1.2 Discrepancies between LAG3 antibodies	114
	8.1.3 Summary	116
	8.1.4 Limitations and opportunities for further study	117
8.2	Regulation of LAG3 expression	119
	8.2.1 LAG3 expression kinetics and regulation by TLR activation	119
	8.2.2 Summary	121
	8.2.3 Limitations and further research opportunities.....	122
8.3	LAG3 in primary T cell activation	123
	8.3.1 Summary	125
	8.3.2 Limitations and further research opportunities.....	125
8.4	A model of LAG3 activity and its use in assessing the impact of LAG3 on T cell receptor signal transduction	126
	8.4.1 Creating a model for studying LAG3	126
	8.4.2 Importance of LAG3's cytoplasmic domain in its translocation to the cell surface	129
	8.4.3 Cell line model reflects LAG3 activity	131
	8.4.4 The impact of LAG3 on the TCR signaling pathway	132
	8.4.5 Summary	136
	8.4.6 Limitations and further research opportunities.....	137
8.5	General discussion.....	141
	8.5.1 LAG3 expression profile, kinetics and mechanisms	141
	8.5.2 LAG3 mechanism of action in T cells	142

8.6	Significance.....	144
	References	146
9	Appendices.....	159
9.1	LAG3 plasmid insert full sequences	159
9.1.1	Full-length LAG3 for creation of J.LAG3.....	159
9.1.2	LAG3 with cytoplasmic domain deletion for creation of J.ΔCY	159

List of Tables

Table 1 Surface staining panel used for flow cytometry of PBMC in ex vivo staining.....	36
Table 2 Antibodies used to stain PBMC in experiments on LAG3 blockade and LAG3 kinetics.	36
Table 3 Antibodies used to stain Jurkat cells.....	37
Table 4 Flow cytometric classification of cell types and subsets by marker	56
Table 5 Phosphorylation events downregulated in J.LAG3 relative to J.Vector or J.ΔCY.	102
Table 6 Phosphorylation events upregulated in J.LAG3 relative to J.Vector or J.ΔCY.....	103
Table 7 Roles of proteins from Tables 4 and 5.....	135

List of Figures

Figure 1 Potential for checkpoint blockade in an HIV functional cure.	29
Figure 2. Representative ex vivo gating for identification of populations.....	58
Figure 3 LAG3 expression across cell populations.....	60
Figure 4 pDC confirmation by CD303.....	62
Figure 5 mAb or pAb staining of LAG3 by cell population.....	65
Figure 6 mAb and pAb staining of transduced Jurkat cells.	68
Figure 7 LAG3 expression on T cells after stimulation.....	73
Figure 8 LAG3 expression after stimulation and rest.	74
Figure 9 Impact of Flagellin or LPS on T cell LAG3 expression.....	76
Figure 10 Proliferation from SEB activation with LAG3 blockade.....	82
Figure 11 Flow cytometry gating for cytokine detection following SEB reactivation.	85
Figure 12 LAG3 expression and cytokine production after removal of SEB.....	86
Figure 13 Cytokine production from SEB activation with LAG3 blockade.	87
Figure 14 Experimental plans regarding LAG3 model.	93
Figure 15 CD3, CD2, CD28, CD4 and LAG3 expression on cell lines.	96
Figure 16 IL-2 production by cell lines stimulated with SEE or SED.	98
Figure 17 Procedure for analyzing changes in peptide phosphorylation.....	101
Figure 18 LAG3 inhibition of ERK1/2 phosphorylation.	105

List of Acronyms

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
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
IFN- γ	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
ITK	Interleukin-2-inducible T-cell kinase

iTreg	Induced T regulatory cells
ITSM	Immunoreceptor tyrosine-based switch motif
KIR	Killer immunoglobulin-like receptor
KLRG1	Killer cell lectin-like receptor subfamily G member 1
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
MR1	MHC-related protein 1
mRNA	Messenger RNA
NFAT	Nuclear factor of activated T-cells
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NLR	Nod-like receptors
nTreg	Natural T regulatory cell
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	Phytoerythrin
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PLC γ 1	Phospholipase C, gamma 1
PRR	Pattern recognition receptors
PTEN	Phosphatase and tensin homolog protein
RLR	Retinoic acid-inducible gene-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
sLAG3-Ig	Soluble LAG3-Ig
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
TGF- β	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
TNF- α	Tumor necrosis factor alpha
TOX	Thymocyte selection-associated high mobility group box protein
Treg	Regulatory T cells
α -syn	α -synuclein fibrils

Overview:

Cancer and infectious agents, such as HIV, downregulate the immune response in several ways, including by increasing the expression of an immune inhibitory protein called LAG3. This thesis explores the expression and mechanisms of LAG3 with the long-term goal of informing interventions directed at LAG3 that could be used to treat these and other diseases.

1 Introduction

1.1 Introduction to mammalian immunology

Humans are constantly exposed to many billions of bacteria, viruses and fungi. To survive, humans need an immune system that protects the body from these microorganisms. The human immune system has evolved into a tremendously complex system with many components. Largely, this system can be categorized into innate and adaptive immune systems, each with specialized cells with the purpose of protecting the body from invading pathogens or cancer without excessively damaging the body.

1.1.1 Innate immunity

Innate immunity is not specific to any particular pathogen and responds generally and rapidly to invading microorganisms. The innate immune system includes 'barriers' such as skin, mucosa, and stomach acid. Innate immunity also includes the ability of cells to trap and kill organisms in vesicles (phagocytosis) and recognize molecules associated with microbes such as lipopolysaccharide (LPS), flagellin, double stranded ribonucleic acid (dsRNA), called pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) that include toll-like receptors (TLRs), nod-like receptors (NLRs) and retinoic acid-inducible gene-I-like receptors (RLRs).

The main specialized cells of the innate immune system include granulocytes (neutrophils, eosinophils and basophils), professional antigen presenting cells (APCs) and natural killer (NK) cells¹.

The main APCs are monocytes, dendritic cells (DCs), macrophages and B cells. When APCs encounter an invading pathogen their PRRs recognize PAMPs of the pathogen and induce the production of chemical messengers and effector molecules called cytokines, chemokines and interferons that activate and recruit other cells of the immune system¹. For example, in response to TLR activation, plasmacytoid dendritic cells (pDCs) produce a large amount of type I interferon with other cytokines and chemokines and upregulate co-stimulatory molecules, thereby activating and recruiting other immune cells². At the same time, APCs will phagocytose the pathogens and present antigens from the pathogens in protein complexes called major histocompatibility complexes (MHCs)¹. MHCs can be one of two classes: class I MHCs (MHC-I) present peptides 8-10 amino acids long typically of intracellular origin, class II MHCs (MHC-II) present peptides of at least 13 amino acids in length that are typically of extracellular origin¹.

Monocytes are typically classified as classical, intermediate or non-classical monocytes. 80-90% of monocytes are classical monocytes, which circulate in the bloodstream for approximately one day before trafficking to tissues and lymph nodes or differentiating into non-classical monocytes, which circulate for approximately 7 days, via intermediate monocytes³. Once in the tissue, monocytes may differentiate into macrophages or DCs, or potentially remain as functioning monocytes³. Monocytes, dendritic cells and macrophages have many overlapping functions including

phagocytosis of pathogens or cell debris, remodeling of capillaries and extracellular matrices, several immunomodulatory roles (both positive and negative) through antigen presentation, the release of chemokines or cytokines and cell-cell interactions³.

NK cells recognize cells, typically infected or cancerous cells, that downregulate inhibitory killer immunoglobulin-like receptor (KIR) ligands (e.g., MHC-I) or upregulate activating KIR ligands⁴. After recognition, NK cells will kill the infected/cancerous cell or produce cytokines to modulate the immune response⁴.

1.2 Adaptive immunity

In contrast to the innate immune system, the adaptive immune system is antigen-specific, initially slower to respond and generates a long-lasting memory response. The adaptive immune system consists of B cells and T cells composing the humoral and cell-mediated branches, respectively¹.

When a B cell recognizes its cognate antigen through its B cell receptor (BCR), a signal is transmitted that leads to proliferation and further differentiation into memory B cells, which are primed for a recall (secondary) response, or plasma cells which produce antibodies¹. Antibodies are important in many aspects of immunity including opsonization, activation of the complement cascade, neutralization of pathogens and antibody-dependent cellular cytotoxicity¹. This B cell activation and differentiation is guided by helper T cells.

1.2.1 T cell immunity

Unlike B cells, T cells recognize antigens through use of their T cell receptor (TCR) only when presented by MHCs. The TCR is composed of a disulfide-linked heterodimer with each chain containing one immunoglobulin-like constant domain and one variable domain¹. The distal sides of the variable domains form the MHC/antigen binding site¹. At this site, the TCR recognizes short peptide fragments presented by an MHC molecule. Each T cell possesses single TCR type produced by recombination of gene segments, allowing it a unique recognition pattern that may correspond to a series of amino acids¹. Through a process of positive and negative selection, developing T cells are selected based on their ability to respond, but not too strongly, to self antigen in the thymus¹. The specificity of TCRs makes T cells crucial components of adaptive immunity.

1.2.1.1 T cell subsets

T cells are generally classified based on expression of either CD4 or CD8. CD4 and CD8 are positive co-receptors that bind to MHCII and MHCI, respectively, and enhance the cells sensitivity to antigen¹. Typically, CD4⁺ T cells are either regulatory T cells (Treg) or helper T (T_h) cells, while CD8⁺ T cells are cytotoxic T lymphocytes (CTL)¹.

T helper cells recognize antigens of at least 13 amino acids in length presented by MHC class II, typically of extracellular origin¹. When recognition occurs, T helper cells differentiate into different effector subsets based on their environment during

activation. These subsets, which include T_{h1} , T_{h2} , T_{h17} , T follicular helper cells (T_{FH}) and others, each express different sets of proteins and produce different cytokines upon subsequent antigen recognition and thereby promote subset-specific immune responses¹. In this way, T_h cells are crucial orchestrators of the immune response.

T regulatory cells can develop naturally in the thymus (nTreg) or can be induced from naïve T_h cells by exposure to TGF- β in the absence of pro-inflammatory cytokines, these are called induced T regulatory cells (iTreg)¹. Treg effectively regulate the immune response through multiple mechanisms, including the production of anti-inflammatory cytokines transforming growth factor β (TGF- β) and interleukin-10 (IL-10)¹. The importance of Tregs is best demonstrated by autoimmune conditions caused by their absence.

CTLs recognize peptides of 8-10 amino acids in length presented by MHC class I, which are typically of intracellular origin¹. These are typically antigens from mutated proteins or intracellular pathogens. Upon recognition of antigen and appropriate co-stimulation, CTLs release effector cytokines (chemical messengers) and kill the cancerous or infected cell by releasing perforin and granzymes, which create holes in the membranes of cells and induce programmed cell death, respectively¹.

Unconventional T cells include $\gamma\delta$ T cells, invariant natural killer T (iNKT) cells, and mucosal-associated invariant T (MAIT) cells. These are innate-like T cells which play roles in both innate and adaptive immunity.

$\gamma\delta$ T cells – While most T cell receptors are composed of α and β chains, some T cells have TCRs made of γ and δ heterodimer. Unlike conventional $\alpha\beta$ T cells,

$\gamma\delta$ T cells can recognize a diverse array of ligands induced by cellular stress or infection which they respond to directly, playing an innate-like role facilitating the transition between innate and adaptive immune responses¹.

NKT cells – This innate characteristic is also true of NKT cells which are CD1d-restricted T cells that respond to lipid antigens. There are two types of NKT cells, type I invariant NKT (iNKT) respond to lipid antigens using the same TCR alpha chain: V α 24-J α 18. NKT cells also express NK cell receptors. NKT cells have many functions and can be further broken down into subtypes, but in general respond rapidly to stimulation and are important links between innate and adaptive immunity⁵.

MAIT – Similarly to iNKT cells, MAIT cells express an invariant alpha chain of the TCR but recognize PAMPs. Specifically, MAIT cells respond to folic acid metabolites produced by bacteria and yeast presented by MHC-related protein 1 (MR1), a non-classical MHC class I molecule^{6,7}.

1.2.1.2 TCR signaling

1.2.1.2.1 Formation of the immune synapse

T cells initially contact potential-target cells through non-specific interactions of Lymphocyte function-associated antigen 1 (LFA-1) and CD2 with Intercellular Adhesion Molecule (ICAM) and CD58¹. This allows the TCR to bind to, and potentially recognize, the peptide:MHC (pMHC) complex. If recognition occurs, the TCRs and co-receptors cluster, forming an immune synapse and inducing TCR signaling.

1.2.1.2.2 Principles of TCR signaling

TCR signaling occurs mainly by the sequential activation of kinases. Kinases catalyze the phosphorylation of proteins, while the removal of the phosphate group is catalyzed by phosphatases^{1,8}. When phosphate groups are added to certain serine, tyrosine or threonine residues of a protein, protein activity may be altered. For example, protein phosphorylation may directly turn a protein activity on or off by changing its conformation, or it may facilitate the recruitment and binding of other signaling molecules¹⁹.

Phosphotyrosines are the most important phosphorylated amino acid in TCR signaling¹. Src homology 2 (SH2) domains, which are present in many components of the TCR signaling pathway, recognize phosphorylated tyrosine and the surrounding amino acids, and are often linked to other functional domains¹. In this manner, a protein with an SH2 domain can bind specifically to its target only when phosphorylated and exert its function.

During TCR signaling, one kinase may phosphorylate many proteins, in some cases a kinase will phosphorylate several other kinases, which are in turn activated to phosphorylate other proteins⁹. In this manner, the TCR activation signal is amplified (amplification may also occur through secondary messengers such as calcium).

1.2.1.2.3 Initiation of TCR signaling

While it is unclear exactly how the pMHC:TCR interaction initiates signaling, clustering of TCRs plays a major role, since cross-linking of TCRs with antibodies alone

can activate T cells⁹. Interestingly, the TCR itself does not induce signaling. Instead, the TCR is associated with CD3 chains which contain several immunoreceptor tyrosine-based activation motifs (ITAMs)^{1,9}. Each ITAM possesses two tyrosines which are phosphorylated with the help of the Src-family kinase lymphocyte-specific protein tyrosine kinase (Lck)¹. Lck is constitutively associated with the intracellular domains of CD8 and CD4 so that when the TCR recognizes its specific pMHC and CD4 or CD8 bind as co-receptors, Lck is colocalized with CD3 ITAMs, allowing phosphorylation^{1,9}. When phosphorylated, these tyrosines recruit the tandem SH2-containing protein Zeta-chain-associated protein kinase 70 (ZAP-70), which is also phosphorylated by Lck (although autophosphorylation can also occur) changing into an active conformation¹. In its active conformation, ZAP-70 phosphorylates linker for activated T cells (LAT), a transmembrane scaffold protein which is linked with SH2 domain containing leukocyte protein of 76kDa (SLP-76) (which is also phosphorylated by ZAP-70) by GRB2-related adaptor downstream of Shc (Gads)¹. This LAT signalosome is central to T cell activation.

In a parallel manner, the CD3 complex and CD28 costimulatory molecule activate phosphoinositide 3-kinase (PI3K)⁹. Active PI3K then phosphorylates the inositol molecule phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP₃) which recruits proteins such as interleukin-2-inducible T-cell kinase (ITK) kinase to the cell membrane. ITK then activates SLP-76 and phospholipase C, gamma 1 (PLC γ 1), which hydrolyzes PIP₂ creating inositol trisphosphate (IP₃) and diacylglycerol (DAG)⁹.

IP₃ then binds to IP₃ receptor in the endoplasmic reticulum, leading to a release of calcium stores, which in turn causes the influx of extracellular calcium. This influx of calcium then activates other signaling proteins, including indirect activation of Nuclear factor of activated T-cells (NFAT).

Meanwhile, DAG activates protein kinase C (PKC) and Ras, which is also activated by the LAT signalosome⁹. The Ras pathway leads to the activation of the mitogen-activated protein kinase (MAPK) pathway, which activates several transcription factors.

1.2.1.2.4 Outcomes of TCR signaling: Transcription factors

An important result of TCR signaling is the activation of transcription factors. Transcription factors are proteins that bind to specific DNA sequences to control the transcription of genes. TCR signaling activates the transcription factors NFAT, Activator protein 1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), JUN, Fos and others^{1,10}. These transcription factors induce the expression of several genes, including the gene for IL-2, an important cytokine that promotes T cell proliferation and differentiation.

1.2.2 T cell exhaustion

When naïve T cells are activated, they proliferate and differentiate into effector cells. Effector cells produce cytokines and effector molecules to further activate and recruit surrounding immune cells and kill the antigen source, whether it be infected or cancerous cells.

In the case of acute infection, after the infection resolves and the antigen is cleared, a small subset of these cells will differentiate into memory cells that remain in the body and are able to respond to re-exposure to their cognate antigen.

In the case of chronic infection or persistent cancer, the antigen source is not cleared, and these effector T cells gradually and progressively lose proliferative and effector function, including the loss of important effector molecules. This typically starts with loss of interleukin (IL)-2 production and eventually progresses as production of interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α) is also lost^{11,12}. These cells with impaired effector phenotype are said to be 'exhausted'.

Immune exhaustion can occur in any scenario of persistent T cell activation, including autoimmunity, immunogenic cancer or chronic infections with *Mycobacterium tuberculosis*¹³, malaria parasite¹⁴, Hepatitis B virus¹⁵, hepatitis C virus¹⁶ or human immunodeficiency virus (HIV)^{17,18}.

The exhaustion phenotype develops progressively as the cell is activated repeatedly. In the late stages of exhaustion, the cell has differentiated completely and potentially irreversibly into an exhausted cell¹⁹. Not even adoptive transfer into an uninfected host rescues an effector or memory phenotype²⁰. However, during the early stages, exhaustion can be reversed either by clearing of the cognate antigen, or by reinvigoration by antibody-mediated blockade of proteins that promote the exhaustion phenotype. Antibody-mediated blockade refers to the use of antibodies that bind to an exposed epitope of the protein that results in the inhibition of that protein's activity,

usually by binding to the receptor binding domain. In the case of reversing exhaustion, the proteins targeted are a functional class of proteins called immune checkpoints.

1.3 Immune checkpoints

Immune checkpoints (ICs) are negative co-receptors expressed after activation of T cells that impair further T cell activation. ICs play an important role in limiting excessive T cell activation which, if left unchecked, could lead to autoimmunity. Exhausted T cells express high levels of ICs including programmed cell death-1 (PD-1), T cell immunoglobulin and mucin domain containing-3 (Tim-3), cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), T-cell immunoreceptor with Ig and ITIM domains (TIGIT), B- and T-lymphocyte attenuator (BTLA), CD244, Killer cell lectin-like receptor subfamily G member 1 (KLRG1) and Lymphocyte activation gene-3 (LAG3).

While not exclusively expressed during exhaustion, ICs contribute to the exhaustion phenotype by impairing activation of immune cells. ICs are expressed on a variety of immune cells, however, they and their contribution to immune exhaustion, as well as the immune exhaustion phenotype itself, are mostly studied in T cells. ICs typically inhibit T cell activation through intracellular motifs that interfere with the TCR signaling pathway, although other mechanisms such as competitive inhibition for positive co-receptors also exist.

Immune exhaustion is thought to have evolved as a protection mechanism from immunopathology during chronic infections and autoimmunity²¹⁻²⁴. In these cases, an elevated immune response can cause significant damage to self, and exhaustion can

be advantageous. Unfortunately, immune exhaustion also arises during cancer and infections when a robust immune response is desired. In these circumstances, bolstering the immune response by inhibiting ICs can greatly improve outcomes²⁵.

Currently, antibodies blocking PD-1 and CTLA-4 are approved for treatment of several forms of cancer in many countries including Canada and the United States. Since combining these therapies can be more effective than each alone, many clinical trials investigating whether PD-1 and CTLA-4 blockade can be combined with the blockade of other ICs. Of those, perhaps the furthest along and most promising is LAG3.

1.4 LAG3

1.4.1 LAG3 structure

LAG3 was discovered in 1990 as a member of the immunoglobulin superfamily and an ancestral homolog of CD4. While LAG3 is likely similar in structure to CD4, it is only 20% similar in amino acid sequence²⁶. Human LAG3 is a 70kDa transmembrane protein with a 54 amino acid-long intracellular domain and with four extracellular domains, labelled D1-D4 in order from most distal to proximal, with four glycosylation sites²⁷.

1.4.2 LAG3 expression

1.4.2.1 Cellular distribution

LAG3 research has been focused on conventional T cells and Tregs, but unconventional T cells, B cells, NK cells, pDC and neurons also express LAG3^{28–31}. However, the proportion of cells expressing LAG3 often varies substantially between studies, and no study has published a comparison of LAG3 protein expression across these cell types. Therefore, inferring a percentage of cells that express LAG3 and interpreting relative expression levels between cell types can be dubious.

1.4.2.2 Transcriptional regulation

Cellular activation through the TCR upregulates LAG3 expression, and this can be amplified by cytokines (especially IL-12, IL-2, IL15, IL-27, and IL-7)^{21,32–36}.

LAG3 transcriptional regulation is not well characterized, with many regulatory and inducer elements theorized to play a role but experimental evidence is lacking. Methylation of the LAG3 promoter is strongly associated with reduced LAG3 expression in renal cell carcinoma and melanoma, while methylation of the CCCTC-binding factor (CTCF) binding site downstream of the LAG3 gene is associated with increased LAG3 expression^{37,38}. Several potential transcription factor binding sites exist upstream of the LAG3 gene and a number of transcription factors known to be involved in immune exhaustion induce LAG3 expression, including NFAT and Thymocyte selection-associated high mobility group box protein (TOX)^{39,40}. T-bet (a T-box transcription factor), an important regulator of immune exhaustion which guides differentiation of

CTLs, is of particular interest since it is inversely correlated with LAG3 expression in a seemingly bidirectionally causal relationship (deletion of LAG3 increases T-bet and vice-versa) in murine T cells^{41–43}. This suggests that while T-bet reduces exhaustion, LAG3 expression may induce exhaustion.

1.4.2.3 Post-translational regulation

Surface expression of LAG3 regulated in two ways. Firstly, and likely most importantly, LAG3 is cleaved by a disintegrin and metalloproteinase (ADAM)10 and ADAM17 at a 20-amino acid connecting peptide on the extracellular side of the transmembrane domain^{44–46}. This cleavage effectively regulates LAG3 activity as shown by increased inhibition of T cell immunity when metalloproteinase inhibitor is given to mice, or when mice are genetically modified to possess a non-cleavable form of LAG3⁴⁴. Cleavage of LAG3 as a regulatory mechanism is also demonstrated by human cancer patients, where a higher LAG3:ADAM10 ratio is associated with poor prognosis and accelerated disease progression⁴⁵.

Cleavage of LAG3 results in a soluble form of LAG3 (sLAG3) that can be found in the serum and potentially serve as a biomarker^{47–49}. Some have speculated that sLAG3 is produced as a splice variant of LAG3, but the evidence for such splice variants is weak⁵⁰. Interestingly, T cells are not the major source of sLAG3, that most likely being pDCs^{46,51}. While it was initially speculated that sLAG3 may have some function, potentially blocking the binding of surface associated LAG3 to its ligand or inducing DC cell maturation by MHC class II crosslinking, to date no biological role for sLAG3 has been discovered and its ability to bind MHC class II or other LAG3 ligands is

questionable^{44,52,53}. These speculated roles for sLAG3 have been demonstrated by a synthetic LAG3-Ig chimeric protein which maintains LAG3 in dimeric form⁵⁰. The potential importance of existing as a dimer will be discussed later.

Not all cell-associated LAG3 is expressed on the cell surface. Roughly half of all LAG3 resides in late endosomes after activation, with a potentially greater proportion before activation⁵⁴. This storage of LAG3 may represent LAG3 that has been recycled from the cell surface in a form of negative regulation by endocytosis. LAG3 endocytosis can occur following interaction with α -synuclein fibrils, but has otherwise not been researched²⁸. This intracellular store of LAG3 also may exist to allow quick translocation to the cell surface after cellular activation, or as another layer of post-translational regulation.

These multiple methods of post-translational regulation make it difficult to infer LAG3 surface expression by measurements of LAG3 transcript or total LAG3 protein and therefore favor direct measurement of surface LAG3 by immunofluorescence or flow cytometry.

1.4.3 LAG3 function

In general, LAG3 is thought to regulate the immune response. LAG3 deficient mice have more NK cells, pDCs and $\gamma\delta$ T cells than wild-type mice, implying a role for LAG3 in reducing their expansion⁵⁵. Interestingly, LAG3 deficient mice also have greater numbers of granulocytes and macrophages, which are thought not to express LAG3 even in wild-type mice⁵⁵.

1.4.3.1 LAG3 on T cells

LAG3 is best studied on T cells, where it is known to inhibit activation through the TCR. This inhibition has been well demonstrated in both humans and mice as LAG3 knockout or blockade increases T cell proliferation and expression of IL-2, IFN- γ and TNF- α ^{56–58}. Similar LAG3 function has been demonstrated on iNKT and MAIT cells, while LAG3 function on $\gamma\delta$ T cells remains uninvestigated^{7,59}.

LAG3 activity is likely similar between Th and CTLs, inhibiting proliferation and activation. While studies are mixed, with one showing that LAG3 blockade does not impact CTL activation and another showing that LAG3-mediated inhibition of CTL is dependent on Th LAG3 expression, several other studies demonstrate a Th-independent inhibitory function of LAG3 on CTLs^{16,60–62}.

LAG3 function on Treg is not as clear as on conventional T cells. Early studies have shown that LAG3 expression on Tregs can enhance Treg suppressor function on responding T cells when antigen is abundant^{21,63}. When antigen concentration is lower, Treg function is enhanced only by LAG3 expression on the responding conventional T cell^{41,61,63,64}. Contrarily, more recent studies have shown that LAG3 inhibits Treg activity and development, leading to enhanced disease in autoimmune models^{41,65,66}. LAG3's impact on Treg suppression of DC immunity is similarly unclear^{67,68}.

1.4.3.2 LAG3 on other cells

One early study showed that in mice, LAG3 knockout or blockade improved the cytotoxic capacity of NK cells, but this has not yet been confirmed despite attempts^{69,70}.

In two different mouse models of cancer, LAG3 blockade after treatment with IL-12 improved NK function and increased their numbers while reducing metastases⁷¹. This implies a similar role for LAG3 on NKs as in T cells. Non-peer reviewed data supports this by showing that LAG3 blockade increases IFN- γ , TNF- α , Macrophage Inflammatory Protein 1 alpha (MIP-1 α) and MIP-1 β secretion from NK cells without affecting cytotoxic activity⁷². Interestingly, a recent study supplement claims that LAG3 inhibits NK cell pruning of virus-specific T cells in infected mice⁷³.

As with other cells, LAG3 promotes an inhibitory activity for pDCs. Indeed, pDCs that express LAG3 produce more IL-6 but less IFN- α , promote Treg generation, and help recruit myeloid-derived suppressor cells²⁹. Interestingly, LAG3 on T cells can inhibit pDC expansion and vice-versa⁵¹.

1.4.4 LAG3 mechanism of action

1.4.4.1 LAG3 ligand binding

LAG3 ligands include MHCII, Fibrinogen-like protein 1 (FGL-1), liver and lymph node sinusoidal endothelial cell C-type lectin (LSEctin) and Galectin-3 (Gal-3). LAG3 inhibition of T cells has been demonstrated upon binding to each of these ligands^{60,74,75}. LAG3 also binds to α -synuclein (α -syn) fibrils, but the immunological relevance of this is unknown²⁸.

1.4.4.1.1 MHC binding

As with CD4, its homolog, LAG3 binds MHCII but with 100 fold higher affinity than CD4⁷⁶. Of the four extracellular domains, only D1 and D2 of LAG3 are involved in MHCII binding, being both necessary and sufficient²⁷. A 30 amino acid loop on the distal side of D1 is involved in binding, potentially representing the entire binding site, with D2 likely providing flexibility of movement²⁷. Deletion of this loop eliminates MHCII binding, while mutations in the loop alter LAG3:MHCII binding strength²⁷. Further demonstration of this loop's importance in binding is that the 17B4 antibody clone that targets this loop is well known to inhibit binding to MHCII^{27,77}.

It is important to note that mutations in another section of D1 can also inhibit the LAG3:MHCII interaction²⁷. It was proposed that this region is important in facilitating oligomerization of LAG3, since when LAG3 with these mutations is co-expressed with wild-type LAG3, it abolishes all LAG3:MHCII binding, including that of the wild-type LAG3²⁷. Later research confirmed that LAG3 dimerization and oligomerization is dependent on D1. This would then suggest that oligomerization of LAG3 is necessary for MHCII binding⁴⁶. This is supported by studies using a dimeric soluble LAG3-Ig (sLAG3-Ig) that show strong interaction with MHCII, but binding of monomeric sLAG3 to MHCII has not been confirmed despite attempts^{27,44,70,76,78-80}.

Recent research has discovered that LAG3 specifically binds to peptide loaded MHCII (pMHCII)⁷⁸. Indeed, this research showed that LAG3 binding is dependent on the antigen affinity for the MHCII and on expression of MHCII accessory molecules, including the invariant chain, which acts as a chaperone for MHCII, and H2-DM, which

acts as a peptide editor. This suggests that LAG3 function is dependent on MHC haplotypes, the peptide presented by the MHC and properties of the APC itself.

1.4.4.1.2 Other ligand binding

As previously mentioned, in addition to MHCII, LAG3 binds to FGL-1, LSECtin, Gal-3 and α -syn. LSECtin and Gal-3 are lectins that contain a carbohydrate-recognition domain, which likely binds glycosylated regions of LAG3 (although no binding site analysis has been performed for these), and an oligomerization domain which may allow LAG3 clustering. Both LSECtin and Gal-3 have been shown to inhibit IFN- γ during CTL activation in a LAG3 dependent manner^{74,75}. Like MHCII, FGL-1 binding depends on both D1 and D2, but the binding profile is not identical, as mutation of the Y77 residue (Y73 in mice) does not reduce binding of FGL-1, whereas it does inhibit LAG3 binding⁸¹. This is further demonstrated as the antibody clone C9B7W that targets murine LAG3 inhibits FGL-1, but not MHCII⁸². As with MHCII and FGL-1, α -syn binds to the extra loop of D1, but also relies on D2, D3 and the intracellular domain for complete binding⁷⁸.

1.4.4.1.3 Functional role of ligand binding

LAG3 inhibits T cell activation upon ligand binding, but the mechanism by which ligand binding induces LAG3 activity is not clear. LAG3's interaction with ligand may help LAG3 colocalize with the TCR or facilitate LAG3 cross-linking.

In support of the colocalization model is the fact that colocalization does occur when LAG3 binds to MHCII during activation. This colocalization of LAG3 with the

immune synapse does not occur with LAG3 mutations that inhibit MHCII binding, such as the P111A mutation, nor when T cell activation occurs through unstable pMHCII (which does not bind LAG3)⁷⁸. While this suggests colocalization occurs during MHCII binding, it does not prove that colocalization is necessary for LAG3 function. However, in a model that allows LAG3 to bind to pMHCII of an APC that is non-cognate to the T cell, while the T cell is activated by cognate pMHCI (to which LAG3 does not bind), LAG3 activity is reduced by two-thirds⁷⁸. Here, LAG3 activity was highest when peptide concentration was low, suggesting that pMHCI may be crowding out LAG3 from the immune synapse at higher peptide concentrations.

The colocalization model is further supported by an experimental model that uses anti-CD3 and anti-LAG3 antibodies with a secondary antibody to crosslink these proteins⁸³. Cross-linking of CD3 activates T cells, while cross-linking CD3 and LAG3 together reduces this activation. Cross-linking each protein separately and simultaneously by using secondary antibody targeting each isotype (anti-IgG1 and anti-IgG2) however, does not reduce activation compared to cross-linking CD3 alone.

The colocalization model implies that LAG3 inhibits the early stages of TCR signaling. Recently, researchers suggested exactly this as inhibitors of general kinases, PKC θ , PKC δ or calcineurin did not affect LAG3 activity in a Jurkat cell line engineered to express LAG3⁵⁸.

Despite this evidence for the colocalization model, it fails to explain how LAG3's non-MHCII ligands inhibit T cell activation, since it's unclear how they would enhance colocalization with the TCR. Nor does it explain how LAG3 could inhibit non-TCR

stimulation, such as IL-7⁸⁴. This activity may be explained by the cross-linking model, although it does not explain the lack of LAG3 activity when LAG3 is engaged while the T cell is activated by MHCI or anti-CD3 antibodies.

Taken together, the LAG3-ligand interaction is necessary for LAG3 function, although how this interaction enables LAG3 function remains unclear.

1.4.4.2 Competitive inhibition of CD4 as a model for LAG3 inhibition

The first speculative explanation for LAG3's mechanism was that it inhibits the CD4:MHCII interaction through stronger interaction with MHCII that displaces CD4^{76,78,82,85}. This remains a popular explanation despite being supported by no evidence and being refuted by many studies that, when taken together, strongly imply that CD4 competitive inhibition is not a major mechanism for LAG3.

These studies refute the CD4 competitive inhibition model of LAG3 activity by establishing four assertions:

1. CD4 can bind to MHCII even in the presence of a sLAG3-Ig. LAG3 can inhibit CD4 binding, but only when the TCR is not engaged⁷⁶. This has been confirmed two independent groups^{76,78}.
2. LAG3 inhibitory activity is entirely dependent on its cytoplasmic domain^{55,57,78,86}.

3. LAG3 activity can be induced independently of MHCII, either by cross-linking LAG3 using antibodies or through the binding of non-MHCII ligands which do not bind CD4^{74,75,81,83}.
4. LAG3 inhibits CTLs^{78,81,87}, which are CD4-negative. This demonstrates that LAG3 can inhibit T cell activation in the absence of CD4.

1.4.4.3 LAG3's intracellular domain

Likely by cross-linking and/or colocalization, ligand binding to LAG3 inhibits T cell activation. However, while other immune checkpoints have ITIM or ITSM domains that recruit phosphatases containing SH2 binding domains that counteract the activating kinases in the TCR signaling pathway, LAG3 has no ITIM or ITSM. Indeed, LAG3 contains no characterized inhibitory domain. This lack of known inhibitory domain contributes to the mystery of the LAG3 mechanism of action.

Rather, LAG3 contains three well conserved sections in its intracellular domain. The first region thought to play a role in LAG3 function consists of the amino acids KIEELE. In three *in vitro* and *in vivo* studies of mice, Workman et al. showed that the lysine (K) of the KIEELE motif was necessary for LAG3 function^{55,57,86}. However, no other groups have confirmed this finding⁵². Contrarily, a recent study determined another of the conserved regions containing the amino acids RRFSALE, but not the KIEELE motif, was essential for LAG3 inhibitory function⁸⁸.

In the RRFSALE region, it seems serine would be the most likely to play a role, since its phosphorylation can activate protein function. Instead, while a serine to alanine mutation had no effect^{57,88}, mutating one or both of leucine (L) or phenylalanine (F) to

alanine halved the inhibitory potential of LAG3 on IL-2 production⁸⁸. This implies a FxxL motif within the RRFSALE region is necessary for at least part of LAG3 function.

Singular deletion of the third conserved region, which consists of several EX repeats – that is glutamic acid followed by another amino acid (most often proline) – on the tip of the cytoplasmic tail that overlaps with the KIEELE motif, does not impact LAG3 function^{57,88}. However, combined mutation of the FxxL motif and deletion of the EX motif increased IL-2 production nearly two-fold when compared to LAG3-negative cells⁸⁸. This suggests that these mutations can convert LAG3 from an inhibitory co-receptor to a positive one. A positive co-receptor role has been shown before with LAG3 lacking its cytoplasmic domain in 3A9 cells⁵⁷.

Roles for FxxL or KIEELE motifs have not been demonstrated outside of the groups that initially proposed their importance, and their role in transmission of the LAG3 negative signal remains uncharacterized.

The EX motif, however, can bind a LAG3 associated protein (LAP) that is 99.8% identical at the amino acid sequence level with the C-terminus of Centromere protein J (CENPJ)⁸⁹. In addition to its participation in organization of centrosomes and microtubules during cell division, CENPJ also enhances activation of NF- κ B and Signal transducer and activator of transcription (STAT) 5^{90,91}. Therefore, one may speculate that LAP and thus the EX motif may be involved in trafficking LAG3 from its intracellular residence in endosomes to lipid rafts on the cell surface. However, deleting only the tail end of the EX motif (called the EP motif) does not reduce surface expression of LAG3. Other speculative roles for the EX motif include sequestration of LAP from its

coactivator activity on STAT5 and NF- κ B. While unproven, this could explain how LAG3 is able to inhibit STAT5 and protein kinase B (PKB) activation upon peptide⁴¹ or IL-7⁸⁴ stimulation or how LAG3 causes cell cycle arrest^{59,92,93}.

1.4.5 LAG3's role during disease

Like other ICs, LAG3's main role is likely to prevent immunopathology. This, combined with the upregulation of LAG3 during immune activation, strongly implies that LAG3 activity is most important when the immune system is activated.

Immune activation occurs as a result of autoimmunity, allergy exacerbations, cancer and infection. Inhibition of persistent immune activation is clearly beneficial during autoimmune disease and allergic responses, where the immune response is overactive and LAG3 deletion or inhibition exacerbates disease. Conversely, this activity is harmful during cancer, wherein a strong immune response can be important and effective in controlling disease and where deletion or blockade of LAG3 can enhance this control.

The picture is more complicated during infection, where a balance is needed between a strong enough immune response to clear infection, but not so strong as to cause excessive immunopathology. This situation is complicated during chronic infection, wherein infectious antigen drives immunopathology, but it is often the immunopathology that is most harmful. In these circumstances, it is unclear whether it's better to have less LAG3, which may improve immune response against the infection and potentially reduce antigen load at the cost of potential off-target damage, or

whether it's better to have more LAG3, which may reduce initial immunopathology at the cost of higher pathogen levels.

It is not difficult to see that this determination of whether LAG3 is beneficial or detrimental during infection is likely highly dependent on many factors specific to each individual, type of infection and potential treatment. While this question is relevant for many infectious diseases, from acute sepsis to chronic hepatitis virus infection. I will use HIV as an example of the considerations that must be taken, since it was the Fowke lab's work on the *GNB3* 825 single nucleotide polymorphism and its relation to HIV progression that led to this thesis on LAG3⁹⁴.

1.4.5.1 HIV

HIV is a retrovirus that uses CD4 and C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) as receptor and co-receptor. After entry, HIV reverse transcribes its RNA genome into DNA, then integrates its DNA into the human host's genome. After integration, HIV can produce more virions or remain latent in the host cell. Antiretroviral treatments (ART) are highly effective at suppressing HIV replication, but there is currently no effective vaccine or cure for HIV.

1.4.5.1.1 LAG3 associated with poor outcomes during HIV-infection.

The Fowke lab and others have found that LAG3 expression is elevated during HIV infection and this elevated expression remains on central memory T cells, NK cells and iNKT cells following treatment with ART^{56,95,96}. LAG3 expression during HIV is associated with unfavourable indications including viral load, disease progression and

faster viral rebound following treatment interruption^{17,56,97}. Furthermore, work by the Fowke lab and others on NK cells shows that LAG3 expression is lower on NK cells of HIV-exposed seronegative populations and elite controllers, while elite controllers and viraemic non-progressors also have less LAG3 on Th cells than appropriate control groups^{98–100}. It is important to note that while it is possible LAG3 expression is driving these associations, it is more likely that immune activation is acting as a strong confounding variable.

1.4.5.2 Treatment potential

LAG3 modulating therapies have much potential across all diseases where LAG3 function is relevant. Most LAG3-targeted therapies are antibodies used to inhibit LAG3 activity, but others can enhance LAG3 activity or deplete LAG3⁺ cells. Because of the similar impact and synergistic activities of LAG3 and PD-1, most LAG3 targeted therapies are being used in combination with PD-1 targeted therapies. These therapies hold promise for treatment of infectious diseases, such as HIV, where LAG3 activity may be beneficial or harmful in different contexts, cancer, where LAG3 activity suppresses a much needed immune response, or autoimmunity, where LAG3 protects against more severe disease.

1.4.5.2.1 Potential in an HIV cure

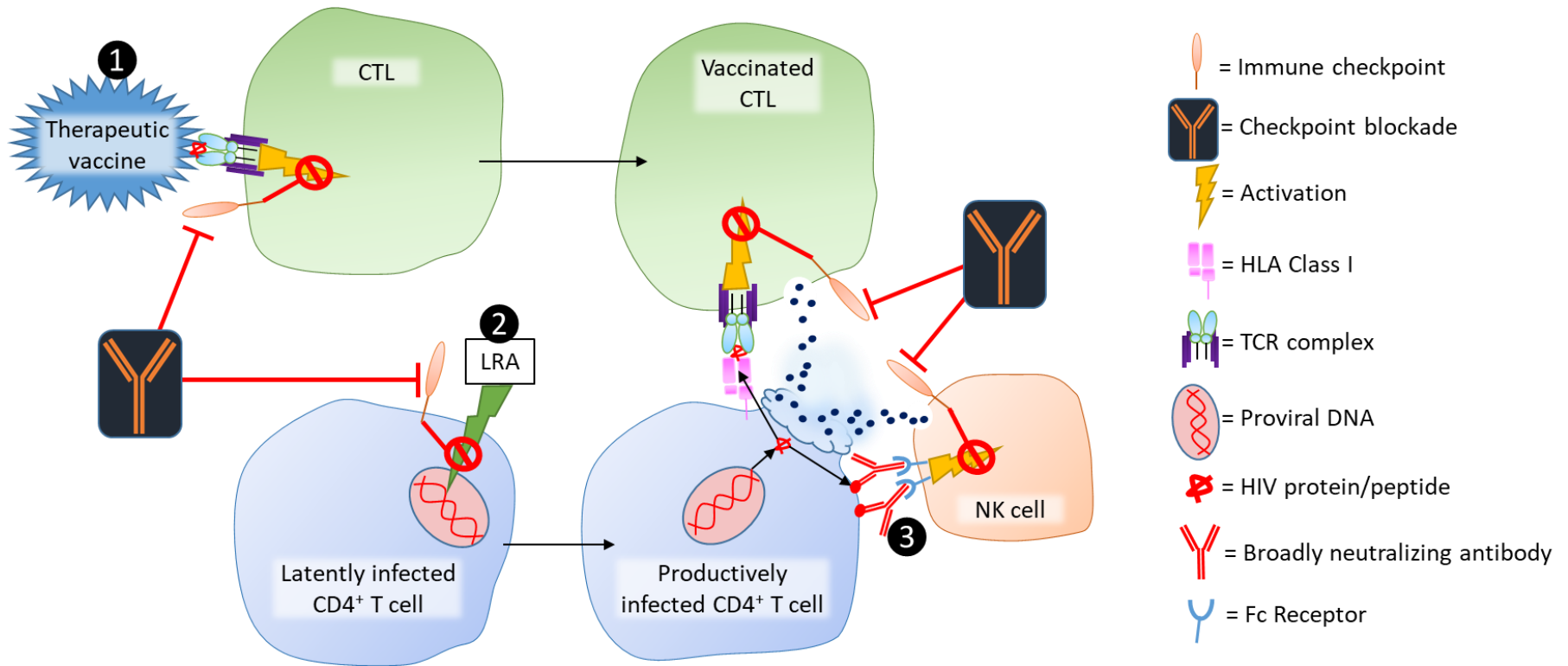
For HIV, there is potential to use LAG3 blockade as part of a functional cure. In contrast to a sterilizing cure, where HIV is eliminated from the body, a functional cure would allow control of HIV replication without use of ART. One functional cure strategy called “shock and kill” seeks to “shock” HIV out of hiding in its latent reservoirs and then

“kill” the HIV-infected cells producing virus. For this “shock and kill” strategy to succeed it is highly likely that multiple tactics addressing both aspects will be needed. LAG3 blockade could conceivably be one such tactic aiding in both aspects.

Firstly, LAG3 is elevated on HIV-latently infected cells with cells co-expressing LAG3, TIGIT and PD-1 8-fold more likely to be harbouring HIV. Secondly, LAG3 may enhance latency by inhibiting NFAT activation and T cell activation, which are both known to induce HIV-transcription. Therefore, LAG3 blockade could enhance the efficacy of a “shock” and do so preferentially on latently infected cells. LAG3 could enhance the “kill”-ing of infected cells by temporarily releasing the brakes on immune cells, especially CTLs, which are necessary to maintain control of HIV¹⁰¹.

PD-1 blockade has shown promise in this regard. In simian immunodeficiency virus (SIV)-infected macaques, PD-1 blockade increased SIV-specific CTL immunity while it reduced viral load and mortality^{102,103}. In humans, case reports show that PD-1 and CTLA-4 blockade can increase cell-associated RNA and reduce latent reservoir size^{104–106} while a clinical trial showed improved CTL responses¹⁰⁷.

Figure 1 Potential for checkpoint blockade in an HIV functional cure.



Checkpoint blockade could play a role in enhancing the efficacy of a therapeutic vaccine (1) and latency reversing agents (2) by augmenting CTL response to antigen and reducing the activation threshold of HIV-infected cells, respectively. Checkpoint blockade could also enhance the killing activity of HIV-specific CTLs and potentially NK cells responding to broadly neutralizing antibodies (3). Together, combinations of these and other potential strategies may result in a smaller viral reservoir and enhanced viral suppression, leading to sustained viral control and constituting a functional cure.

1.4.5.2.2 Potential in cancer treatment

Many forms of cancer are immunogenic. However, cancer cells are more difficult for the immune system to detect and clear compared to pathogens due to their similarity to healthy cells and the general lack of specific cancer-associated molecules. This leads to a more limited immune response in which T cells are frequently exhausted. Increased expression of proteins associated with immune exhaustion are frequently associated with poor prognosis. LAG3 in particular has been associated with tumor progression, poor prognosis and unfavourable outcomes in melanoma^{38,108}, renal cell carcinoma^{37,109}, head and neck squamous cell carcinoma¹¹⁰, colorectal cancer¹¹¹, non-small cell lung cancer^{48,112,113}, follicular lymphoma¹¹⁴, diffuse large B cell lymphoma¹¹⁵ and breast cancer¹¹⁶. Indeed, many studies have found that LAG3 blockade can delay tumor growth, increase proliferation and cytokine production of tumor infiltrating T cells, and overall have therapeutic effect (especially when combined with PD-1 inhibition or deletion) in *in vivo* and *in vitro* models of several forms of cancer^{110,117–121}.

Following the successes of PD-1 and CTLA-4 blockade, dozens of clinical trials are currently examining the use of LAG3 blockade in cancer therapy. To date, data from only one phase III clinical trial and interim reports from two phase I/II trials examining anti-LAG3 combination with anti-PD-1 are available. The completed phase III trial, RELATIVITY-047 (NCT03470922), examined the combination of anti-LAG3 with anti-PD-1 in comparison with anti-PD-1 alone in 714 patients with untreated and unresectable or metastatic melanoma¹²². This trial found that duration of progression free survival, more than doubled to 10.1 months when anti-LAG3 was added to anti-PD-1¹²². One trial (NCT0198609) that examined the combination in patients with advanced

melanoma who failed previous PD-1 blockade, most of whom had also been treated with CTLA-4 blockade, discovered an objective response rate of 11.5% in 61 patients¹⁰⁸. In patients with tumor-associated immune cell LAG3 expression $\geq 1\%$, the response rate was 3.5-fold higher than for patients with $< 1\%$ LAG3 expression (18% vs 5%). The other study showed a durable response in 9.9% of patients, with higher rates in triple-negative breast cancer (2/5 patients) and mesothelioma (2/8 patients)¹²³.

1.4.5.2.3 Autoimmunity

LAG3 blockade is being investigated for treatment of cancer and some infections because LAG3 can be harmful in these contexts. Contrarily, LAG3's inhibitory effect is beneficial during autoimmunity. Therefore, enhancing, not inhibiting, LAG3 activity is the desired impact of therapies. Such a treatment would act upstream of current therapies (e.g., anti-TNF antibodies) for treatment of autoimmunity. For this purpose, an antibody with agonistic activities (IMP761) has been described and shown to inhibit proliferation and activation of T cells. Further, IMP761 reduced T cell infiltration and inflammatory gene signature in a primate model of delayed-type hypersensitivity⁸⁷. Since LAG3 expression and activity are beneficial in many models of autoimmunity, including graft versus host disease⁶¹, colitis⁴¹, myocarditis¹²⁴, diabetes⁶⁵ and others^{24,124,125}, a LAG3 agonistic antibody could have wide-reaching implications. Another approach for the treatment of autoimmunity is to use the expression of LAG3 as a marker of auto-reactive T cells and delete LAG3 expressing cells. This approach was recently used in a human trial for treatment of psoriasis¹²⁶.

Unfortunately, autoimmune disorders can be chronic diseases requiring long-term therapies. Antibodies are not well suited for long-term therapies due to the substantial cost and the frequent generation of anti-immunoglobulin antibodies that neutralize therapeutic antibodies. Therefore, while LAG3 has proven itself as an excellent target for treatment of autoimmune diseases, new types of LAG3-targeted therapies will be needed.

1.4.5.2.4 Safety

LAG3 blockade is expected to be safer than PD-1 or CTLA-4 blockade as, unlike PD-1 and CTLA-4-knockout mice, LAG3-knockout mice do not spontaneously exhibit immunopathology¹²⁷⁻¹²⁹. However, LAG3 inhibition as treatment is likely to be used in combination with blockade of other ICs, especially PD-1. Fortunately, results from RELATIVITY-047 show only a small increase in adverse effects when anti-LAG3 was added to anti-PD-1, and preliminary results of dual-blockade in previously treated patients show similar adverse events as PD-1 blockade alone, indicating that it may increase efficacy without greatly increasing harm^{108,122}.

1.5 Rationale, and objectives

1.5.1 Rationale

To achieve its primary purpose of protecting the body from infection and cancer without excessive damage to the host, the immune system must achieve an optimal immune balance. LAG3 and other immune checkpoints are crucial to regulating an overactive immune response. LAG3 itself is key in regulating immunity during

autoimmunity, cancer and infectious diseases. While this regulation may be desirable in cases of autoimmunity and some infectious diseases, such as HIV, it may be harmful during cancer or other infectious diseases.

The clearest case of LAG3 being harmful is cancer, in which case blocking LAG3 can substantially improve outcomes in animal models and many human clinical trials are underway. Alternatively, for treatment of autoimmunity, LAG3 activity can be enhanced, or as an indicator of auto-reactivity, LAG3-expressing cells may be depleted. At least 13 of these LAG3 targeting therapies are undergoing clinical trials, mostly for treatment of cancer¹³⁰.

Despite these investigations, much about LAG3 remains unknown. Perhaps the biggest gaps in knowledge for LAG3 are its relative expression and its functional mechanism.

LAG3 expression is mostly studied on T cells, despite also being expressed on B cells, NK cells, pDCs and neurons. While RNA sequencing has allowed mRNA comparisons, no comparative study of LAG3 protein expression on these different cell types has been published. Therefore, it is unclear what LAG3 expression levels are on different cells and how they compare to LAG3 expression on T cells. This lack of comparison between cells is particularly bleak because even within T cells, reported cell surface expression of LAG3 can vary dramatically between studies. Comparative and absolute LAG3 expression is important to know because of the many therapies designed to target LAG3. For example, it is important to know what types of cells the

LAG3 depleting antibody GSK2831781 may target, so that investigators can be aware of and anticipate possible side-effects or mechanisms of the treatment.

LAG3 activity inhibits cytokine production and proliferation of the T cells that express it. However, LAG3 function on other cells is unknown. Rather than indiscriminately searching for LAG3 function on every cell type on which it is expressed, it would be helpful to first characterize the mechanism on T cells. Understanding the LAG3 mechanism would also help inform the optimal combination of therapies. Unfortunately, since LAG3 lacks a known signaling motif, its signaling mechanism is largely unknown, even on T cells.

This thesis focuses on two overarching themes:

- 1) LAG3 expression profile, kinetics and mechanisms
- 2) LAG3 mechanism of action in T cells

1.5.2 Objectives

- 1) Design and deploy a flow cytometry panel with the ability to evaluate LAG3 expression on multiple cell types.
- 2) Design a model of LAG3 inhibition of T cell activation and appropriate comparable control.
- 3) Use the model of LAG3 inhibition to evaluate the impact of LAG3 on the TCR signaling pathway.

2 Materials and Methods

2.1 General reagents

2.1.1 Solutions

Phosphate buffered saline (PBS): Gibco PBS pH 7.4 (Fisher Scientific)

FACS Wash: PBS + 2% Fetal Calf Serum (FCS; inactivated at 56°C for 30 min; Corning)

R10 cell culture media: RPMI-1640 (Hyclone) + 10% FCS + 1% Penicillin/Streptomycin (Thermo Fisher Scientific)

R30 cell culture media: RPMI-1640 (Hyclone) + 30% FCS + 1% Penicillin/Streptomycin (Thermo Fisher Scientific)

Freezing media: 10% dimethyl sulfide (DMSO, tissue culture grade, Sigma) + 90% FCS

2.1.2 Plasmids

The plasmids packaged in the lentivirus and used to transduce the Jurkat clone E6-1 cell line were purchased from Origene. The full LAG3 open reading frame (ORF) (cat# RC220269) (cat# CW303487) or the ORF with the cytoplasmic domain deleted (cat# CW303484) was inserted into the pLenti-P2A-Puro lentiviral gene expression vector (cat# PS100109) using restriction endonucleases SgfI and MluI. The full sequences of each LAG3 insert can be found in appendix 9.1.

2.1.3 Antibodies and dyes

2.1.3.1 Ex vivo surface expression of LAG3

Table 1 Surface staining panel used for flow cytometry of PBMC in ex vivo staining

Marker	Clone	Host species	Fluorochrome	Volume used (µl) or dilution
CD3	UCHT1	Mouse	PeCy7	2.5
CD14	M5E2	Mouse	APC-Fire	2.5
HLA-DR	G46-6	Mouse	APC	2.5
CD16	3G8	Mouse	PE-Dazzle	5
CD19	HIB19	Mouse	BV605	1.25
CD123	7G3	Mouse	PeCy5.5	5
CD8	RPA-T8	Mouse	BV650	4µl of 1:20
CD56	NCAM16.2	Mouse	APC-700	1.25
CD38	HIT2	Mouse	BV711	5
iNKT TCR	6B11	Mouse	BV421	5
PD-1	EH12.2H7	Mouse	BV785	1.25
LIVE/DEAD			BV510	1:100 (stain 5µl)
LAG-3	Polyclonal	Goat	PE	10
LAG-3	17B4	Mouse	FITC	2.5
Isotype	IgG1 ctrl	Mouse	FITC	10
Isotype	Polyclonal	Goat	PE	10

2.1.3.2 All other flow cytometry of PBMC

Table 2 Antibodies used to stain PBMC in experiments on LAG3 blockade and LAG3 kinetics

Marker	Clone	Host species	Fluorochrome	Volume used (µl) or dilution
CD3	UCHT1	Mouse	Alexa Fluor 700	5
LAG-3	17B4	Mouse	FITC	5
CD4	RPA T4	Mouse	APC-H7	2.5
CD8	RPA T8	Mouse	V500	2.5
TNF-α	Mab11	Mouse	BV650	5
IFN-γ	B27	Mouse	Alexa Fluor 647	5
IL-2	5344.111	Mouse	BV421	5
Vβ3	JOVI.3	Mouse	PE	5
Vβ14	REA557	Human	PE	10
Vβ17	E17.5F3.15.13	Mouse	PE	20
Secondary	polyclonal	Goat	PE	1
LIVE/DEAD			Far Red	1:20 (stain 5µl)
CFSE				

2.1.3.3 Flow cytometry for Jurkat cells

Table 3 Antibodies used to stain Jurkat cells

Marker	Clone	Host species	Fluorochrome	Volume used (μ l) or dilution
CD3	UCHT1	Mouse	Alexa Fluor 700	5
LAG-3	T47-530	Mouse	PE	5
CD4	RPA T4	Mouse	APC-H7	2.5
CD2	RPA-2.10	Mouse	APC	5
CD28	CD28.2	Mouse	PE	5
LIVE/DEAD			AquaVivid	1:100 (stain 5 μ l)
CFSE				

2.1.4 Toxins and stimulants

Staphylococcus enterotoxin B: Purchased from Toxin Technology Inc

Recombinant Staphylococcus enterotoxin E: Purchased from Cusabio

Recombinant Staphylococcus enterotoxin D: Purchased from Toxin Technology Inc

Flagellin (Fla-ST ultrapure): Purchased from Invivogen

Lipopolysaccharide (LPS): Purchased from Invivogen

Anti-CD3/anti-CD28 beads: Dynabeads Human T-Activator CD3/CD28 for T cell

Expansion and Activation (Gibco)

2.2 Biological samples

2.2.1 Ethics

Ethical approval was obtained from the University of Manitoba research ethics board for all studies presented in this thesis. Participants were informed of the studies being performed and gave informed consent.

2.2.2 Source of peripheral blood

All samples used in this project were collected from local donors at the University of Manitoba.

2.2.3 Processing of peripheral blood

Peripheral blood samples were collected in heparin vacutainers and processed for peripheral blood mononuclear cell (PBMC) collection through density gradient centrifugation. Whole blood samples were centrifuged for 7 minutes at 600xg and plasma was removed and discarded. The remaining volume was diluted in FACS wash, layered on Ficoll (Lymphoprep; MJS BioLynx Inc.) and centrifuged for 25 minutes at 400xg with no brake. PBMC were collected and washed in FACS wash, then centrifuged for 10 minutes at 600xg. PBMCs were then washed in R10 cell culture media and centrifuged for 10 minutes at 400xg. Finally, PBMCs were resuspended in R10 cell culture media and counted using trypan blue (HyClone) dye exclusion. PBMC

were rested for between 2 and 4 hours in R10 cell culture media before being assayed or activated.

2.2.4 Cell lines

The Jurkat E6-1 and Raji B cell lines were purchased from ATCC and maintained in R10 media + 2.5 ug/ml of Plasmocin™ (Invivogen). Jurkat cells and the created derivative cell lines were maintained at a cell density of 100,000-1,000,000 cells/ml, while Raji B cells were maintained at a density between 500,000 and 2,000,000 cells/ml. Both cell lines were cultured in a T-75 culture flask at 37°C and 5% carbon dioxide and culture medium was replaced every 2-3 days.

After initial culture, cells were grown for two weeks before being frozen into working stocks, which were then thawed and used for up to four months before being discarded.

2.3 General methods

2.3.1 Flow cytometry

2.3.1.1 Surface staining

Cells were washed with FACS wash and transferred to 5ml polystyrene FACS tubes or 96 well V-bottom plates (Sarstedt) for staining. Between 5×10^4 and 1×10^6 cells were stained per well in a volume of 50µl or 100µl. Cells were pelleted by centrifugation at 500xg for 3 minutes with no brake. The supernatant was vacuumed for disposal and

cells were resuspended and washed in FACS wash (centrifuged at 500xg for 3 minutes with no brake). Each well of cells was then resuspended in 50 μ l or 100 μ L of surface staining antibody master mix and incubated for 30 minutes in the dark at 4°C. Cells were then washed twice in FACS wash before being resuspended in 300 μ L of 1% paraformaldehyde fixation buffer (BD Bioscience) or permeabilized for intracellular staining. The fixed cells were transferred to 5mL FACS tubes for acquisition by the LSRII flow cytometer.

2.3.1.2 Cytokine staining

Surface stained cells were resuspended in permeabilization buffer (CytoPerm; BD Bioscience) at 4°C for 20 minutes in the dark. Cells were centrifuged at 500xg for 3 minutes with no brake. Next, after each well of cells was resuspended in 50 μ L intracellular cytokine staining antibody mastermix, cells were incubated for 30 minutes in the dark at 4°C. Cells were then washed with Permeabilization wash buffer, resuspended in FACS wash, and transferred to 5mL FACS tubes for acquisition by the LSRII flow cytometer.

2.3.1.3 CFSE staining

Cells were suspended at 0.5-10x10⁶/ml in 1ml of R10 at room temperature in a 15ml conical tube. Next, 1 μ l of 5mM CFSE was added to the lid of the conical tube. The tube was inverted and mixed by vortex and incubated in the dark for 5 minutes at room temperature. Cells were then washed in 10ml of R10 three times before being returned to culture.

2.3.1.4 Compensation

The use of multiple fluorochromes in flow cytometry results in the potential of overlapping emission spectra between fluorochromes. Compensation allows a reduction from the signal obtained in the channel of interest resulting from a secondary channel. The BD FACS Diva software has an algorithm capable of compensating for the spectral overlap using single stained samples for each fluorochrome.

Compensations for surface and intracellular antibody stains were performed using single stained CompBeads (BD Bioscience) that bind mouse antibodies for these experiments. One drop of mouse CompBeads and 1 drop of negative control CompBeads was added to 400 μ L of FACS wash and mixed. One hundred μ L of this bead mixture was transferred to a 5mL FACS tube for each marker in the panel. Each tube had 1 μ L of a single antibody added to the bead mixture and were incubated for 10 minutes at room temperature in the dark. The bead mixtures were then diluted to a final volume of 300 μ L with FACS wash and acquired on the LSRII flow cytometer.

Compensations for the amine reactive live/dead viability dye were prepared using ArC Beads (Invitrogen). One drop of ArC amine beads and 1 μ L of Live/Dead stain was mixed together in a 5mL FACS tube and incubated for 30 minutes at room temperature in the dark. In a separate tube, one drop of ArC negative beads was added along with 200 μ l of FACS wash before being acquired on the LSRII flow cytometer.

Compensation for CFSE staining was performed using CFSE stained and unstained cells.

2.3.1.5 Data acquisition and analysis

Cells were acquired on the LSRII flow cytometer (BD Bioscience). Gate placement was defined through the use of fluorescence minus one (FMO) controls. FMO controls were made by staining samples with all antibodies in the panel except for the target antibody. This allows the gate to accommodate background and spillover fluorescence in the respective channel.

Data acquisition and compensation was performed using BD FACS Diva software (v6.1.2; BD Bioscience) and was exported and analyzed using FlowJo software (v7.6.5; Tree Star Inc.).

2.4 Surface expression of LAG3 on cell subsets

2.4.1 Flow cytometry optimization

Optimal voltages for this panel were determined by voltage titration first with unstained cells, ensuring a robust SD fluorescence for each channel of 3x the electronic noise robust standard deviation (rSD).

Antibody titration was performed for each antibody to determine the optimal dilution of antibody with which to stain the cells as determined by maximizing staining index without losing positive population. Staining index = (median fluorescence of positive population – median fluorescence of the negative population) / 2x rSD of the negative population.

Finally, voltage titration was performed by adjusting laser voltages to maximize staining index without losing positive population.

2.4.2 Analysis

PBMC from each donor were stained with the full panel as indicated in Table 1. In parallel, PBMC from each donor were stained to obtain fluorescence minus one (FMO) conditions for CD38, HLA-DR, CD123 to facilitate gating. Additionally, PBMC from each donor were stained with isotype control antibodies (mouse IgG1, FITC, and Goat IgG, PE) to control for Fc receptor binding. LAG3 expression was determined as the amount of binding above the corresponding isotype control.

Isotype control antibodies were also used for comparison of monoclonal and polyclonal antibody binding to Jurkat cells.

2.5 LAG3 expression kinetics

2.5.1 Activation with anti-CD3/anti-CD28 beads

PBMC isolated from healthy donors were rested for 2-4 hours. 25 μ l of Anti-CD3/anti-CD28 beads per million PBMC were then placed in a 5ml FACS tube. 2ml of PBS was added to the beads, then the tube was vortexed and placed in a magnetic separator for one minute. With the tube in the magnetic separator, the remaining volume was discarded. R10 was then added to the beads and the beads were then

added to the cells in a final volume of 0.5ml in a 24 well plate. The cells were then incubated for the designated time, with or without the addition of LPS or Flagellin.

After the stimulation, cells were added to a 5ml FACS tube. The tube was vortexed and then placed in the magnetic separator for 1 minute. The supernatant was poured into a separate 5ml FACS tube, then 1ml was added to the tube with the beads remaining. The tube was once again vortexed and placed in the magnetic separator for 1 minute before the supernatant was poured into the same separate 5ml FACS tube. The cells were then either replaced in culture for the designated duration in fresh R10, or they were counted and 1×10^6 cells were stained.

2.6 Proliferation and cytokine detection

2.6.1 Proliferation measurement

PBMC isolated from healthy donors were rested for 2-4 hours. Cells were then stained with CFSE as in section 2.3.1.3. 500,000 PBMC were then placed in 500 μ l of R10 + 0.01ng/ml SEB in each well of a 24 well plate for 96 hours in the presence of the blocking antibody or an isotype control. After the stimulation, cells were stained with CD3, CD4, CD8, TCR V β 3, TCR V β 14 and TCR V β 17 and flow cytometry was performed.

2.6.2 Cytokine and LAG3 expression measurement

Healthy donor PBMC were incubated in R10 + 0.1ng/ml or 0.01ng/ml of SEB for three days. Cells were then placed in a 5ml FACS tube and centrifuged at 600g for 5

minutes. Next, 2ml of R10 was added to the cells and they were centrifuged again. Cells were then washed and rested for up to three days. Six hours before being removed and stained Golgi stop (BD Biosciences) was added to the cells at the manufacturer's recommended concentration.

2.7 Creation of LAG3⁺ cell lines

2.7.1 Lentiviral production

Replication incompetent VSV-G pseudotyped HIV-1 based lentiviral vectors were produced by the University of Manitoba Lentiviral Core Facility using a second-generation packaging system. Briefly, 293T cells were transfected with packaging and envelope plasmids along with the transfer plasmid described in section 2.1.2 using calcium phosphate-based transfection methods. Supernatants were then ultracentrifuged to concentrate lentiviral particles and frozen at -20°C until transduction.

2.7.2 Lentiviral transduction

One day prior to lentiviral transduction of Jurkat cells, media was changed to ensure log phase growth. The next day, 1×10^5 Jurkat cells were transferred to a 15ml conical tube and centrifuged at 400g for 7 minutes. The supernatant was disposed of and 250µl of lentivirus mixture was added. Lentivirus mixture is composed of 1×10^5 viral particles and 4ug/ml of polybrene.

Cells were then incubated at 37°C for 2 hours before being washed in 5ml of R10. Cells were then resuspended in 0.5ml and plated in 24 well plate for 72 hours. Next, cells were transferred to 0.4ug/ml of puromycin (Sigma) for selection. 72 hours later, all cells in the untransduced condition were dead by trypan blue staining. Transduced and untransduced cells were then placed in R10 for further culture. Untransduced cells were kept for one week to ensure no growth occurred before being disposed.

2.8 LAG3 function on cell lines

2.8.1 Activation for IL-2 detection

Cell lines were passaged one day prior to activation. On the day of activation, 5×10^4 Raji B cells were plated in a 96 well plate. SEE or SED was added to each well at 2x the concentration indicated for each condition. After 10 minutes, 5×10^4 cells of each Jurkat cell line were added to the appropriate wells, bringing the total volume to 250 μ l and the concentration of SEE/SED to the concentration indicated. Cells were then incubated for 24 hours before the supernatant was removed for IL-2 enzyme-linked immunosorbent assay (ELISA).

2.8.2 IL-2 ELISA

ELISA MAX™ Deluxe set Human IL-2 ELISA kit was purchased from BioLegend. The BioLegend protocol was followed. Each sample was plated in duplicate, including

IL-2 standard. Many of the samples were diluted up to 1/100 to be in range of the standard curve. The average value of the duplicates was used in the final data.

2.8.3 Activation for kinomics array

Cell lines were passaged one day prior to activation. On the day of activation, Jurkat cell lines were transferred to serum-free RPMI and incubated for three hours. After the incubation, 1×10^6 Raji B cells were placed in 62.5 μ l of RPMI in a 1.5ml capped tube conical tube. SEE or SED was added to each tube at 10ng/ml and 2000ng/ml, respectively. After 10 minutes, 1×10^6 cells of each Jurkat cell line were added to the appropriate tubes, bringing the total volume to 250 μ l and the concentration of SEE/SED to 5ng/ml or 1000ng/ml, respectively. Tubes were immediately centrifuged for 1 minute at 150g. After centrifugation, tubes were either incubated at 37°C for the designated time, or immediately submerged in a bath of dry ice and ethanol to flash freeze the cells.

2.8.4 Kinomics array

Cell pellets were thawed on ice, then immediately lysed in 150 μ l of Phosphoarray lysis buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton-X100; 1X Pierce protease/phosphatase inhibitor) on ice for 10 minutes. The tube was then centrifuged at 10,000g for 10 minutes at 4°C. Supernatant was removed and placed in a new tube. Protein concentration was then determined by BCA assay using a BSA standard. While the remaining volume was kept on dry ice.

The lysates were again thawed and 14 μ l of activation mix (50% glycerol, 50 μ M adenosine triphosphate (ATP), 60 mM MgCl₂, 0.05% Brij-35, 0.25 mg/mL BSA, 1x Pierce protease/phosphatase inhibitor) was added to 90 μ l of lysate. Sample mixture was then evenly added to the PepStar peptide microarrays (JPT Innovative Peptide Solutions). The peptide array was then covered with a microscope slide and incubated at 37°C for 2 hours. The cover slip was then removed in PBS + 1% Triton X and arrays were washed in distilled H₂O. Arrays were then submerged in ProQ Diamond Phosphostain and incubated while rocking in dark for 1 hour. Arrays were then destained with 20% acetonitrile and 50mM sodium acetate. Arrays were then washed while rocking with dH₂O and air dried for 10 minutes before spin drying in a centrifuge for 10 minutes. Arrays were then scanned on a PowerScanner microarray scanner (Tecan) with a 580-nm filter imaging arrays and Array-Pro Analyzer version 6.3 software (Media Cybernetics) collecting signal intensity.

2.8.5 Phospho-flow cytometry

Cell lines were passaged one day prior to activation. On the day of activation, Jurkat cell lines were transferred to serum-free RPMI and incubated for three hours. After the incubation, 1x10⁶ Raji B cells stained with CFSE were placed in 62.5 μ l of RPMI in several wells of a 96-well U-bottom plate. SEE or SED was added to each well at 10ng/ml and 2000ng/ml, respectively. After 10 minutes, 1x10⁶ cells of each Jurkat cell line were added to the appropriate wells, bringing the total volume to 125 μ l and the concentration of SEE/SED to 5ng/ml or 1000ng/ml, respectively. Plates were immediately centrifuged for 1 minute at 150g and incubated at 37°C.

After designate time point, an equivalent amount of 4% paraformaldehyde was added to the wells and the plate was incubated for a further 10 minutes at 37°C. The cells were then moved to 5ml polystyrene tubes and washed twice with PBS before 500µl of ice-cold methanol was added slowly. Cells were kept at -20°C for 30 minutes before being stained with Pacific Blue™ labelled succinimidyl ester (ThermoFisher Scientific) dissolved in DMSO, or in DMSO alone for fluorescent barcoding. Immediately after succinimidyl ester was added, 500µl of PBS was added and cells were incubated for 20 minutes. Cells were then washed three times with PBS before being combined into a single tube for each stimulation condition. Cells were then stained with 20µl of PE labelled anti-ERK1/2 phospho (Thr292/Tyr204) (clone: 6B8B69) in FACS wash for one hour at room temperature before being washed three times with FACS wash and run on the BD LSRII flow cytometer.

2.9 Statistics

All statistical analysis, other than for kinomics, was performed using GraphPad Prism (v 6.0; GraphPad Software). Wilcoxon Rank Sum test (non-parametric, paired, continuous variables) was used to compare cytokine production and LAG3 expression on primary cells, including the comparison of monoclonal antibody to polyclonal antibody. A student's t test was used to compare cytokine production from cell lines.

For the kinomics array, the Platform for Integrated, Intelligent Kinome Analysis (PIIKA 2) software was used to obtain fold changes and p-values by t test (<http://saphire.usask.ca.uml.idm.oclc.org/saphire/piika/index.html>).

3 Thesis-related publications

3.1 Peer reviewed articles

1. Graydon CG, Mohideen S, Fowke KR. LAG3's Enigmatic Mechanism of Action. *Front Immunol.* 2021;11:3444. doi:10.3389/fimmu.2020.615317

Description:

This paper examines mechanisms regulating LAG3 expression and investigates the LAG3 mechanism of action, including ligand binding and the role of the cytoplasmic domain.

2. Graydon CG, Balasko AL, Fowke KR. Roles, function and relevance of LAG3 in HIV infection. Hobman TC, ed. *PLOS Pathog.* 2019;15(1):e1007429. doi:10.1371/journal.ppat.1007429

Description:

This paper examines the potential roles for LAG3 within the context of HIV infection, including a consideration of its role on different cells and its potential as a target in a functional cure.

3.2 Poster presentations

Graydon C, Mohideen S, Fowke KR. The use of LAG3+ cell lines in the study of the LAG3 mechanism. Poster session presented at: Canadian Student Health Research Forum; 2021 June 11-15; Online.

Graydon C, Mohideen S, Fowke KR. Development of LAG3+ cell lines and their use for studying the LAG3 mechanism. Poster session presented at: The Canadian Association for HIV Research (CAHR); 2021 May 5-7; Online.

Graydon C, Kowatsch M, Balasko A, Lajoie J, Fowke KR. Differences in LAG3 detection by flow cytometry. Poster session presented at: Canadian Student Health Research Forum; 2020 August 24-28; Online.

Graydon C, Kowatsch M, Balasko A, Lajoie J, Fowke KR. Detecting LAG3: antibody discrepancies in flow cytometry. Poster session presented at: The Canadian Association for HIV Research (CAHR); 2020 May 1-2; Online.

Graydon C, Kowatsch M, Balasko A, Lajoie J, Fowke KR. TLR stimulation downregulates the immune checkpoint LAG3. Poster session presented at: Canadian Student Health Research Forum; 2019 June 10-14; Winnipeg, Canada

Graydon C, Lajoie J, Kindrachuk J, Fowke KR. T cell receptor signaling is inhibited by the immune checkpoint LAG3, which impairs immunity during chronic infectious disease. Poster session presented at: Canadian Society of Microbiologists; 2018 June 18-21; Winnipeg, Canada

Graydon C, Lajoie J, Kindrachuk J, Fowke KR. Lymphocyte Activation Gene-3 inhibits proximal T cell Receptor Signaling. Poster session presented at: Canadian Student Health Research Forum; 2018 June 11-15; Winnipeg, Canada

Graydon C, Lajoie J, Kindrachuk J, Fowke KR. T cell kinase activity is affected by LAG3, an immune checkpoint that impairs immunity during chronic infectious disease. Poster session presented at: AMMI-CACMID national conference; 2018 May 2-5; Vancouver, Canada.

Graydon C, Lajoie J, Kindrachuk J, Fowke KR. Kinome changes associated with LAG3 stimulation. Poster session presented at: The Canadian Association for HIV Research (CAHR); 2018 April 26-29; Vancouver, Canada.

Graydon C, Kindrachuk J, Lajoie J, Fowke KR. Deciphering the mechanics of the LAG-3 immune brake in T cells. Poster session presented at: CanCURE Annual Meeting; 2017 Oct 19-20; Montreal, Canada.

Graydon C, Stalker AT, Juno JA, Lajoie J, Fowke KR. Deciphering the mechanics of the LAG-3 immune brake in T cells. Poster session presented at: Mucosal Immunology Symposium; 2017 Sep 27-28; Winnipeg, Canada

Graydon C, Stalker AT, Juno JA, Lajoie J, Fowke KR. LAG-3 Inhibition of T Cell Activation. Poster session presented at: Canadian Student Health Research Forum; 2017 Jun 6-9; Winnipeg, Canada

Graydon C, Stalker AT, Juno JA, Boily-Larouche G, Lajoie J, Fowke KR. LAG-3 Inhibition of T cell Activation. Poster session presented at: The Canadian Association for HIV Research (CAHR); 2017 April 6-9; Montreal, Canada.

Graydon C, Stalker AT, Juno JA, Boily-Larouche G, Lajoie J, Fowke KR. Assessing the LAG-3 inhibitory mechanism on T cell activation. Poster session presented at: CanCURE Annual Meeting; 2016 Nov 8-11; Montreal, Canada.

Graydon C, Stalker AT, Juno JA, Boily-Larouche G, Lajoie J, Fowke KR. Assessment of the LAG-3 inhibitory mechanism on T cell activation. Poster session presented at: Canadian Student Health Research Forum; 2016 Jun 7-9; Winnipeg, Canada.

Graydon C, Stalker AT, Juno JA, Boily-Larouche G, Lajoie J, Fowke KR. Assessing the LAG-3 mechanism and inhibition of T cell activation. Poster session presented at: The Canadian Association for HIV Research (CAHR) Conference; 2016 May 12-14; Winnipeg, Canada

Graydon C, Stalker AT, Juno JA, Boily-Larouche G, Lajoie J, Fowke KR. Assessing the LAG-3 mechanism and inhibition of T cell activation. Poster session presented at: CancerCare Research Days; 2016 May 9; Winnipeg, Canada

Graydon C, Stalker AT, Juno JA, Boily-Larouche G, Lajoie J, Fowke KR. Assessment of the LAG-3 mechanism on the inhibition of T cell activation. Poster session presented at: CanCURE Annual Meeting; 2015 Nov 12-13; Montreal, Canada.

Graydon C, Stalker AT, Juno JA, Boily-Larouche G, Lajoie J, Fowke KR. Assessment of the LAG-3 mechanism on the inhibition of T cell activation. Poster session presented at: Canadian Student Health Research Forum; 2015 Jun 2-5; Winnipeg, Canada.

Graydon C, Stalker AT, Juno JA, Boily-Larouche G, Lajoie J, Fowke KR. Assessment of the LAG-3 mechanism on the inhibition of T cell activation. Poster session presented at: CanCURE Annual Meeting; 2014 Nov 20-21; Montreal, Canada.

4 Ex vivo surface expression of LAG3

4.1 Rationale

LAG3 research has focused on T cells. However, LAG3 is also expressed on B cells, NK cells, plasmacytoid dendritic cells (pDC) and even neurons²⁸⁻³¹. An inconsistency in the literature is that the proportion of cells expressing LAG3 often varies substantially between studies, and no study has published a comparison of LAG3 protein expression across these cell types. Due to the post-translational mechanisms regulating LAG3 surface expression, including intracellular storage, endosomal recycling and cleavage, such a comparison must specifically measure LAG3 expressed on the cell surface, where it is functional.

4.2 Hypotheses

1. LAG3 is expressed at low levels on resting lymphocytes and at higher levels on monocytes.
2. The variation in LAG3 expression reported in the literature is due to use of different antibodies.

4.3 Objectives

1. Create a broad panel to detect LAG3 expression across lymphocytes and monocytes.
2. Compare different antibodies targeting LAG3.

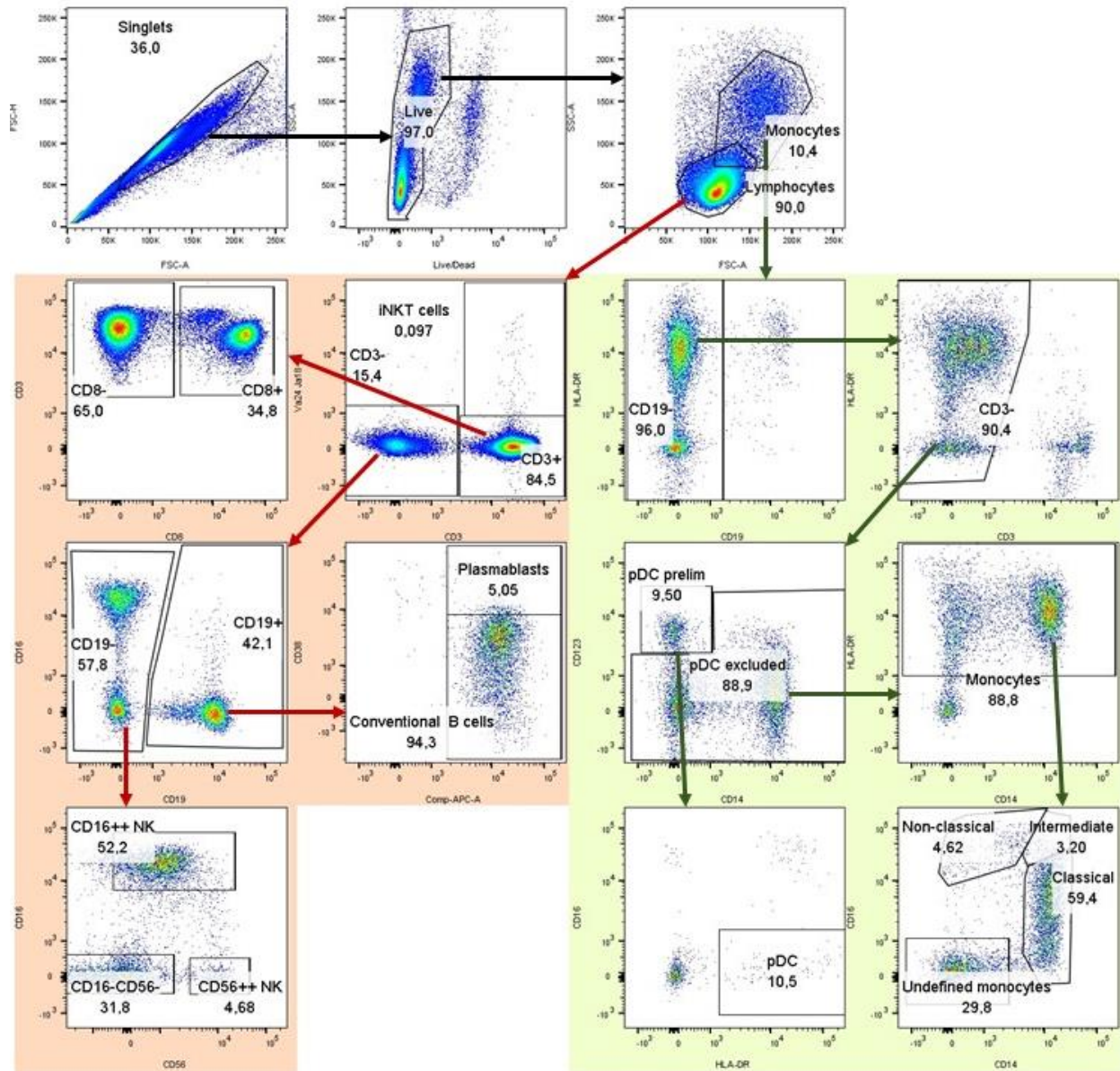
4.4 Results

4.4.1 LAG3 across cell subsets

4.4.1.1 *Panel*

PBMC were isolated from the blood of eight healthy blood donors from Winnipeg, Manitoba. Cell types and subsets were classified as shown in

Table 4 and gating strategy is shown in Figure 2. Representative ex vivo gating for identification of populations.



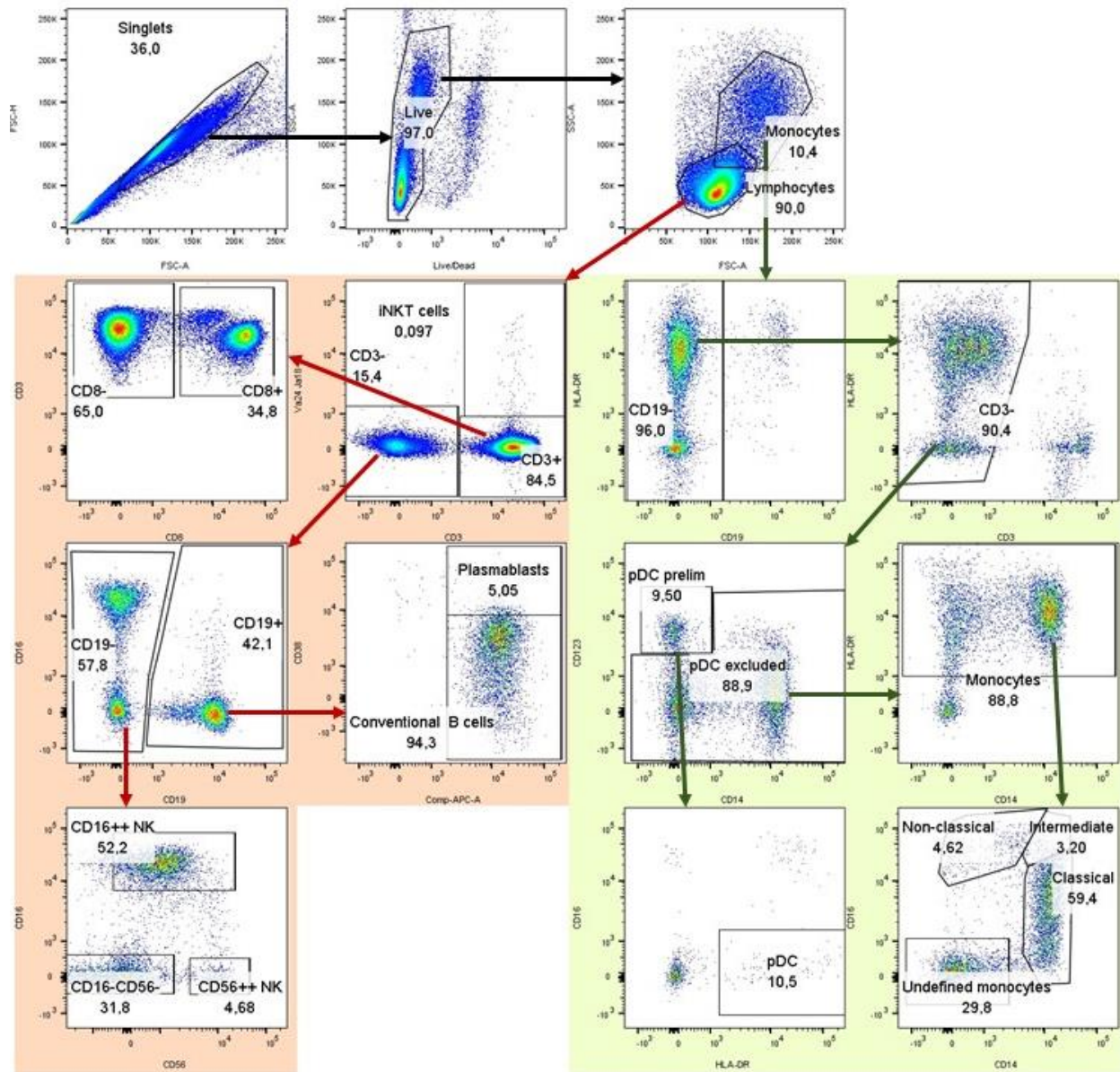
. Monocyte and NK types are separated by expression of CD14 and CD16, and CD16 and CD56, respectively, as is typical¹³¹, while plasma cells were separated from conventional B cells by high expression of CD38 as per OMIP-24¹³².

Table 4 Flow cytometric classification of cell types and subsets by marker

Cell subset	Markers used to define
iNKT cells	CD3 ⁺ Vα24 Jα18 TCR ⁺
Classical monocytes	CD3 ⁻ CD19 ⁻ CD123 ⁻ HLA-DR ⁺ CD14 ^{hi} CD16 ⁻
Intermediate monocytes	CD3 ⁻ CD19 ⁻ CD123 ⁻ HLA-DR ⁺ CD14 ^{hi} CD16 ⁺

Non-classical monocytes	CD3 ⁻ CD19 ⁻ CD123 ⁻ HLA-DR ⁺ CD14 ^{med/low} CD16 ⁺
Plasmacytoid dendritic cells	CD3 ⁻ CD19 ⁻ CD123 ⁺ CD14 ⁻ CD16 ⁻ HLA-DR ⁺
CD56⁺⁺ NK	CD3 ⁻ CD19 ⁻ CD16 ⁻ CD56 ^{hi}
CD16⁺⁺ NK	CD3 ⁻ CD19 ⁻ CD16 ⁺
Plasma cells	CD3 ⁻ CD19 ⁺ HLA-DR ⁺ CD38 ^{hi}
Conventional B cells	CD3 ⁻ CD19 ⁺ HLA-DR ⁺ CD38 ^{med/low}
CD8⁺ T cells	CD3 ⁺ CD8 ⁺
T cells	CD3 ⁺

Figure 2. Representative ex vivo gating for identification of populations.



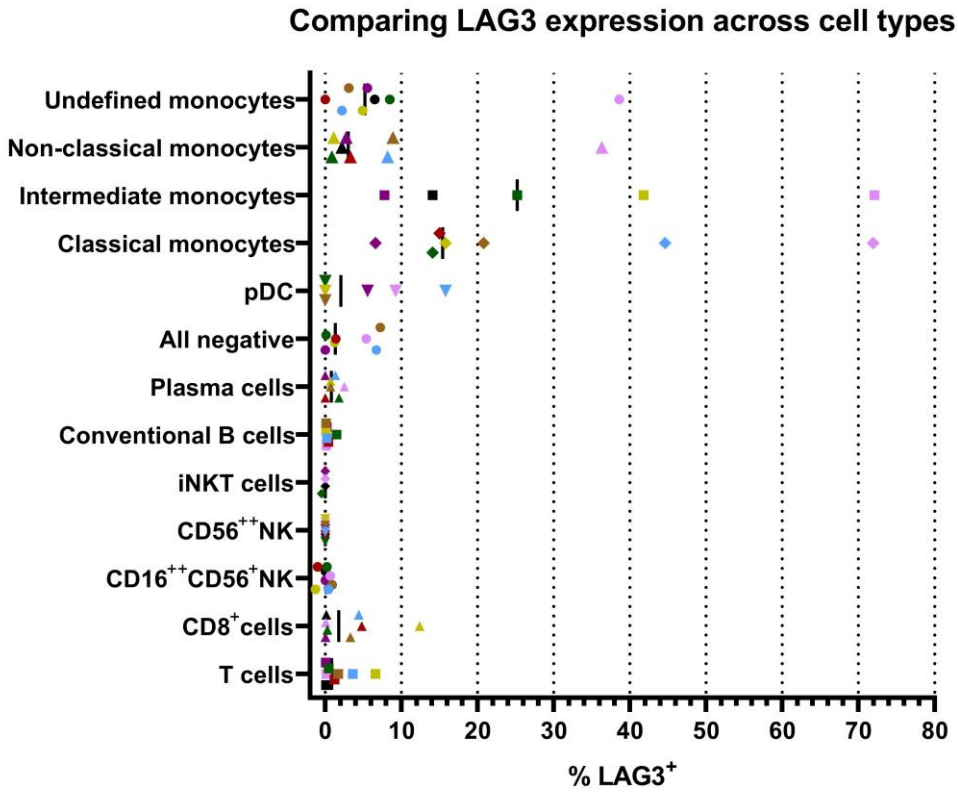
Red arrows show the order of gating. Singlets were identified by FSC-area (FSC-A) versus FSC-height (FSC-H), then live cells were selected based on negative staining for Live/Dead™ Fixable Aqua Dead Cell Stain kit. From there, lymphocytes were separated from monocytes based on forward and side scatter gating. From the Lymphocyte gate (red background), T cells and iNKT cells were identified as CD3⁺ and CD3⁺ Va24 Ja18⁺ staining, respectively. CD3⁻ cells were separated into CD19⁺ and CD19⁻ gates to allow further discrimination of B cells into plasma cells and conventional B cells by CD38, or of NK cells into CD16⁺⁺ or CD56⁺⁺ subsets, respectively. From the monocyte gate (green background), CD19⁻CD3⁻ cells were analyzed by CD123 expression and CD14 exclusion for further identification of pDCs by HLA-DR staining and CD16 exclusion, while other CD19⁻CD3⁻ cells were separated into monocyte subsets by CD14 and CD16 gating after HLA-DR⁺ cells were selected.

4.4.1.2 LAG3 expression across cell populations

PBMC LAG3 expression for each cell population was determined by staining with a monoclonal antibody that recognizes LAG-3 and assessing events occurring above a gate based on an isotype control. The percent of each cell population that were detected as LAG3⁺ were subtracted by the percent LAG3⁺ in the isotype-stained control.

As shown in Figure 3, LAG3 expression was highest in monocyte populations, with the highest expressers overall being classical and intermediate monocytes, where an average of 25% and 32% of cells express LAG3, respectively. LAG3 expression on lymphocytes was uniformly low, with means of 3.1% and 1.7% and medians of 1.8% of and 0.83% of CD8⁺ T cells and total T cells being LAG3⁺, respectively. All other lymphocyte populations were <1% LAG3⁺ (Figure 3). pDCs, which can arise from either myeloid or lymphoid progenitors, had intermediate LAG3 expression averaging 4.7%. Interestingly, 2.8% of cells that were negative for all tested markers expressed LAG3, higher than all lymphocytes except CD8⁺ T cells.

Figure 3 LAG3 expression across cell populations.

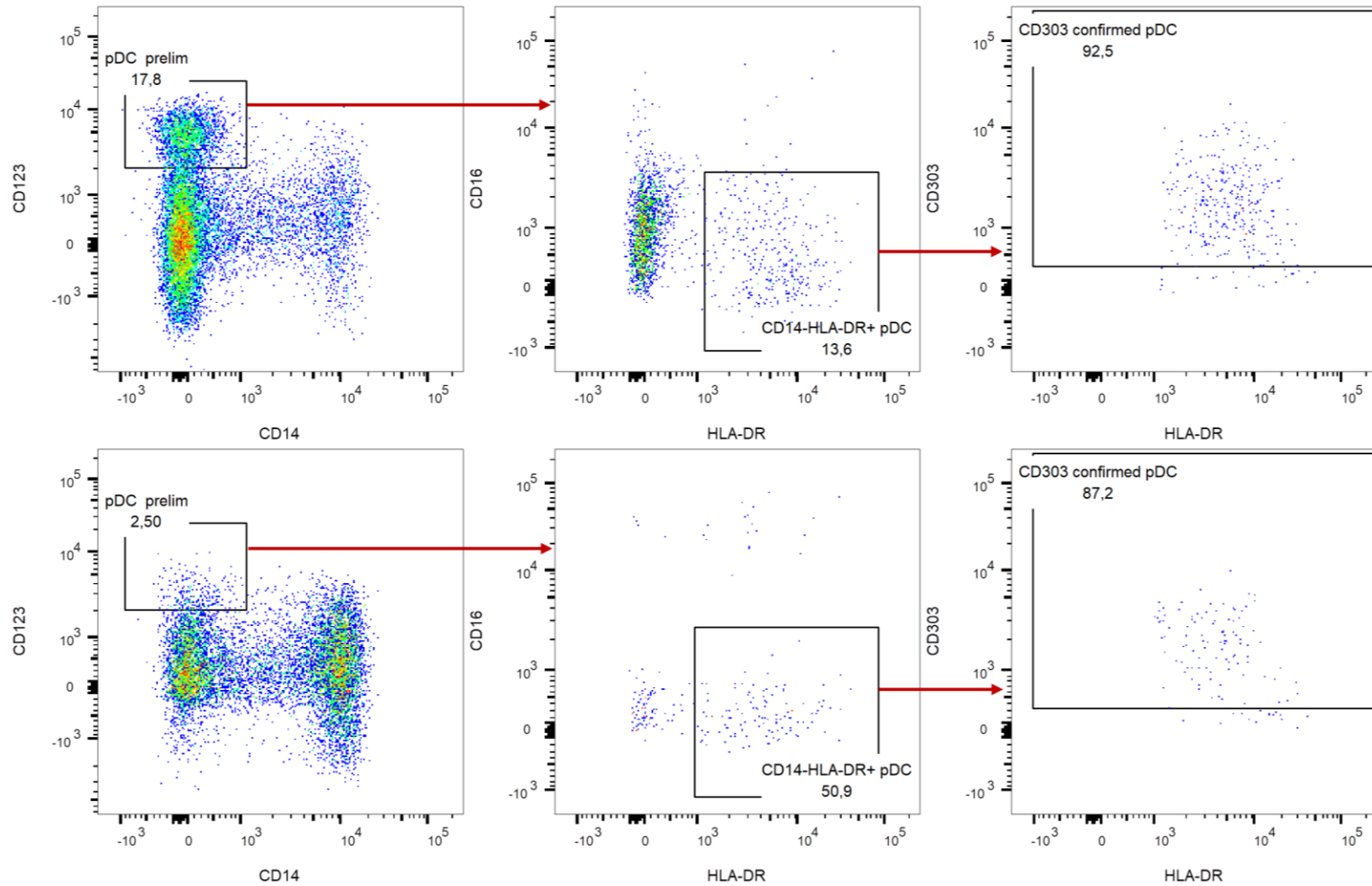


Populations outlined in Figure 2 identified from PBMC of healthy donors (n=8) were assessed for LAG3 expression based on staining by monoclonal antibody (clone: 17B4, FITC). Percent of cells staining positive were subtracted by the percent of cells in each population stained positive with an isotype control. Populations with less than 30 events were excluded. Mean +/- SD is shown by error bars

4.4.1.2.1 Confirmation of pDC population

While the identification of pDCs used in this panel has been confirmed before in OMIP-44¹³³, usually a classical pDC marker such as CD303 or CD304 is used to identify these cells². To confirm that the cells gated as pDC do express the classical pDC marker CD303, LAG3 was substituted for CD303 in our panel and applied this to the PBMC of two healthy donors. In both donors, approximately 90% of the cells were identified as pDC in our panel express CD303 and are, therefore, confirmed pDC. This is adequate specificity for our purposes.

Figure 4 pDC confirmation by CD303.



PBMC from two healthy donors were stained with the full panel previously described, but with LAG3-PE substituted by CD303-PE. Staining for each donor is shown above.

4.4.2 Monoclonal and polyclonal antibody discrepancies

4.4.2.1 Discrepancies in the literature

Reports of LAG3 expression in the literature are inconsistent. Limiting these to measurement of LAG3 expression on healthy donors, average expression on T cells has been reported as low as <1% or as high as 8%^{56,95,96,134–139}. A pattern emerges upon deeper reading of these reports, with those reporting high LAG3 expression often using a goat polyclonal antibody (pAb) from R&D Systems Inc. (Cat #: FAB2319P), while those reporting low LAG3 expression use a monoclonal antibody (mAb) (usually clones 17B4, 3DS223H or T47-530). mAb binding is generally more specific than that of pAbs, while pAbs can be more sensitive due to the ability to bind multiple epitopes on the same protein¹⁴⁰.

4.4.2.2 Differences in LAG3 detection based on antibody

Because of differences in LAG3 expression reported in the literature, detection of LAG3 by pAb was compared to detection by mAb across cell populations using an optimized flow cytometry panel.

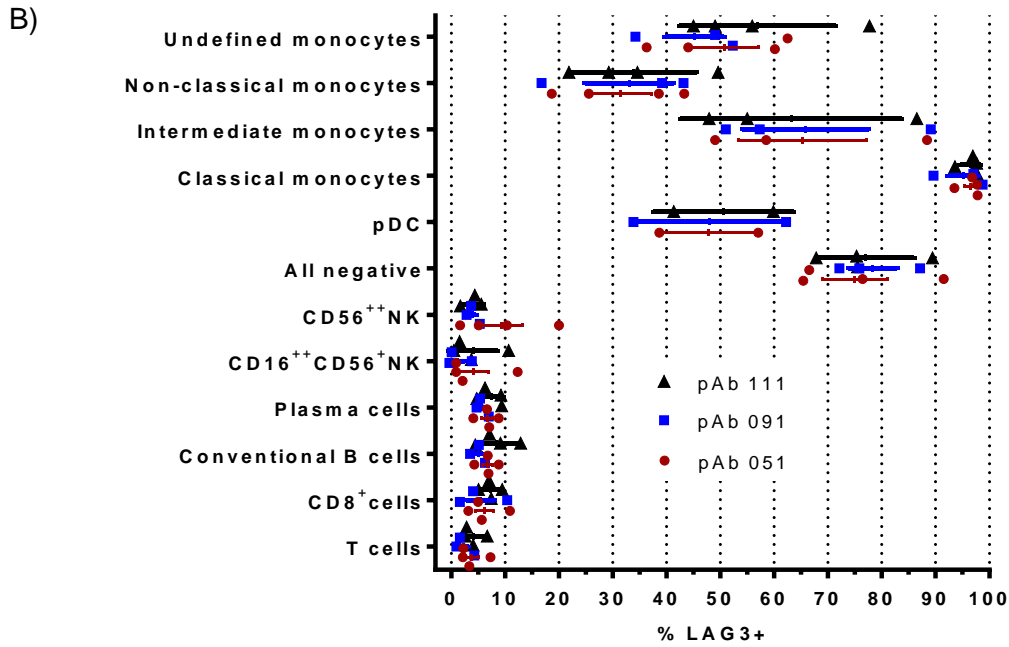
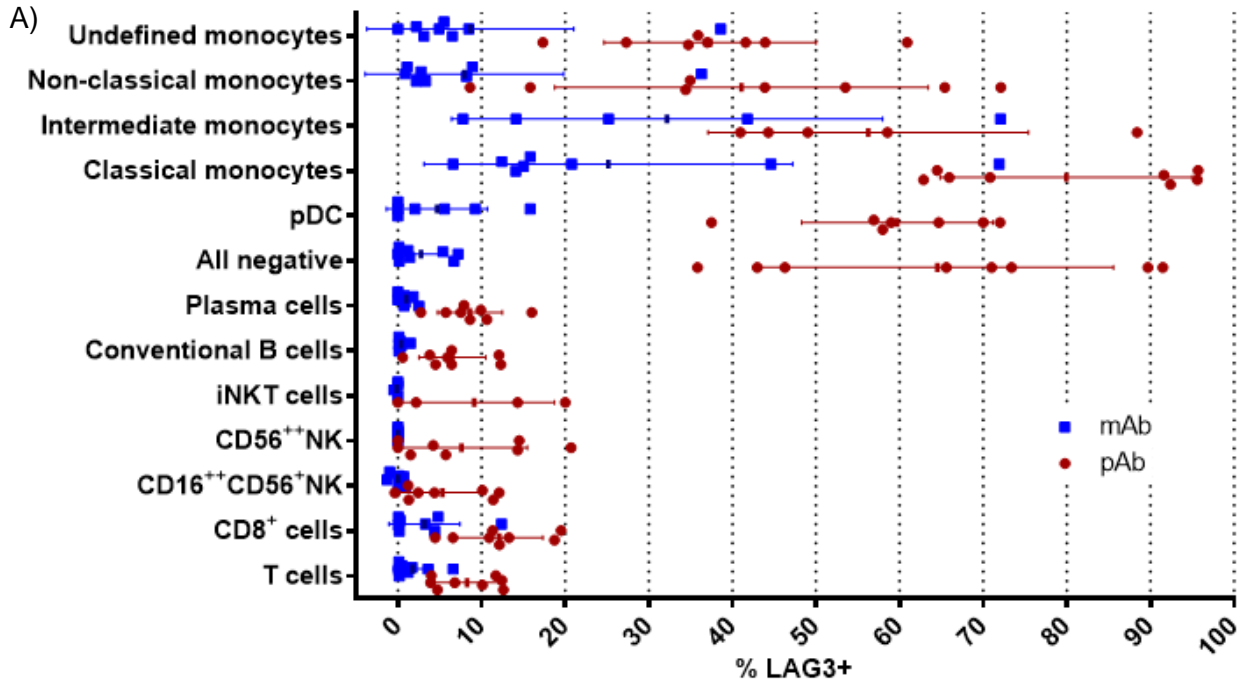
Compared with the mAb, average pAb staining was significantly higher for all populations other than iNKT cells and intermediate monocytes (Wilcoxon's matched pairs, $\alpha=0.05$), which are rarer populations where only four and five participants had more than 30 events, respectively (Figure 5a). Like the mAb, pAb exhibited the lowest %-positive staining amongst lymphocytes, with an average of 8% of T cells staining LAG3⁺. The general trend of LAG3 expression was similar by mAb and pAb. As with the

mAb, pAb also detected LAG3 expression on monocytes, to an even higher degree, with 80% of classical monocytes staining positive, compared to the 25% stained by the mAb.

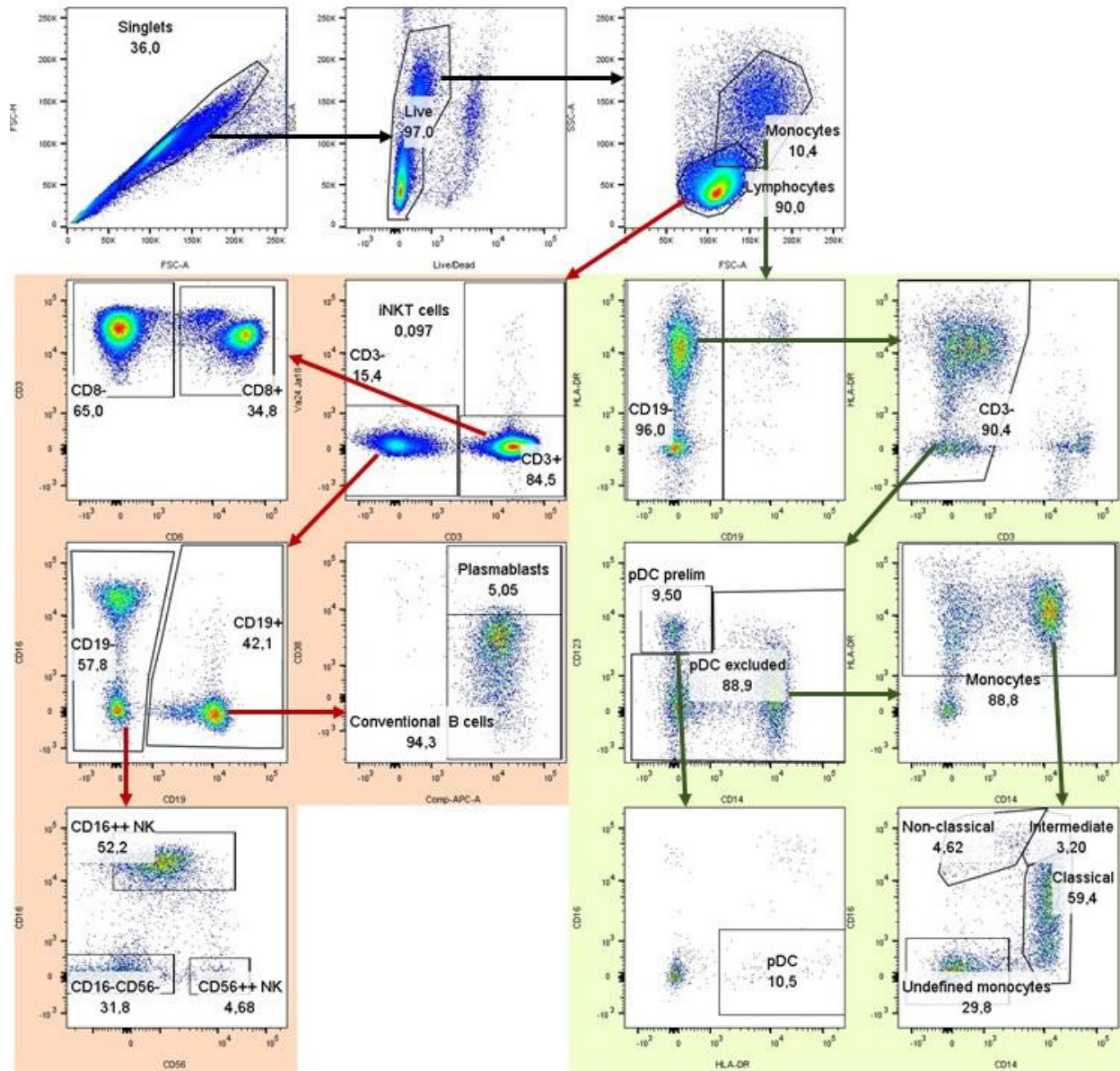
While the difference between pAb and mAb was notable in all populations, the largest was in cells that were negative for all tested markers, where 64% of cells were stained by pAb and only 2.8% stained by mAb.

Due to the potential for variability in polyclonal antibody lots, three different lot numbers were compared to one another in the panel and produced very consistent results, indicating that lot inter-variability is low (Figure 5b).

Figure 5 mAb or pAb staining of LAG3 by cell population.



Populations outlined in Figure 2. Representative ex vivo gating for identification of populations.



identified from PBMC of healthy donors ($n=8$) were assessed for LAG3 expression based on staining by a) monoclonal antibody (clone: 17B4, FITC) or polyclonal antibody (goat polyclonal, PE) or b) three different lots of polyclonal antibody. Percent of cells staining positive were subtracted by the percent of cells in each population stained positive with an isotype control. Populations with less than 30 events were excluded.

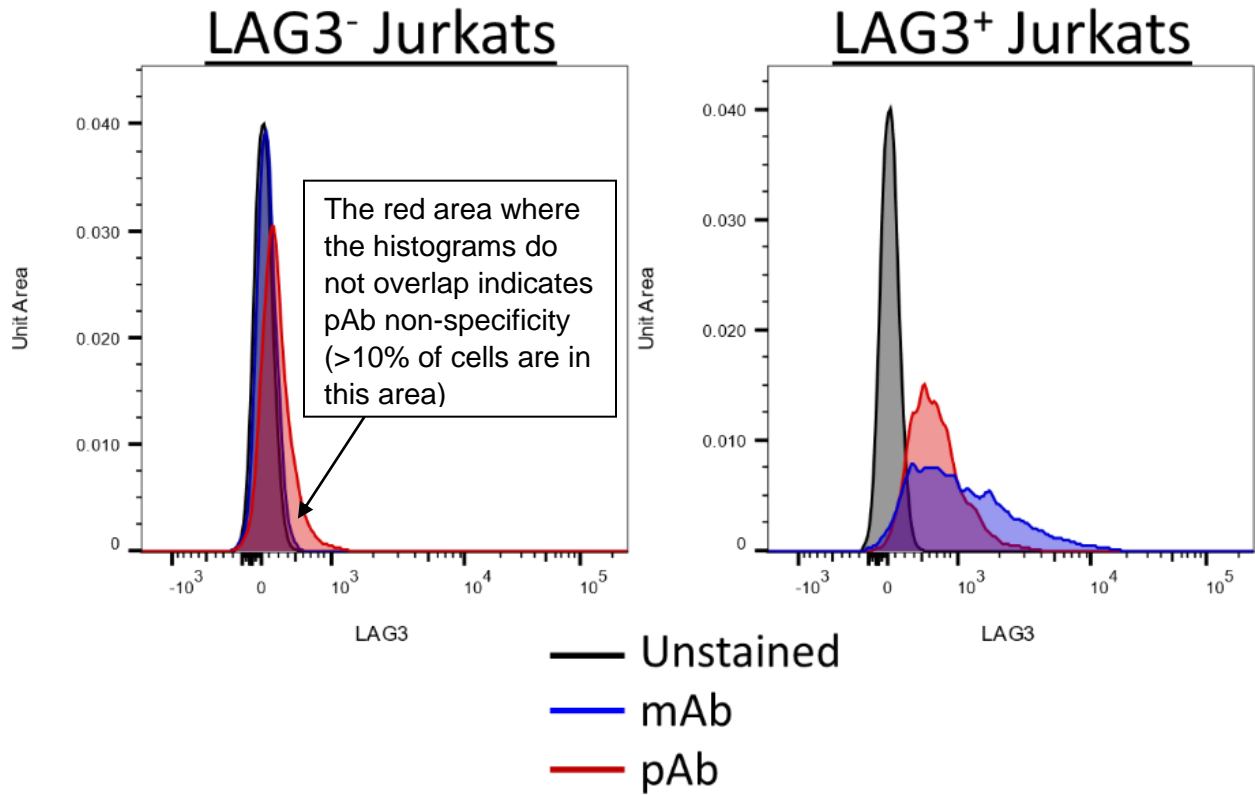
4.4.2.3 Comparing antibodies on LAG3⁺ and LAG3⁻ cell lines

This discrepancy between antibodies raises the question of which antibody is preferable for flow cytometric detection of LAG3. To answer this question, a comparison of sensitivity and specificity of mAb to that of pAb was performed.

To this end, the LAG3-negative Jurkat cell line was used. Using flow cytometry, mAb and pAb staining of Jurkats made to express LAG3 (LAG3⁺) by transduction and Jurkats transduced with the vector alone (LAG3⁻) were compared. As shown in Figure 6 pAb, but not mAb, stained LAG3⁻ cells, indicating non-specific binding. Furthermore, while pAb does bind LAG3 (as shown by increased binding of LAG3⁺ cells) it does so with less sensitivity than mAb (as shown by less pAb staining than mAb on LAG3⁺ cells).

This suggests that the monoclonal antibodies are preferable to polyclonal antibody for LAG3 staining.

Figure 6 mAb and pAb staining of transduced Jurkat cells.



LAG3⁺ and LAG3⁻ Jurkat cells were stained with either an anti-LAG3 mouse IgG monoclonal antibody (BD, clone: T47-530) (blue), or the anti-LAG3 goat polyclonal antibody (R&D, Cat #: FAB2319P) (red), or left unstained (black). In this histogram, LAG3 staining is represented by a shift of the curve to the right. The y-axis represents in arbitrary units the proportion of cells at each intensity of LAG3 staining.

4.5 Summary

Immune phenotyping panels were designed and compared LAG3 expression across several cell populations. This study detected LAG3 expression on monocytes, at high levels relative to LAG3 expression on lymphocytes, whereon LAG3 is typically studied. It was determined that by comparing anti-LAG3 mAb to pAb, the antibodies bound at very different levels on the same cell populations, with the pAb binding higher. This discrepancy, which can be observed in the literature by examining patterns of reported LAG3 expression and comparing with antibody use, existed across cell populations, although some cell populations showed bigger discrepancies than others. mAb and pAb were then compared on LAG3⁻ and LAG3⁺ Jurkat cell lines. Increased binding to LAG3⁻ Jurkats and reduced binding to LAG3⁺ Jurkats by the pAb implies reduced specificity and sensitivity compared with the mAb.

4.6 Discussion

While LAG3 expression on cell types including T cells and pDCs matches previous findings^{29,95,96}, the detection of LAG3 binding to monocytes conflicts with the previous thinking that LAG3 is not expressed on monocytes^{33,51,141}. However, some recent studies have preliminarily shown LAG3 expression on monocytes, but these studies were incomplete, with two showing *LAG3* RNA expression in monocytes and macrophages and another showing LAG3 protein on CD68 macrophages in tissue, without excluding the possibility of other CD68⁺ cell types, such as pDCs^{38,115,142,143}.

This discovery of LAG3 on monocytes through use of flow cytometry adds evidence to the assertion that LAG3 is expressed on monocytes. If true, many questions arise, including what function LAG3 has on these cells and the relevance and differential expression of LAG3 on these cells during disease. Monocytes are important multifunctional cells, with roles in homeostasis and response to infection. Understanding LAG3's potential role in this regulation of monocyte activity may be an important area of further research.

The discovery of discrepancies between the binding of monoclonal and polyclonal antibodies and the apparent non-specificity of the polyclonal antibody also has several consequences and prompts several questions to be answered in future studies. The first question it warrants is what conclusions from previous studies that used the polyclonal antibody should be re-interpreted? Another question is what other proteins is the pAb targeting? These are potential areas of further research. This discovery may also establish a new best practice for LAG3 detection in support of monoclonal antibodies.

5 Regulation of LAG3 expression on T cells

5.1 Rationale

LAG3 expression is regulated by several mechanisms, including post-translationally by endocytosis and cleavage by metalloproteases. Surprisingly, little has been published on LAG3 expression kinetics after activation. Furthermore, some very limited evidence suggests that LAG3 may be downregulated by TLR activation, perhaps via increased cleavage by ADAM10/17, although there is other data suggesting no impact of TLR ligands on LAG3 expression^{144–146}.

5.2 Hypotheses

1. LAG3 expression declines at a constant rate after stimulus is removed.
2. TLR activation reduces LAG3 expression.

5.3 Objectives

To study the LAG3 mechanism of action and mechanisms of expression, we first needed to characterize LAG3 expression kinetics on primary T cells after activation.

1. Determine rate of LAG3 downregulation after removal of stimulus.
2. Determine whether TLR ligand exposure reduces LAG3 expression.

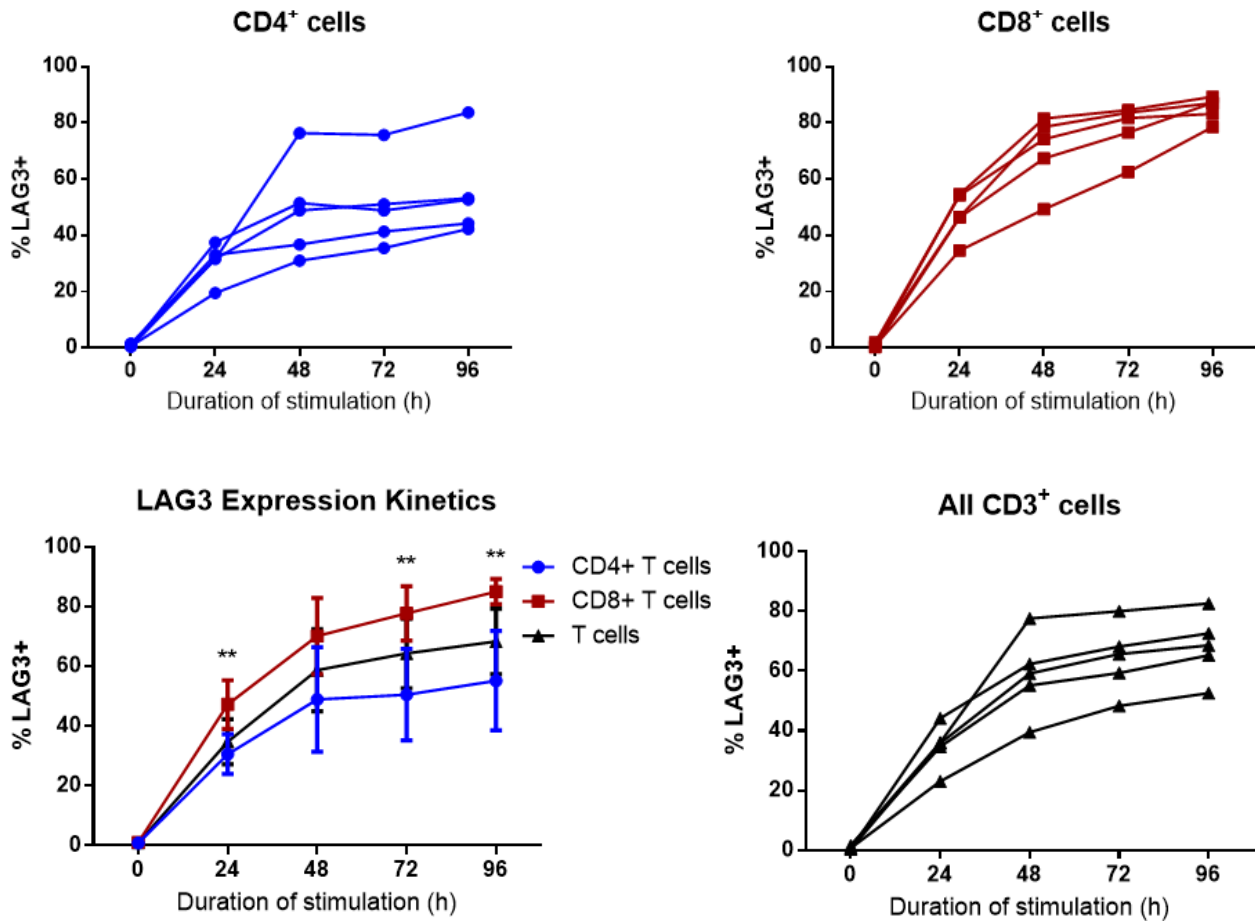
5.4 Results

5.4.1 LAG3 expression kinetics on primary T cells

PBMC were persistently activated with anti-CD3/anti-CD28 coated magnetic beads for up to 96 hours and LAG3 was measured on T cells every 24 hours (Figure 7). LAG3 expression was significantly higher on CD8⁺ T cells than CD4⁺ T cells at all time points other than 48 hours, where it trended higher ($p=0.060$). LAG3 expression mostly plateaued after 48 hours of activation, after which LAG3 expression increased by 16.3%, 21.15% or 12.92% over the following 48 hours on all T cells, CD8⁺ T cells or CD4⁺ T cells, respectively.

To study how LAG3 is downregulated after activation, PBMC were activated with anti-CD3/anti-CD28 coated magnetic beads for 24 or 48 hours and LAG3 expression was assessed on T cells up to 72 hours after stimulus was removed (Figure 8). On T cells activated for 48 hours, LAG3 persistently declined at a constant linear rate. In contrast, LAG3 continued rising after stimulus was removed from T cells activated for 24 hours such that 24 hours after removing stimulus and thereafter, expression on these cells was no different than cells activated for 48 hours.

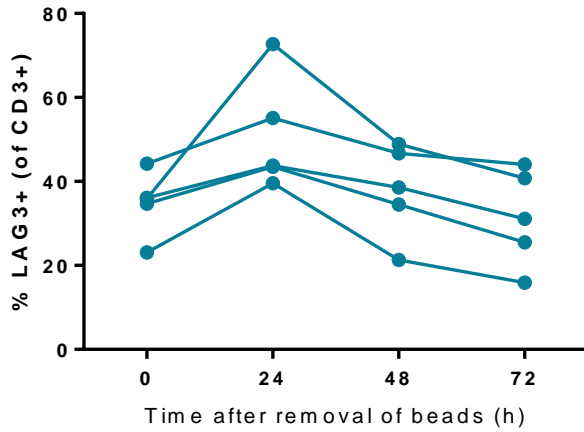
Figure 7 LAG3 expression on T cells after stimulation.



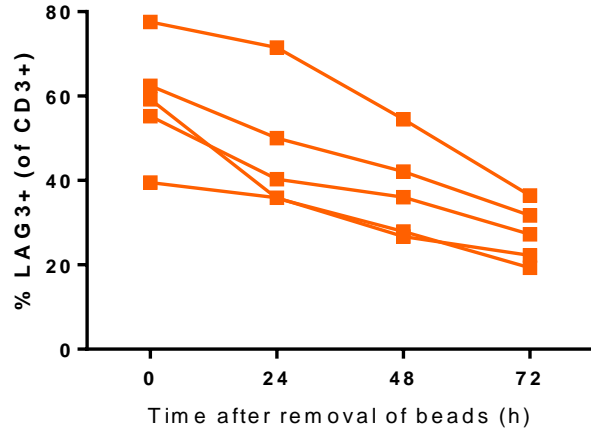
PBMC from 5 donors were stimulated with anti-CD3/anti-CD28 coated magnetic beads for either 24, 48, 72 or 96 hours. Cells were stained with Live/Dead marker along with antibodies targeting CD3, CD4, CD8 and LAG3 and measured by flow cytometry. Summary data or trends at the individual level are shown for CD4+ and CD8+ T cells or the average proportion of total T cells that express LAG3. Wilcoxon signed-ranks test was used to determine statistical significance. ** = $p < 0.01$.

Figure 8 LAG3 expression after stimulation and rest.

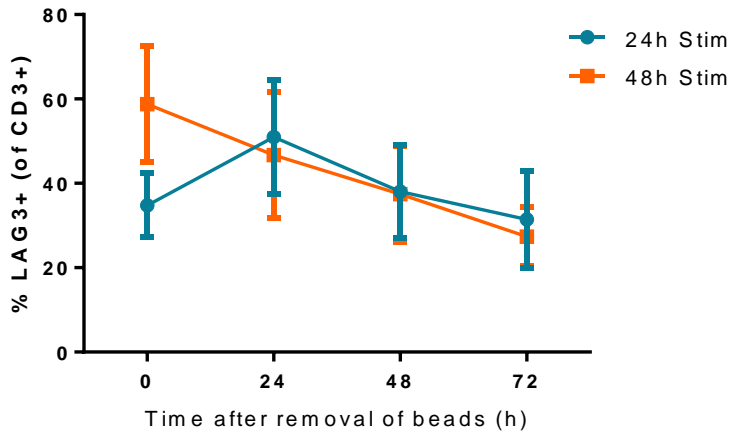
LAG3 Expression after 24 hour stimulation



LAG3 Expression after 48 hour stimulation



Combined LAG3 Expression Kinetics Post-stimulation

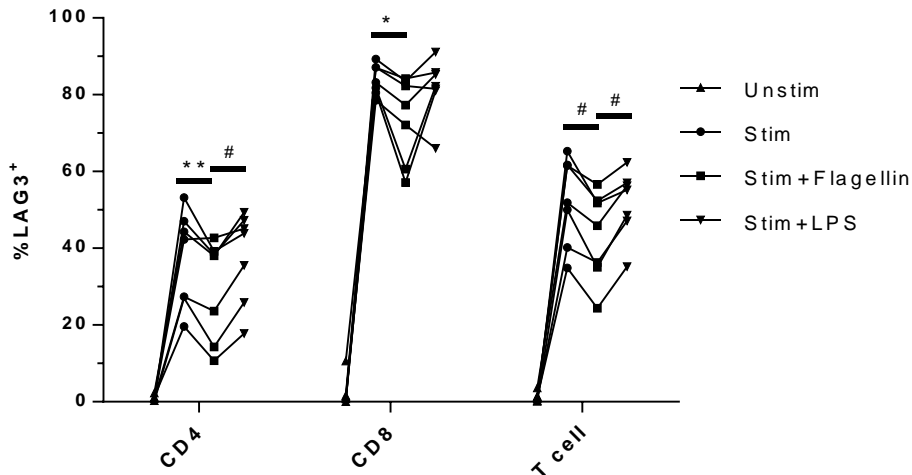


PBMC from 5 donors were stimulated with anti-CD3/anti-CD28 coated magnetic beads for either 24 (blue circles) or 48 (orange squares) hours. Beads were then magnetically removed, and cells were left in fresh media for up to 72 hours. LAG3 expression was measured immediately following removal of beads and then every 24 hours thereafter.

5.4.2 Impact of TLR activation on LAG3 expression

One mechanism of regulating LAG3 expression is by cleavage of LAG3 by the metalloproteases ADAM10 or ADAM17⁴⁴. This mechanism has been shown to be important in regulating LAG3 expression in the tumor microenvironment⁴⁵. Since ADAM10 and ADAM17 are induced by TLR activation, the TLR4 and TLR5 ligands LPS and flagellin (from *Salmonella typhimurium*), respectively, were added to PBMC at the start of a 96 hour activation with anti-CD3/anti-CD28 coated magnetic beads and LAG3 expression was measured on T cells (Figure 9). LAG3 expression was unimpacted by LPS addition but was significantly lower (22% lower on CD4⁺ T cells, 12% lower on CD8⁺ T cells and 17% lower on total T cells) when flagellin was added to cell cultures. Viability of T cells in all conditions, as measured by negative staining for LIVE/DEAD, met or exceeded 99%, suggesting this difference was not due to activation-induced cell death.

Figure 9 Impact of Flagellin or LPS on T cell LAG3 expression.



PBMC isolated from healthy donors ($n=7$) were stimulated with anti-CD3/anti-CD28 beads with or without 100ng/ml of LPS or Flagellin. After 96 hours, cells were assessed for LAG3 expression by flow cytometry. Wilcoxon signed-ranks test was used to determine statistical significance. * = $p < 0.05$, ** = $p < 0.01$, # = $p < 0.005$.

5.5 Summary

In primary cells, most of the increase in LAG3 expression after activation occurs within the first 48 hours. This expression is significantly higher on CD8⁺ T cells than CD4⁺ T cells. Once stimulus is removed after 48 hours of activation, LAG3 expression starts a constant rate decline for at least 72 hours, whereas if stimulus is removed after only 24 hours of activation, LAG3 expression will continue increasing over the next 24 hours, such that it matches cells activated for 48 hours until it declines, matching the rate of decline in cells activated for 48 hours.

Activated primary cells also showed a consistent reduction in LAG3 expression when flagellin was present during activation. This same reduction was not observed with LPS, indicating that TLR5, but not TLR4 activation reduces LAG3 expression.

5.6 Discussion

The results showing the rapid expression of LAG3 and continued increase if stimulus is removed within 24 hours, suggest that the mechanisms involved in reducing LAG3 expression require up to 48 hours to reach a steady state. These mechanisms may be the production of metalloproteases known to cleave LAG3 from the cell surface, or simply cell division.

In studying LAG3 mechanism on primary cells, the low baseline expression of LAG3 on T cells, as shown here and in section 4.4.1 demonstrates the need for previous activation to induce LAG3 expression. The results shown in this chapter show

that LAG3 expression steadily declines during a resting period after activation. These factors could pose challenges to studying the LAG3 mechanism.

The literature is conflicting on whether TLR activation reduces LAG3 expression¹⁴⁴⁻¹⁴⁶. Our finding that Flagellin, but not LPS, reduces LAG3 expression suggests that some, but not all TLR activation can reduce LAG3 expression. However, since higher LPS concentrations were not tested, one cannot be sure that this result of LPS showing no effect is a true result. These differences may also be due to the different models used, with all previous studies being done in mice.

Further research using metalloprotease inhibitors may suggest this as the mechanism by which TLR ligands reduce LAG3 expression, or alternative research may show that induction of T-bet transcription factor is the mechanism, since TLR activation can induce T-bet and metalloprotease activation^{62,145,146}. LAG3 downregulation in the presence of PAMPs would be an effective way to reduce LAG3 expression during infection, when LAG3 could inhibit an immune response, while allowing LAG3 to remain elevated in autoimmunity, or after infection has cleared, cases where IC expression may be desired.

The ability for TLR ligands to reduce LAG3 expression, along with the expression of other ICs, make them attractive adjuvants for preventative or therapeutic vaccines, especially against cancer, to which immune cells may be less sensitive.

6 LAG3 and primary T cell activation

6.1 Rationale

LAG3's best characterized inhibitory activity is reduction in cytokine production and proliferation after activation^{92,113}. A model for assessing LAG3's mechanism of action must first demonstrate LAG3 activity. Staphylococcal enterotoxin B (SEB) is a superantigen that binds outside the peptide binding cleft, recognizing TCR V β 3, V β 12, V β 14 and V β 17 chains to activate the 10-20% of T cells with this TCR repertoire¹⁴⁷. Since SEB is able to activate a large fraction of T cells in comparison with peptide activation while maintaining co-receptor activity, it has been used with LAG3 blockade (antibody that inhibits the binding of LAG3 to MHCII) to characterize LAG3 inhibition of cytokine production and proliferation⁹².

To characterize how LAG3 inhibits TCR signaling, LAG3 must be expressed on a large fraction of T cells to increase signal:noise ratio. Furthermore, while LAG3 expression is high, TCR signaling levels must be reduced to the level where reactivation allows detection of potentially subtle differences. Herein, cytokine production was used as a surrogate for TCR signaling to determine whether such a model is feasible in primary T cells.

6.2 Hypothesis

1. LAG3 blockade augments proliferation during T cell activation with SEB.

2. LAG3 blockade augments cytokine production during T cell activation with SEB.

6.3 Objectives

1. Measure proliferation following T cell activation with SEB.
2. Find optimal resting period for cells after SEB activation for re-activation
3. Measure cytokine production following T cell activation with SEB.

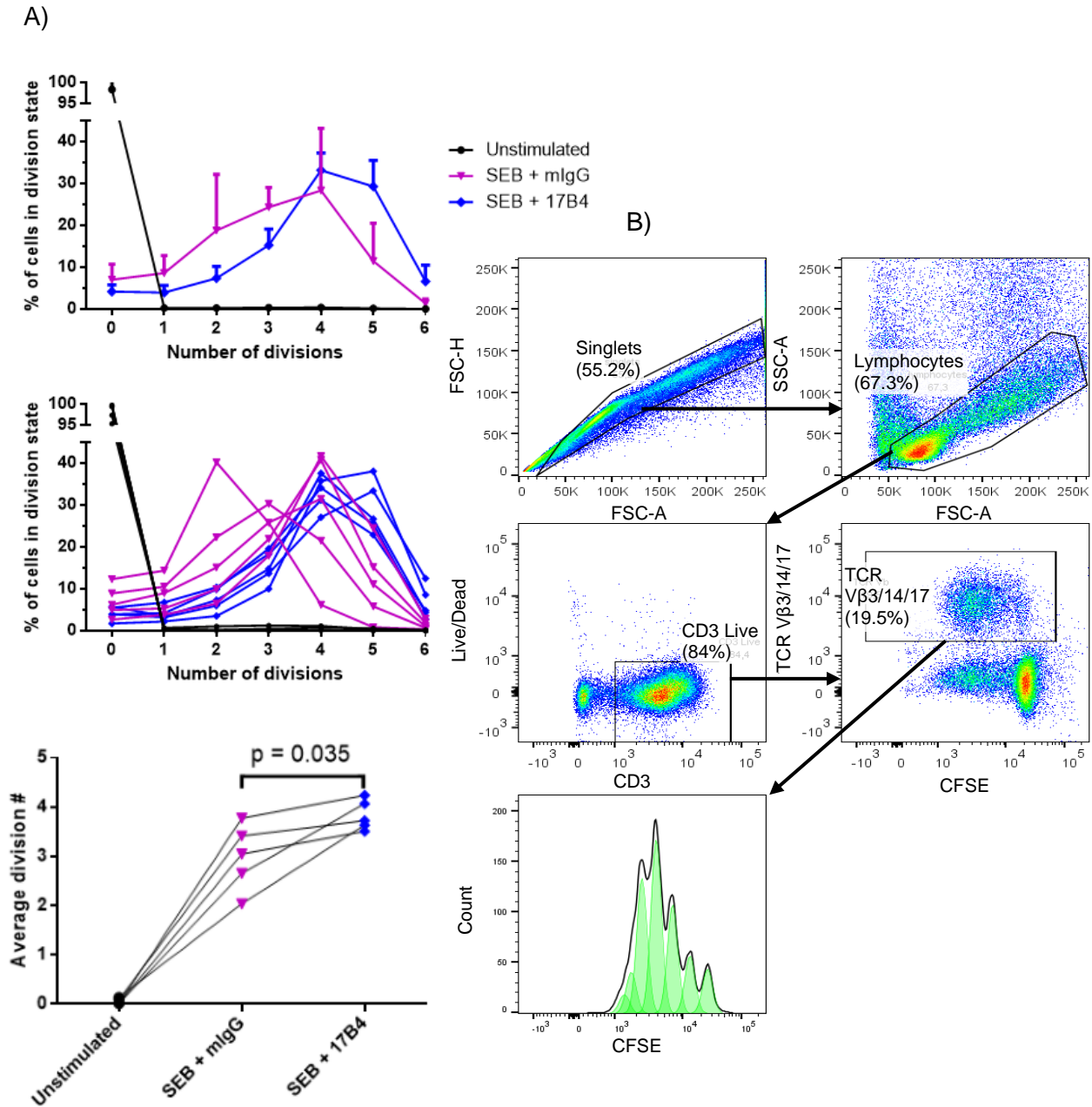
6.4 Results

6.4.1 Proliferation

T cell activation induces both proliferation and cytokine production, but the mechanisms involved in each are distinct¹⁴⁸. Furthermore, while cytokine production occurs within a few hours, proliferation occurs over days. LAG3 blockade can augment both cytokine production and proliferation in response to SEB stimulation⁹². To confirm LAG3's proliferation enhancing properties and reaffirm SEB stimulation as a model for LAG3 function and confirm the efficacy of the LAG3 blocking antibody (clone 17B4), PBMC were stained with CFSE and activated with 0.01ng/ml SEB for 96 hours in the presence of the blocking antibody or an isotype control. For each cell division, the CFSE in stained cells is divided in roughly equal halves. Proliferation can then be measured by dilution of CFSE. Often, proliferation is interpreted by the percentage of divided cells. However, during SEB activation, such a large fraction of SEB-responsive cells divide

that it prevents accurate measurement in this way. Instead, with antibodies against specific SEB-responsive TCR V β chains, one can measure what proportion of SEB responsive cells have divided a specific number of times. This allows excellent visual comparison between conditions and statistical interpretation by comparing the average division number in each condition. In this way, proliferation of SEB activated cells was compared between LAG3-inhibited (SEB+17B4) and uninhibited (SEB+mIgG) and was significantly higher when LAG3 was blocked (Figure 10).

Figure 10 Proliferation from SEB activation with LAG3 blockade.



Healthy donor PBMC were stained with CFSE and activated with 0.01ng/ml SEB for three days with 10ug/ml LAG3 blocking antibody (clone: 17B4) or isotype control. Live TCR Vβ3/14/17⁺ lymphocytes were assessed for proliferation by CFSE dilution (gating strategy shown in B). Mean with SD error is shown for the percent of cells at each division number (top), along with individual data (middle). A t test comparing the average division number of cells was used to test for statistical significance (bottom).

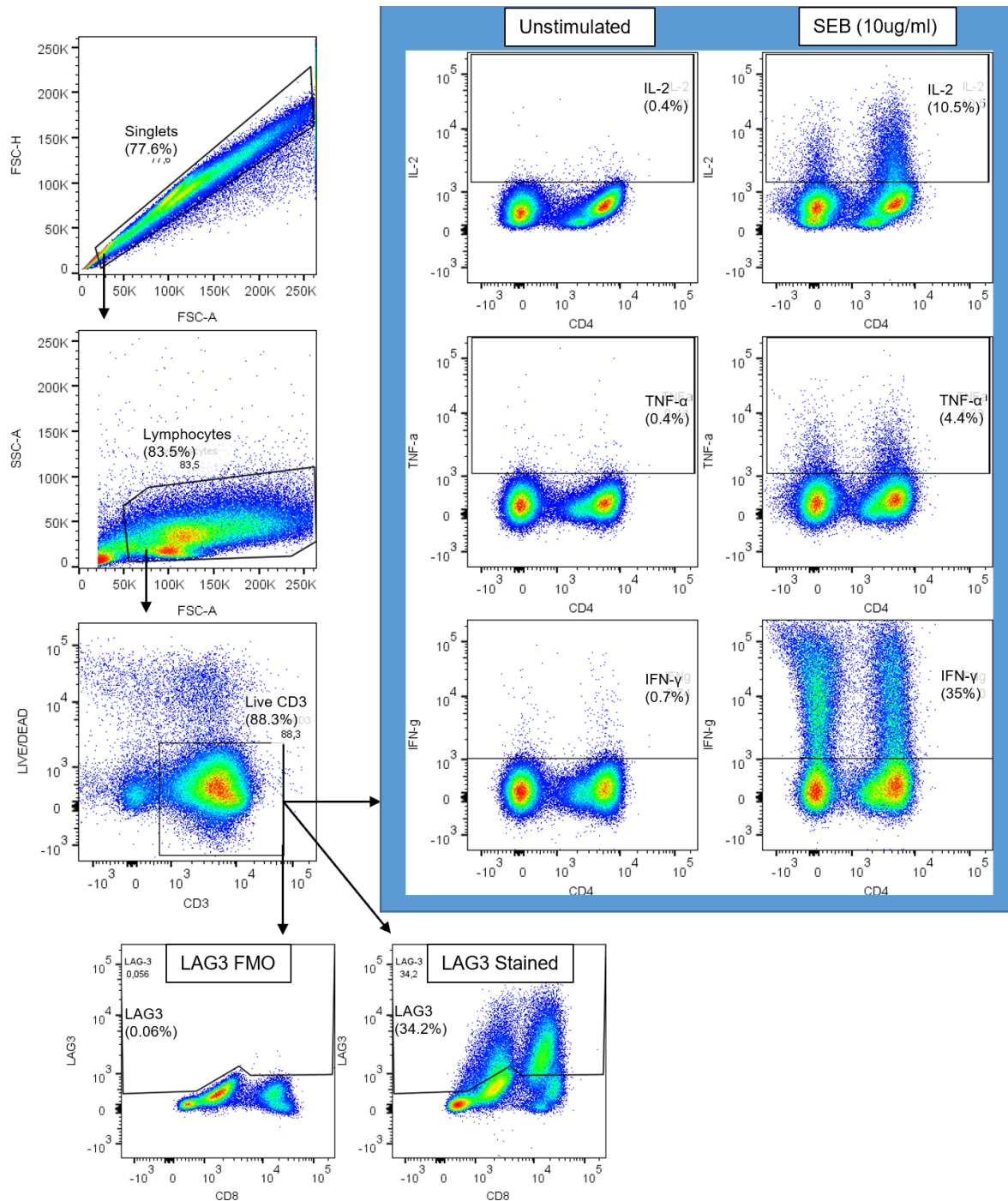
6.4.2 Cytokines

To determine the effect of LAG3 on T cell cytokine production or signaling in response to a stimulus, LAG3 must first be expressed on the T cells. Since LAG3 is expressed at very low levels on resting T cells, the T cells must be activated to induce LAG3 expression. In addition to upregulating LAG3, an initial activation would serve to expand the proportion of T cells that are responsive to SEB. To this end, healthy donor PBMCs were activated with SEB for three days. Then, cells were washed and rested for up to three days to lower cytokine production. One day after removal of stimulus, cytokine expression (particularly IFN- γ) remained slightly elevated, whereas by two days cytokine expression had returned to baseline (Figure 12). As with stimulation by anti-CD3/CD28 coated beads (Figure 8), LAG3 expression decreased progressively after stimulus was removed (Figure 12) but was still elevated two days later. Again, similarly to stimulation by anti-CD3/CD28 coated beads, LAG3 was higher in CD8⁺ T cells than CD4⁺ T cells, where after two days LAG3 was expressed on 42% of CD8⁺ T cells, but only 9% of CD4⁺ T cells activated with 0.1ng/ml of SEB.

To determine the impact of LAG3 on cytokine production and the restorative effect of LAG3 blockade healthy donor PBMCs were activated with SEB for three days, washed and rested for two days before 6 hour reactivation with SEB with or without LAG3 blockade (clone 17B4). Cells were then fixed, permeabilized and stained for LIVE/DEAD, TCR V β 14, TCR V β 17, CD3, CD4, CD8, IL-2, TNF- α and IFN- γ . Flow cytometry was performed using the gating strategy outlined in Figure 11.

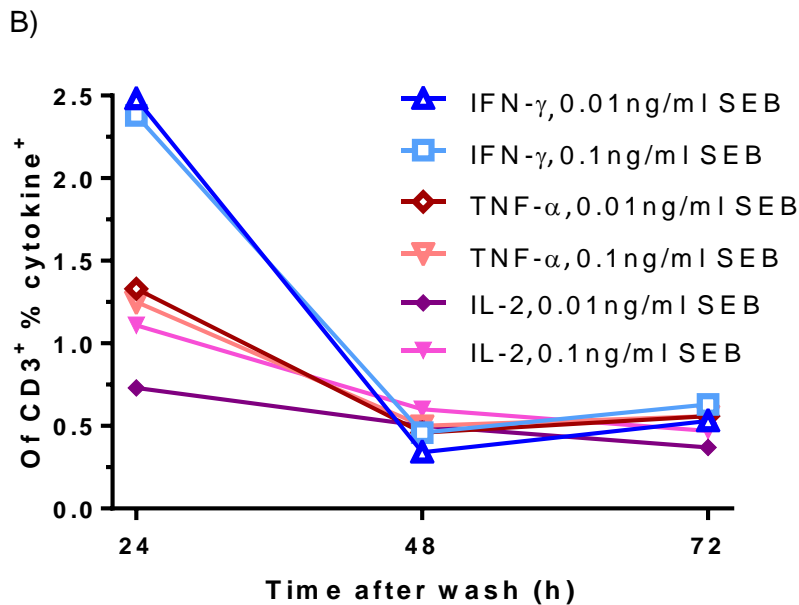
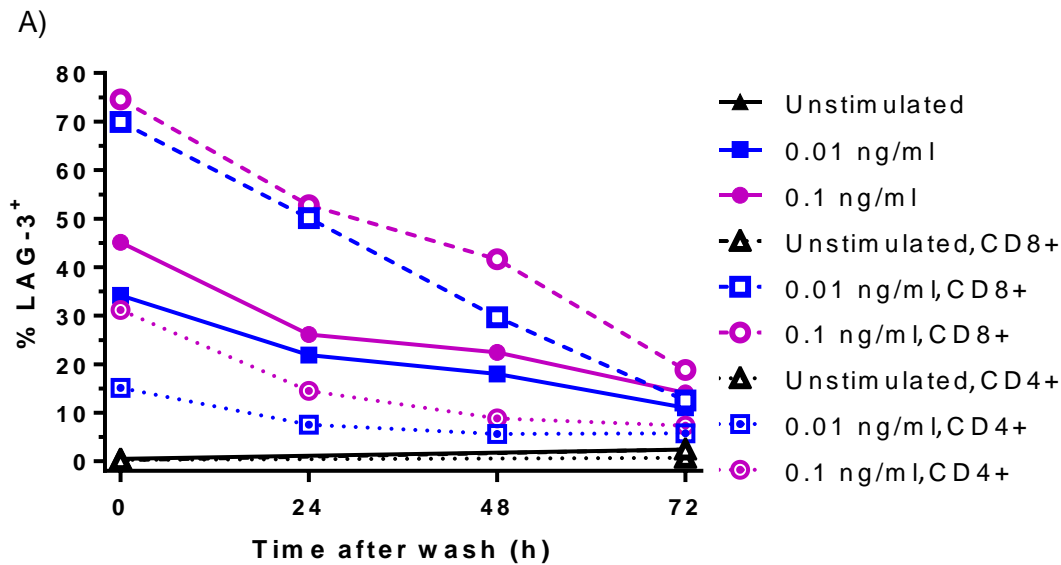
Anti-LAG3 is used at concentrations of 10ug/ml to block LAG3 activity. Here, three different concentrations were used. While robust production of IL-2, TNF- α or IFN- γ occurred after restimulation, levels were similar between cells reactivated in the presence of isotype control or LAG3 blockade (Figure 13), demonstrating that LAG3 blockade in this model is ineffective at increasing cytokine production.

Figure 11 Flow cytometry gating for cytokine detection following SEB reactivation.



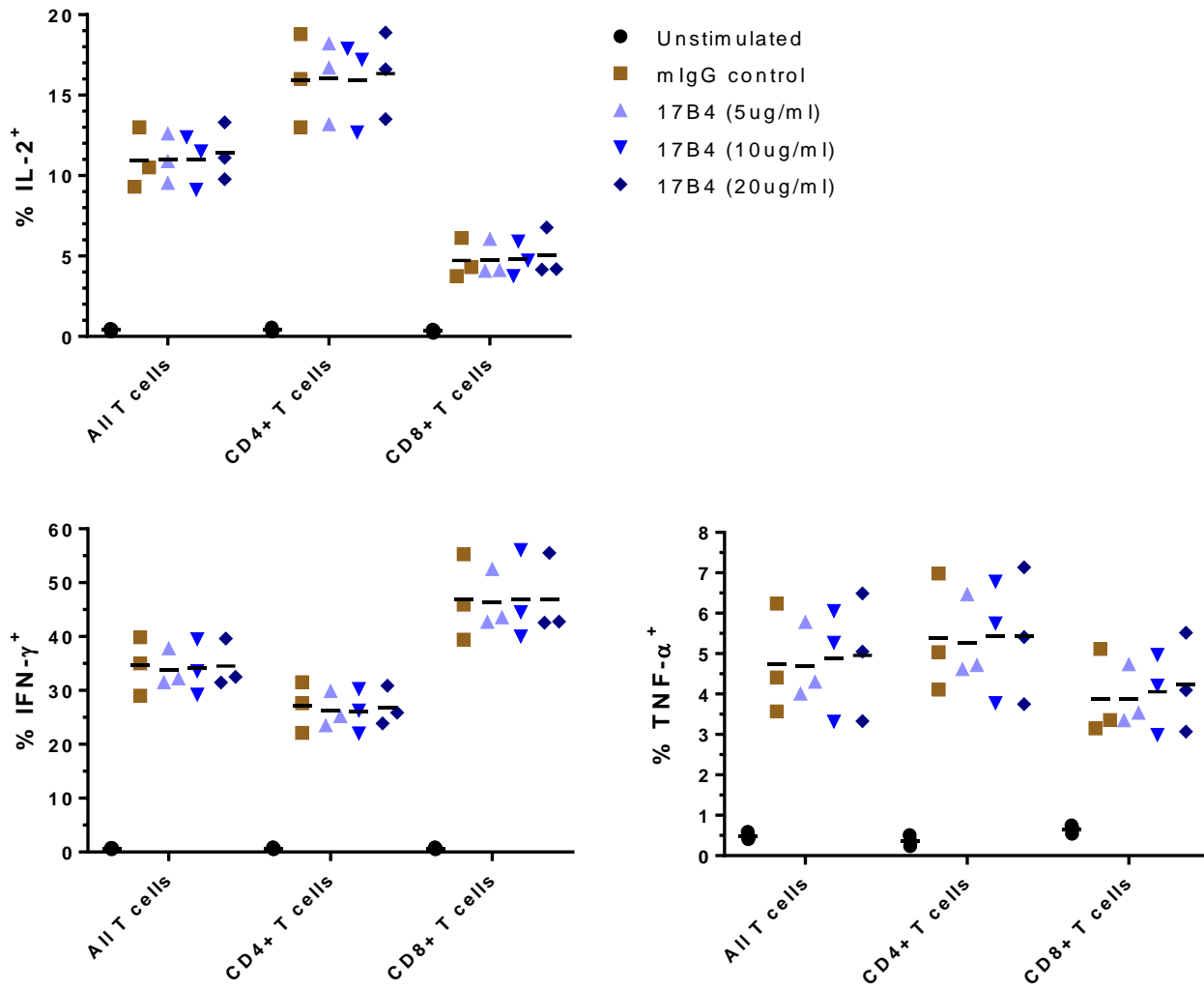
Healthy donor PBMC were activated with either 0.1ng/ml of SEB for three days. Cells were then rested in fresh media 72 hours before 6 hour reactivation with 10ng/ml SEB in the presence of BD GolgiStop™. Cells were fixed and stained for LIVE/DEAD, CD3, CD4, CD8 and LAG3, or fixed, permeabilized and stained for LIVE/DEAD, CD3, CD4, CD8, IL-2, IFN-γ and TNF-α before flow cytometry was performed. Single cells are first gated by FSC-area and FSC-height followed by lymphocyte gating by SSC-area and FSC-area. Live CD3⁺ Lymphocytes are then analyzed for cytokine expression.

Figure 12 LAG3 expression and cytokine production after removal of SEB.



Healthy donor PBMC were activated with either 0.1 ng/ml or 0.01 ng/ml of SEB for three days. Cells were then rested in fresh media for the designated duration and with BD GolgiStop™ for the final six hours. Cells were fixed and stained for LAG3 and phenotype markers (a), or fixed, permeabilized and stained for IL-2, IFN- γ and TNF- α (b) before flow cytometry was performed. A) shows LAG3 expression on total T cells (solid line), CD8+ T cells (dashed line) or CD4+ T cells (dotted line).

Figure 13 Cytokine production from SEB activation with LAG3 blockade.



Healthy donor PBMC were activated with 0.1ng/ml SEB for three days, then rested for two days in fresh media before 6 hour reactivation with 10ng/ml SEB in the presence of BD GolgiStop™. Cells were fixed, permeabilized and stained for IL-2, IFN-γ and TNF-α before flow cytometry was performed.

6.5 Summary

The effect of LAG3 blockade on proliferation was measured by CFSE dilution in SEB-responsive cells after SEB activation. In this assay, proliferation was significantly increased by LAG3 blockade.

Since resting T cells have very low levels of LAG3, activating cells with LAG3 present requires prior activation to induce LAG3 expression. To achieve this, PBMC were activated for 3 days and rested for two days before reactivation with SEB +/- LAG3 blockade. Two days was chosen as a resting period because this was determined experimentally to achieve baseline cytokine expression while keeping relatively high levels of LAG3 expression. This reactivation induced strong cytokine production, but no change was observed when LAG3 was inhibited.

6.6 Discussion

These experiments were completed to confirm findings from the literature that LAG3 inhibits proliferation and cytokine production in T cells and determine the feasibility of using a similar model for evaluation of the impact of LAG3 on TCR signaling. The low baseline expression of LAG3 on T cells necessitates pre-activation of T cells to induce LAG3 followed by a rest period to bring signaling back to baseline. While LAG3 blockade does increase proliferation during SEB activation of T cells, this section shows that this model is likely not feasible for the study of the LAG3 mechanism since LAG3 blockade had no impact on cytokine production, likely due to the somewhat

low proportion of T cells expressing LAG3 two days after activation, a reasonable duration for lowering signaling protein activation to a new baseline for reactivation.

7 Impact of LAG3 on T cell receptor signal transduction

7.1 Rationale

As an immune checkpoint, LAG3 inhibits cytokine production and proliferation of the T cells that express it. Unlike other ICs, LAG3 does not possess a known signaling motif and its signaling mechanism is largely unknown. LAG3 is known to inhibit NFAT activity following activation^{58,87,113}, but what other transcription factors, signaling proteins or signaling pathways LAG3 are involved in is unknown. This gap in knowledge inhibits the progress of LAG3-related research, making it more difficult to characterize LAG3 activity on non-T cells or to find the optimal therapeutic to combine with LAG3 targeting therapies. Furthermore, since antibodies as therapeutics have drawbacks, especially for use in chronic diseases, other therapeutics that mimic LAG3 activity may be effective, but are less likely to be discovered when LAG3's mechanism of action is unknown.

In vitro modifications of LAG3 genomic structure (mutants) are often used to study the LAG3 mechanism of action in a classical structure/function analysis. Cells with a truncated form of LAG3, where LAG3 lacks its cytoplasmic domain, are often used as a comparator. When LAG3 expression on these cells is assessed, its expression is usually similar or slightly lower than that of wild-type LAG3 (personal communication with D. Vignali and C.J. Workman, May 20, 2017)⁸⁸. However, one study using Jurkats transfected with LAG3 lacking the cytoplasmic domain showed reduced surface expression of LAG3, while maintaining similar total cellular levels as full-length

transfectants¹⁴⁹. Therefore, there is somewhat conflicting evidence in the literature on the role of the cytoplasmic domain in the trafficking of LAG3 to the cell surface.

7.2 Hypothesis

1. Surface LAG3 expression requires the cytoplasmic domain.
2. LAG3 expressing Jurkats exhibit impaired cytokine production during superantigen stimulation.
3. LAG3 broadly inhibits TCR signaling in Jurkat cell line model.

7.3 Objectives

1. Create a model of LAG3 inhibitory function in T cell line
2. Ensure LAG3 expression on transduced cells and comparable expression of other proteins
3. Characterize the model
4. Apply model to kinomics array and phospho-flow cytometry

7.4 Results

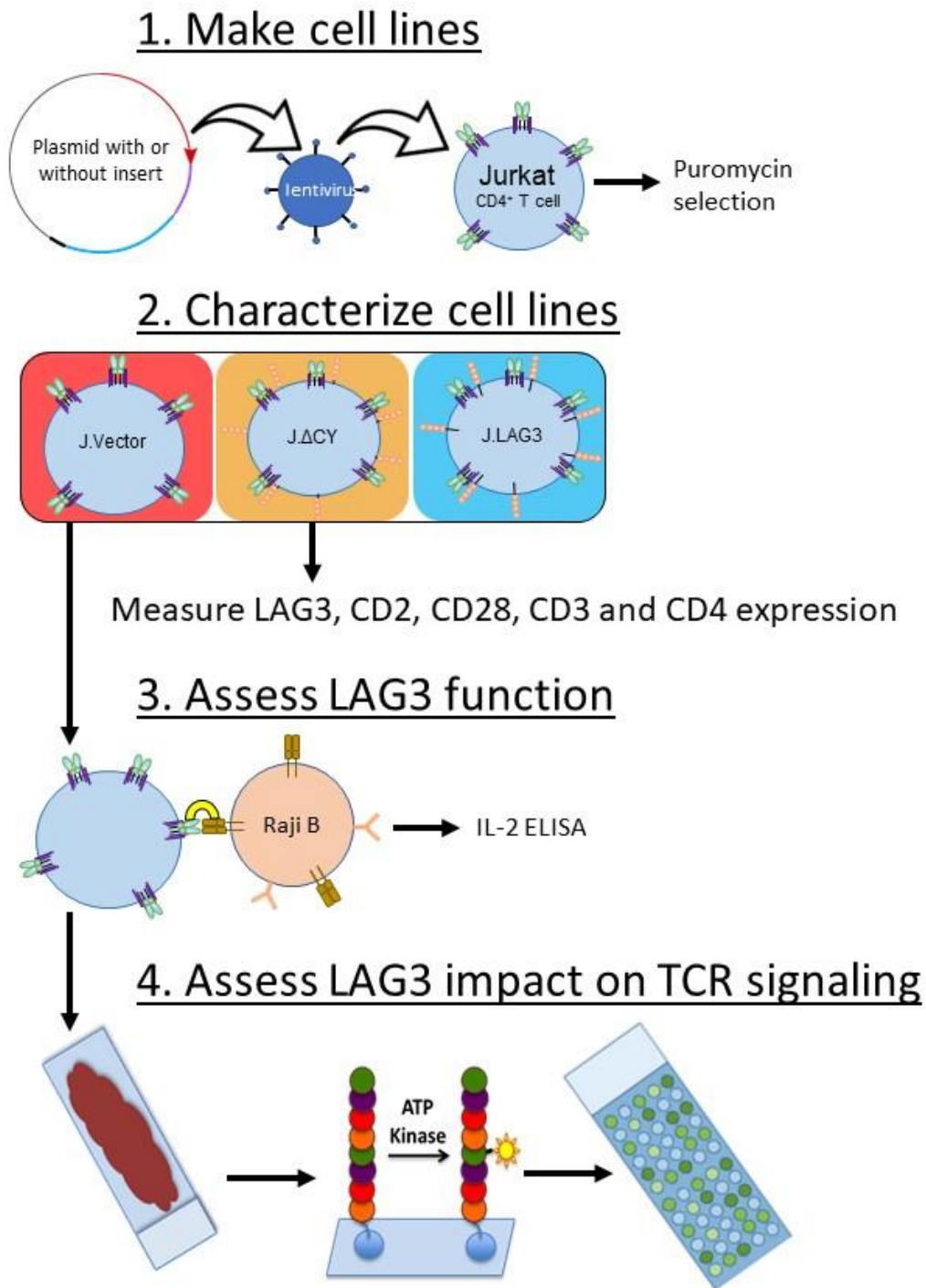
7.4.1 Creation of cell line model

Until 2018, there was no model of LAG3 in human cells. This inhibited the in-depth study of the LAG3 mechanism. The objectives of this section were to create a model of LAG3 activity, characterize the model, then use the model to determine the impact of LAG3 on TCR signaling (Figure 14).

To build a model of LAG3 activity, the Jurkat cell line was used. The Jurkat cell line is a CD4⁺ T cell line derived from the peripheral blood of a 14-year boy with T cell leukemia¹⁵⁰. The Jurkat cell line has been instrumental in bolstering the understanding of TCR signaling mechanisms, allowing the characterization of Lck, Zap-70, LAT, SLP-76 and CD45¹⁵¹. The well characterized signaling mechanism and the ease of activation makes Jurkat cells a good, but not ideal, model for studying LAG3's effect on TCR signaling.

Plasmids containing inserted full-length LAG3 gene, a truncated LAG3 gene lacking the cytoplasmic domain or no insert, were obtained commercially and packaged in lentivirus using HEK293 cells. Next, Jurkat E6-1 cells were transduced with these lentiviruses. Then, cells that have translated the insert region are selected by puromycin.

Figure 14 Experimental plans regarding LAG3 model.



First (1) The cell lines were made by lentiviral transduction of Jurkat E6-1 cells with a plasmid containing LAG3 followed by puromycin selection. Next, (2) cells are characterized by measuring LAG3, CD4, CD2, CD28 and CD3 expression and (3) measuring IL-2 after activation with superantigen coated Raji B cells. Finally (4) activated cells were lysed and added to a slide printed with peptides corresponding to regions of signaling proteins with phosphorylation sites. Then the slide was stained and scanned for phosphorylation.

7.4.2 Characterization of cell line model

To determine the effect of LAG3 on the activation of the cell lines, LAG3 expression must be the only relevant variable. To confirm that cell lines express similar levels of cellular activation receptors, CD2, CD28, CD3 and CD4 expression were assessed on each cell line (Figure 15A). Each cell line expressed similar levels of each of these proteins, as measured by percent of cells expressing each marker. Mean and median fluorescence intensity is shown for CD2, CD28 and CD3, since a high proportion of cells express these markers. The similar expression of co-activation proteins indicates that lentiviral transduction and puromycin selection did not change the cell profiles relative to each of the other cell lines and supports the interpretation that any difference in IL-2 production between cell lines is due to LAG3 activity.

7.4.3 Role of cytoplasmic domain on LAG3 expression in Jurkat cell line

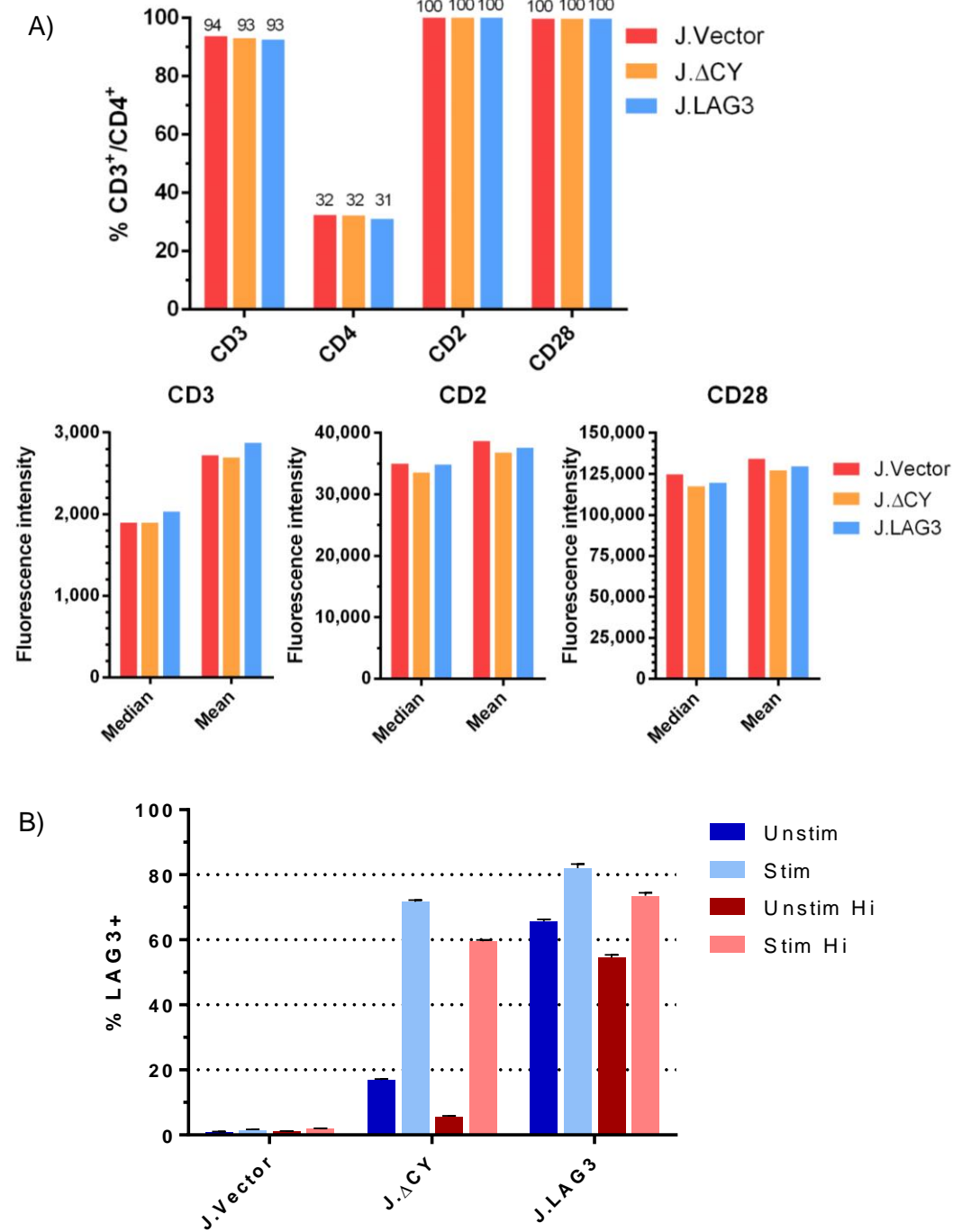
To confirm LAG3 expression on cell lines and determine how it changes based on cell densities in culture or activation, cell lines were cocultured at low (1×10^5 cells/ml) or high (1×10^6 cells/ml) density with equivalent number of Raji B cells +/- staphylococcus enterotoxin E superantigen (1ng/ml). After 24 hours in coculture, LAG3 expression was measured by flow cytometry (Figure 15B). Cells were cultured at high and low density because future experiments would use the cells at low or high density for IL-2 measurement and TCR signaling, respectively.

As expected, cells transduced with the vector alone (J.Vector) did not express meaningful levels of LAG3, while cells transduced with vector containing full-length

LAG3 gene (J.LAG3) expressed high levels of LAG3. Interestingly, cells transduced with vector containing LAG3 without its cytoplasmic domain (J. Δ CY) expressed LAG3, but at much lower levels than J.LAG3 cells. This is similar to results of Bae et al. where transfection with LAG3 lacking its cytoplasmic domain exhibited reduced surface expression of LAG3 as compared with full-length LAG3. Interestingly, when J. Δ CY cells were activated by superantigen, LAG3 expression was significantly increased to a level comparable with J.LAG3 cells. While LAG3 expression also increased on J.LAG3 cells after activation, the increase was more moderate than with J. Δ CY.

Culturing the cells at high density reduced LAG3 expression consistently by an absolute margin of between 8.4% and 12.2% in all conditions. Due to the consistency of the reduction in expression, the expression pattern remained similar between cells cultured at low and high density. However, because of the large difference between J. Δ CY and J.LAG3 expression of LAG3, the change in relative expression is more striking ranging from a 11.5% reduction to a 66.1% relative reduction in LAG3 expression in cells cultured at high density compared with the same cells cultured at low density (Figure 15B).

Figure 15 CD3, CD2, CD28, CD4 and LAG3 expression on cell lines.



Jurkat cells were transduced by lentivirus with plasmid containing sequence corresponding to full-length LAG3 (J.LAG3), LAG3 lacking its cytoplasmic domain (J.ΔCY) or no LAG3 sequence (J.Vector). After puromycin selection, (A) cells were stained with antibodies targeting CD3, CD4, CD2 or CD28. (B) Next, cells were cultured at low (1×10^5 cells/ml) or high (1×10^6 cells/ml) (Hi) concentration along with equal number of Raji B cells in a 24 well plate. Cells were cultured alone (Unstim) or stimulated by SEE (1ng/ml) (Stim) for 24 hours before being stained for LAG3 (n=3).

7.4.4 IL-2 production after activation with SEE and SED

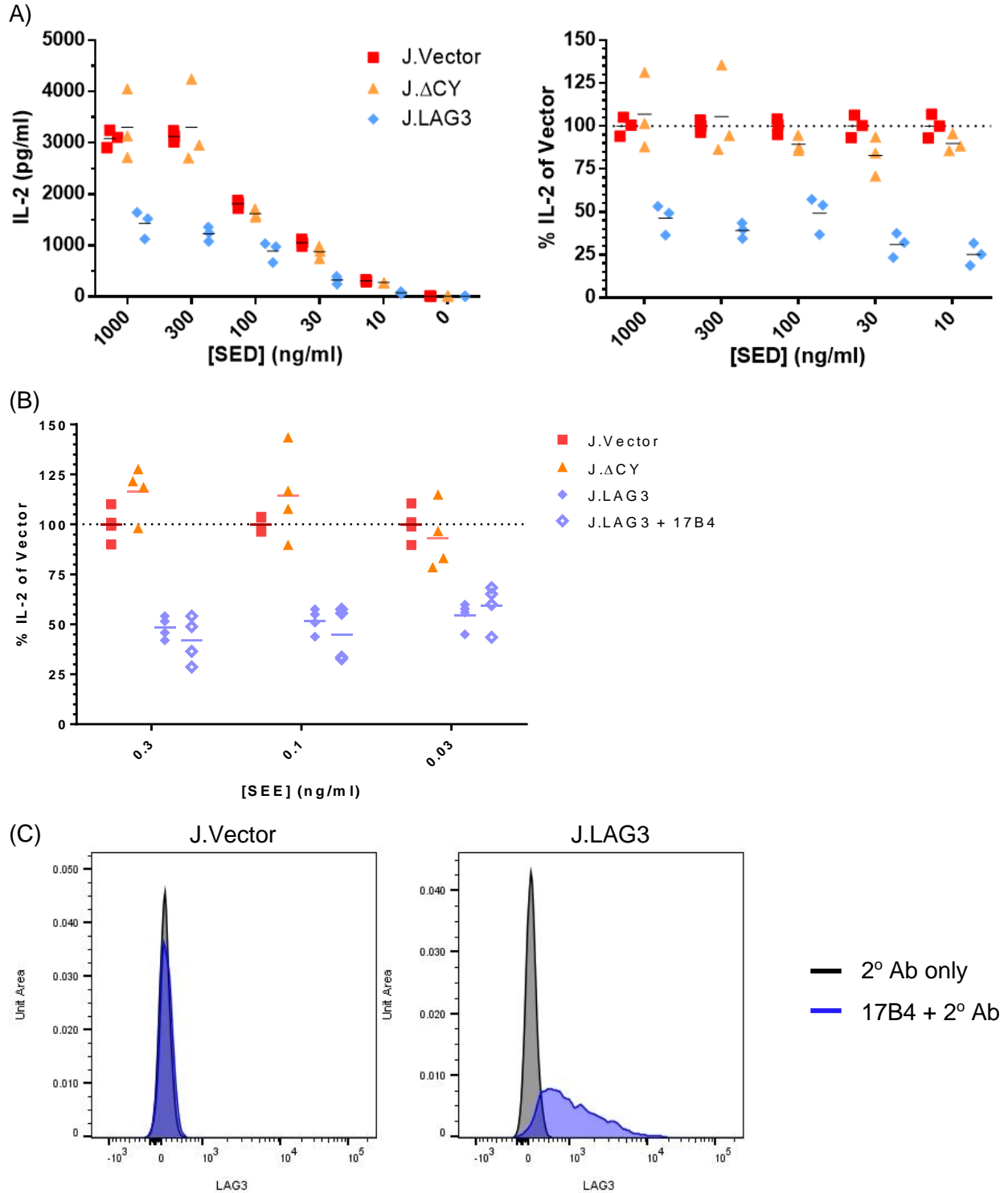
Inhibition of IL-2 is one of the best characterized aspects of LAG3 activity. Since Jurkat cells can be induced to produce large quantities of IL-2 in response to activation, IL-2 production was used as a measurement of LAG3 activity.

To activate the Jurkat cell lines, 50,000 cells of each cell line were incubated for 24 hours with an equivalent number Raji B cells with SEE or SED in a 96 well plate with 250µl /well. Supernatant levels of IL-2 were measured by ELISA.

As expected, J.Vector, which lacks LAG3, and J.ΔCY, which possesses non-functional LAG3, produce similar amounts of IL-2 in response to activation. In contrast, J.LAG3 produced approximately half the quantity of IL-2 as the J.Vector and J.ΔCY. This was the case after activation with either SEE or SED and occurred to a similar degree at all SEE and SED concentrations tested (Figure 16).

Notably, when Jurkat cells were pre-incubated with LAG3 blockade (clone 17B4), no restoration of IL-2 production was observed (Figure 16B). This was despite the ability of the blockade antibody to bind LAG3 in these cells (Figure 16C).

Figure 16 IL-2 production by cell lines stimulated with SEE or SED.



Transduced cell lines were cocultured with Raji B cells loaded with (A) SED as absolute amount or as a percent of J.Vector or (B) SEE at varying concentrations for 24 hours with or without blockade (17B4) as a percent of J.Vector. IL-2 was detected by ELISA. (C) Binding of the 17B4 blockade antibody to LAG3 was tested by secondary staining with a PE-labelled anti-mouse antibody.

7.4.5 Impact of LAG3 on signal transduction

7.4.5.1 Kinomics

To obtain a broad picture of how LAG3 activity may disrupt TCR signaling, each cell line was activated with Raji B cells preincubated with SEE or SED superantigens for 2, 15, 45 and 90 minutes. Cell pellets were flash frozen immediately after activation. At a later date, cell pellets were lysed and the lysate was added to a slide with 1294 peptides printed in spots that correspond to regions of signaling proteins that have the potential to be phosphorylated. The active kinases in the lysate are then able to phosphorylate the peptides on the slide. The slide is then stained with a phosphoprotein stain and scanned to detect fluorescence, indicating phosphorylation.

For each time point, the mean fluorescence of each peptide in each cell line was compared to the others and fold-changes and p values are obtained. When comparing J.Vector or J. Δ CY to J.LAG3, peptides that had an absolute value fold-change of greater than 1.5 and a p-value of less than 0.05 in both conditions were carried forward, and fold-change was averaged. Next, since J.Vector and J. Δ CY should behave similarly, peptides that had an absolute value fold-change of greater than 1.5 and a p-value of less than 0.05 when these two cell lines were compared were excluded. Finally, since SEE and SED both activate the cell lines in a nearly identical manner, peptides that had not yet been excluded in either SEE or SED activated conditions were carried forward and fold-changes were once again averaged (Figure 17). The proteins that these peptides belong to and average fold-changes are listed in Table 5 and Table 6.

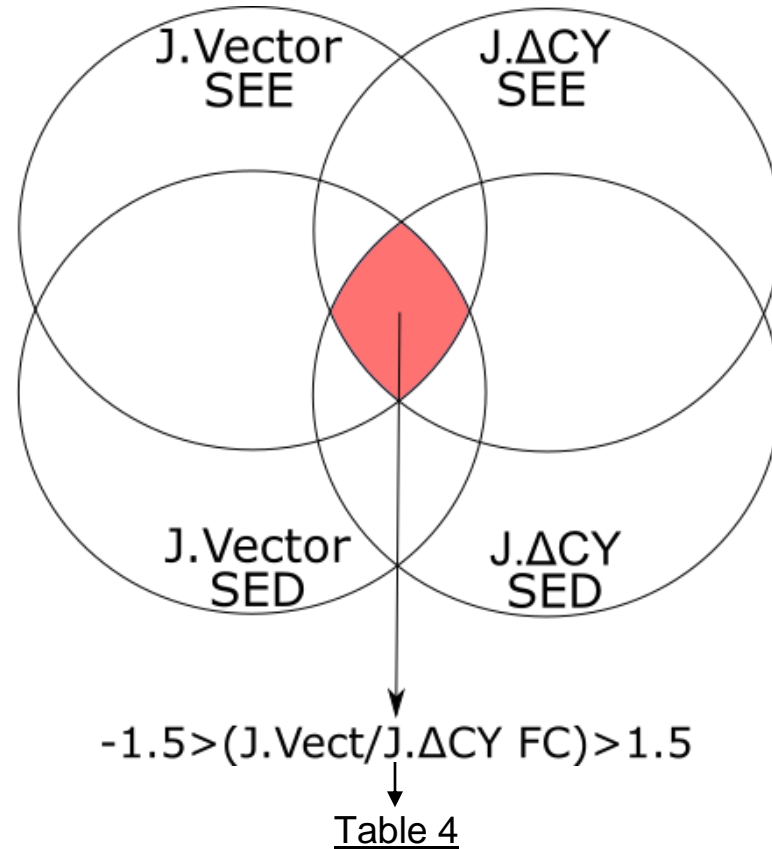
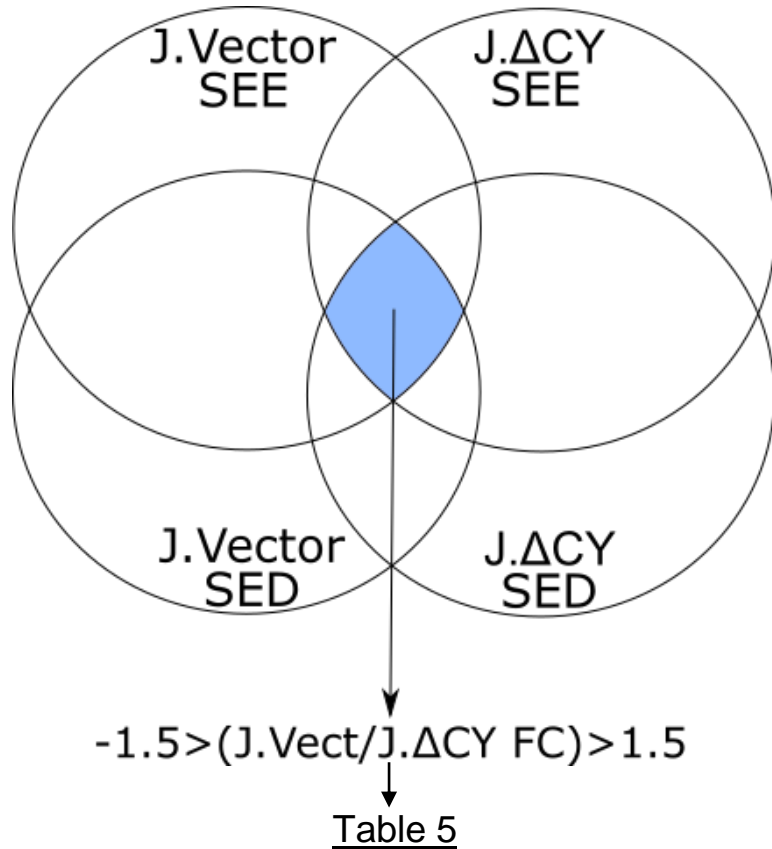
The proteins in Table 5 and Table 6 were then cross-referenced to TCR signaling or MAPK signaling pathways of NetPath¹⁵², reactome¹⁵³ and KEGG¹⁵⁴ using the InnateDB¹⁵⁵ platform. Proteins that are involved in the TCR or MAPK pathway are labelled red and blue respectively, while those upregulated in both are purple. Phosphorylations downregulated in the J.LAG3 condition in comparison with the other cell lines include cyclic AMP-responsive element-binding protein 1 (CREB-1), Growth factor receptor-bound protein 2 (GRB2), Glycogen synthase kinase-3 beta (GSK3 β), B-cell lymphoma/leukemia 10 (Bcl-10) (Table 5). Meanwhile, phosphorylations of proteins involved in the TCR pathway that are upregulated in J.LAG3 compared with the other cell lines lacking LAG3 activity include cyclic AMP-dependent transcription factor (ATF-2), cyclin-dependent kinase 1 (CDK1) and Serine/threonine-protein kinase (PAK2) (Table 6).

Proteins of the MAPK pathway that have downregulated phosphorylation in J.LAG3 include Mitogen-activated protein kinase kinase kinase 5 (MAP3K5) and Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit delta isoform (PPP2R5D) in addition to CREB-1 and GRB2 which are also involved in TCR signaling (Table 5). All those that are upregulated are also involved in the TCR pathway and include CDK1, ATF-2 and PAK2 (Table 6).

Figure 17 Procedure for analyzing changes in peptide phosphorylation.

Peptide phosphorylations downregulated compared to J.LAG3

Peptide phosphorylations upregulated compared to J.LAG3



For each time point, fold changes in peptide phosphorylation between J.LAG3, J.Vector and J.ΔCY were analyzed. Changes with p-value of greater than 0.05 were excluded from analysis. From the remaining peptides with p-values of less than 0.05, those that were upregulated or downregulated with a fold change (FC) of greater than 1.5 or less than -1.5, respectively, in J.LAG3 compared with J.ΔCY and J.Vector in SEE and SED activations, but not those with FC of greater than 1.5 or less than -1.5 in J.Vector compared with J.ΔCY are presented in Table 5 and Table 6

Table 5 Phosphorylation events downregulated in J.LAG3 relative to J.Vector or J.ΔCY.

2 minute		15 minute		45 minute		90 minute	
Protein (phosphorylation)	FC	Protein (phosphorylation)	FC	Protein (phosphorylation)	FC	Protein (phosphorylation)	FC
Phosphoglucomutase-2 (T34)	-2.04	Calpastatin (S223)	-2.70	Mitogen-activated protein kinase kinase kinase 5 (S75)	-2.67	Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit delta isoform (S85)	-9.48
Mothers against decapentaplegic homolog 2 (S467)	-1.88	E3 ubiquitin-protein ligase TRIM36 (Y178)	-2.65	Cyclic AMP-responsive element-binding protein 1 (S257)	-2.13	E3 ubiquitin-protein ligase TRIM36 (Y178)	-2.91
Long-chain-fatty-acid--CoA ligase 4 (S151)	-1.78	Phosphoglucomutase-2 (T34)	-2.21	Cytoplasmic dynein 1 light intermediate chain 1 (S207)	-2.11	Myosin light chain kinase, smooth muscle (S1073)	-2.53
		B-cell lymphoma/leukemia 10 (S171)	-2.19	Steroidogenic acute regulatory protein, mitochondrial (S195)	-1.99	Eukaryotic elongation factor 2 kinase (S364)	-2.35
				Eukaryotic elongation factor 2 kinase (S364)	-1.96	Cytoplasmic dynein 1 light intermediate chain 1 (S207)	-2.14
				Histone deacetylase 4 (S467)	-1.86	Rab GDP dissociation inhibitor alpha (Y333)	-2.12
				Growth factor receptor-bound protein 2 (Y52)	-1.78	Glycogen synthase kinase-3 beta (S133)	-1.80
						MS4A1 (S36)	-1.73

Bold text indicates proteins with differences at multiple time points.

Red, blue and purple text indicates involvement in TCR signaling pathway, MAPK signaling pathway or both, respectively

Table 6 Phosphorylation events upregulated in J.LAG3 relative to J.Vector or J.ΔCY

2 minute		15 minute		45 minute		90 minute	
Protein (phosphorylation)	FC	Protein (phosphorylation)	FC	Protein (phosphorylation)	FC	Protein (phosphorylation)	FC
Frizzled-1 (Y605)	2.43			TLR4 interactor with leucine rich repeats (S156)	4.74	Acyl-CoA synthetase family member 4 (Y195)	7.79
				Phosphorylase b kinase regulatory subunit beta (T693)	2.67	Glyceraldehyde-3-phosphate dehydrogenase (Y40)	4.37
				Phosphorylase b kinase regulatory subunit alpha, skeletal muscle isoform (S759)	2.42	Potassium-transporting ATPase alpha chain 2 (S71)	3.91
				Calcium/calmodulin-dependent protein kinase kinase 1 (S458)	2.39	Serine/threonine-protein kinase PAK 2 (S197)	3.58
				Calpastatin (S558)	2.13	SH3 domain-containing RING finger protein 3 (S376)	3.17
				Integrin alpha-6 (T677)	2.13	Toll/interleukin-1 receptor domain-containing adapter protein (Y117)	2.74
				Cyclic AMP-dependent transcription factor ATF-2 (S326)	2.06	Cyclin-dependent kinase 1 (S39)	2.22
				Cyclin-dependent kinase 2 (T160)	1.95	M-phase inducer phosphatase 1 (T508)	1.80
				Utrophin (Y3157)	1.85		

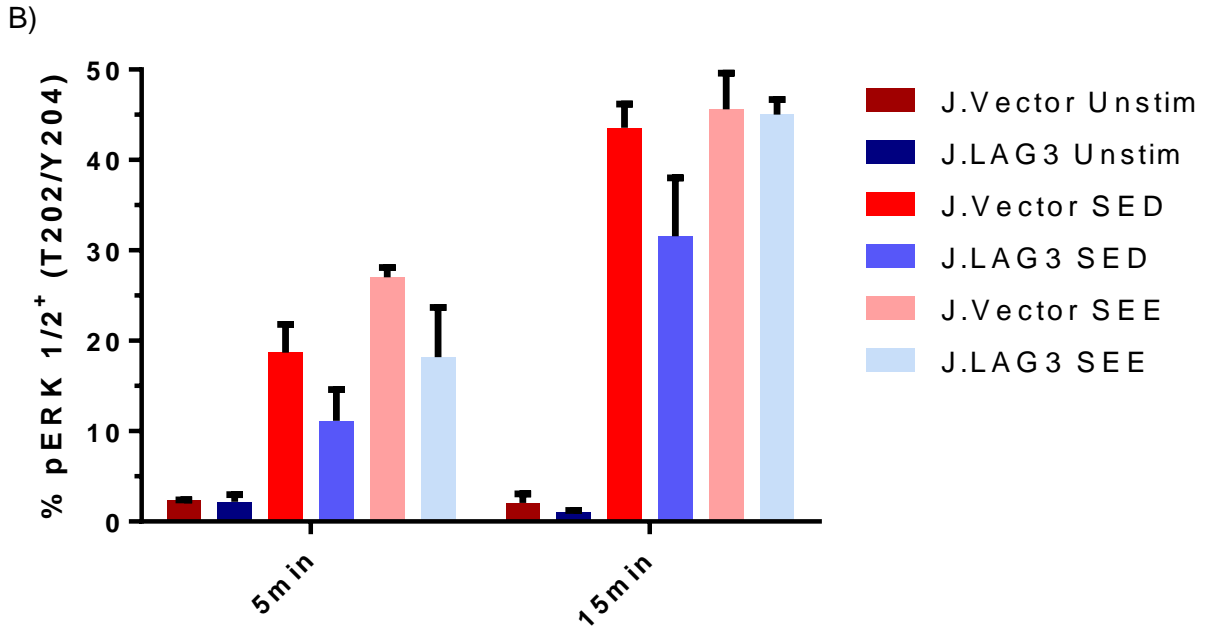
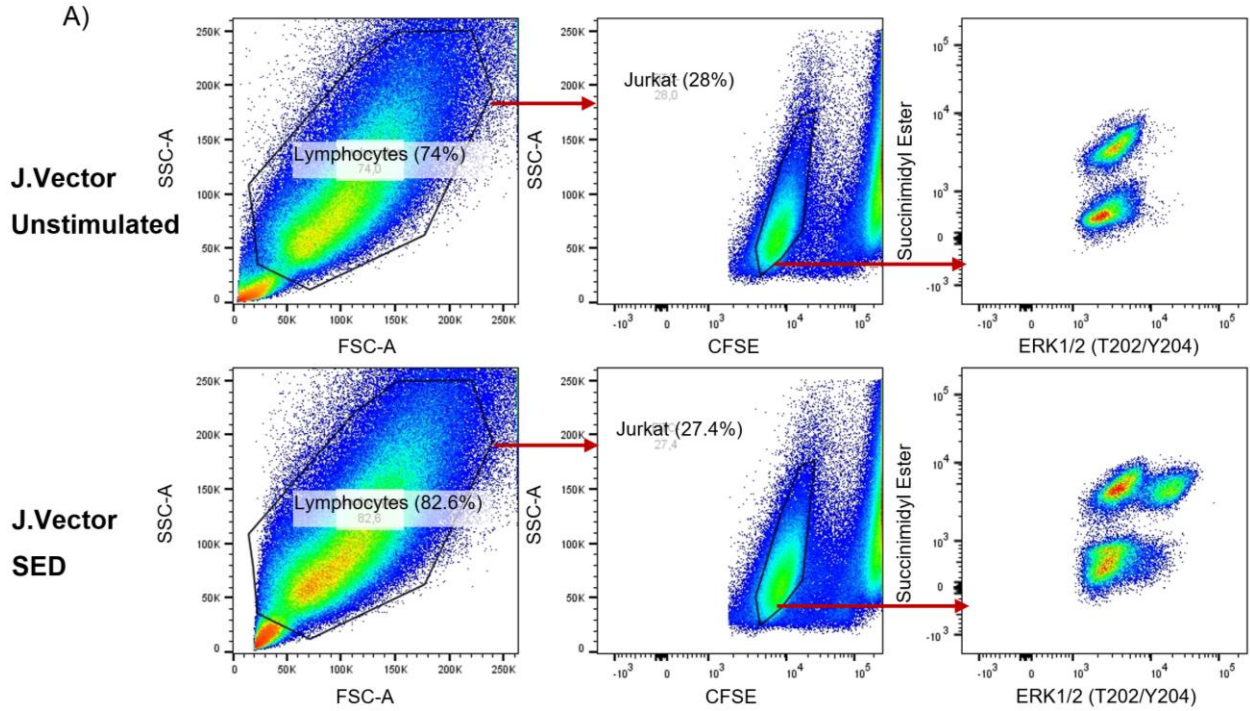
Red, blue and purple text indicates involvement in TCR signaling pathway, MAPK signaling pathway or both, respectively

7.4.5.2 Phospho-flow cytometry

The kinomics approach provides a broad picture of what branches of the TCR pathway may be particularly impacted by LAG3 activity by looking at kinase activity. To confirm LAG3's inhibition of the MAPK pathway, phospho-flow cytometry was used. Phospho-flow cytometry uses antibodies that bind specifically to the phosphorylated residue of a protein, but not the unphosphorylated residue. ERK1 (extracellular signal-regulated kinase 1) and ERK2, also known respectively as MAPK3 and MAPK1, are two closely related MAPKs with similar amino acid sequence and functionality¹⁵⁶. ERK1/2 phosphorylation on tyrosine 204/187 and threonine 202/195 activates the kinases and is the central outcome of the MAPK pathway¹⁵⁷. After activation, ERK1/2 can phosphorylate hundreds of cytoplasmic and nuclear substrates, including several transcription factors, leading to increased transcription, cell cycle progression, proliferation, migration and differentiation¹⁵⁷.

We therefore targeted ERK1/2 using phospho-flow cytometry to determine the impact of LAG3 activity.

Figure 18 LAG3 inhibition of ERK1/2 phosphorylation.



Five and fifteen minutes after SEE or SED activation, cells were fixed, permeabilized and stained for ERK1/2 T202/Y204 phosphorylation. A) Representative gating of unstimulated and SED activated J.Vector cells are shown where Raji B cells were removed by excluding CFSE and time points were separated by fluorescent barcoding. B) Summary data from experiments (n=2) are shown.

7.5 Summary

The Jurkat E6-1 cell line was chosen to create a model of LAG3 activity that would allow the study of the LAG3 mechanism of action. These cells were made to express full-length LAG3 or a truncated mutant of LAG3 lacking the cytoplasmic domain. Transduction with the vector alone, lacking any insert, was used as a control. After transduction, the cells that translate the inserted region were selected for by puromycin.

Next, CD3, CD2, CD28 and CD4 expression were shown to be similar between each cell line, suggesting no bias in transduction or puromycin selection. Then, LAG3 was measured on each cell line after being cultured at low or high cell density and activated or not by SEE. Full-length LAG3 was highly expressed and increased marginally after activation, whereas LAG3 lacking its cytoplasmic domain is expressed at very low levels, unless cells are activated, at which point its expression is similar to that of full-length LAG3. In both cell lines, culturing at higher cell density reduced LAG3 surface expression.

To ensure LAG3 activity, cell lines were activated with different concentrations of SEE or SED. In all concentrations tested, IL-2 was reduced by approximately half in cells transduced with full-length LAG3, whereas those transduced with the truncated mutant showed no impairment in IL-2 production. This suggests that the LAG3 expression on these cells is inhibiting IL-2 production. However, LAG3 blockade did not restore IL-2 production.

Finally, cells were activated with SEE or SED before being lysed and analyzed by a kinomics array. Proteins were then identified that have up/downregulated phosphorylation in J.LAG3 condition compared to the other cell lines. Of the 40 proteins that fulfil the stringent selection criteria, 22 were downregulated while 18 were upregulated. After cross-referencing these with signaling databases, 4/22 of the downregulated and 2/18 of the upregulated were identified as playing a role in the TCR signaling pathway, while 4/22 and 3/18 were identified as playing a role in the MAPK pathway.

The inhibitory role of LAG3 in the MAPK pathway was confirmed by measuring phospho-ERK1/2 using phospho-flow cytometry. This inhibition was observed as soon as 5 minutes after activation and was maintained, albeit to a lesser extent, at 15 minutes after activation.

7.6 Discussion

The model of LAG3 in Jurkats while not without limitations, which are discussed in section 8.4.6.1, provides the replicability and flexibility needed to study the LAG3 mechanism. Since this model was created, two other studies have published similar but different models. These models and the differences that make our model unique are discussed in detail in section 8.4.1.

The low level of LAG3 expression in the J.ΔCY cell line and subsequent increase in expression after activation observed in Figure 15b suggest that LAG3 is trafficked to the cell surface via at least two distinct mechanisms: at least one mechanism is

activation-independent and requires the cytoplasmic domain, while another is activation-dependent but does not rely on the cytoplasmic domain. The residence of LAG3 in intracellular vesicles hint that natural secretory pathways are responsible for this second activation-dependent mechanism^{54,149}.

LAG3 activity in the cell lines was tested and validated by superantigen activation in the presence of Raji B cells. This model confirms the ability of LAG3 to inhibit IL-2 production and the need for LAG3's cytoplasmic domain to exert its inhibitory function (Figure 16a-b). Unfortunately, the LAG3 blocking antibody (clone 17B4), while able to bind to LAG3 and block LAG3 in primary cells as shown in Figure 10 and previous studies^{16,92}, did not restore IL-2 production (Figure 16c). This contrasts with the other models of LAG3 in Jurkats which successfully blocked LAG3 using antibodies not commercially available^{58,158}. The antibody choice may be the distinguishing factor.

This model may be used to study the LAG3 mechanism in detail. Section 7.4.5 is the start of such research. Analysis of the kinomics array employed in this section identified peptides from proteins involved in the MAPK pathway, along with proteins generally involved in the TCR signaling pathway, as being differentially phosphorylated by lysates of J.LAG3 compared with J.ΔCY and J.Vector. Identification of ERK1/2 inhibition using phospho-flow cytometry confirms this result. Identifying the mechanism by which LAG3 functions has many potential downstream impacts including the better understanding of LAG3 contextual function and relevance, and the potential to improve LAG3-targeting therapies.

Future work in understanding the role of LAG3 in the TCR pathway may use MAPK inhibitors or a kinase inhibitor library with this model to determine whether inhibition of certain kinases can abrogate the LAG3 inhibitory function. Future work may also use these cells to identify LAG3-interacting proteins while LAG3 is engaged, a crucial component of understanding how LAG3 exerts its inhibitory function. The use of alternative LAG3 blocking antibodies could also provide an additional comparator for these studies.

8 Discussion

The Fowke lab has previously discovered that the immune checkpoint protein LAG3 is upregulated during HIV on T cells, NK cells and iNKT cells, and is correlated with dysfunction of iNKT cells⁹⁶. Moreover, the Fowke lab discovered that HIV-exposed seronegative (HESN) women, who have a phenotype of HIV resistance, express lower levels of LAG3 on T cells and NK cells than other HIV-negative women⁹⁸. Interestingly, in these studies, LAG3 was highest on a CD56⁻CD16⁺ population, which may consist of dysfunctional NK cells, but may also contain monocytes or other cell types¹⁵⁹. These studies reinforce the importance of LAG3, and that relatively little that is known about its cellular distribution.

The lack of data on LAG3 expression patterns is particularly notable since several LAG3 targeting or mimicking therapeutics are being tested in clinical trials for treatment of many forms of cancer or autoimmunity. Among these is an antibody that depletes LAG3-expressing cells. The promising early data on LAG3-targeting therapies in both animal models and humans continues to support the importance of LAG3 and its potential as a target in cancer, autoimmunity and infectious diseases, including sepsis and as part of a functional cure for HIV.

Another major gap in knowledge of LAG3 is its mechanism of action. LAG3 lacks an ITIM, ITSM or other known signaling domain. Although studies have tried to find a signaling domain, they are conflicting, with several mouse studies from the same group claiming the lysine (K) in the KIEELE motif to be necessary for LAG3 function^{55,57,86},

whereas more recent data could not confirm a role for the KIEELE motif, but instead found that the FxxL region of the RFFSALE was necessary⁸⁸. The KIEELE studies, each by Workman et al., used multiple models to show the necessity of the KIEELE motif in LAG3 function, including one in vitro model using murine T cell hybridomas expressing mutated LAG3 to produce IL-2 that was then measured by proliferation of an IL-2-dependent T cell line, and two atypical mouse models where proliferation was the outcome. While multiple models were used, there are caveats, including that LAG3 expression was not shown in any of these models, that only one or few conditions were used and that the models themselves are questionable. This is in contrast to the FxxL study, which used a well-established model of murine DO11.10 T cells expressing multiple LAG3 mutants, measured and presented LAG3 expression, and adjusted the measurement of LAG3-inhibition (IL-2 production) by LAG3 expression⁸⁸. Overall, it is unclear whether the KIEELE domain inhibits LAG3 function, however it is the opinion of the thesis author that due to the conflicting data and the issues mentioned above, it cannot reliably be assumed. In all cases, the cytoplasmic domain was crucial to LAG3 function. To date, only one protein has been found to bind the cytoplasmic domain of LAG3 – LAG3 associated protein (LAP). However, LAP binds to the EX repeat of LAG3, a region that may play a role in LAG3 function, but is not necessary for it^{57,88,89}. Due to the lack of a confirmed signaling motif or interacting proteins, the LAG3 mechanism remains uncharacterized.

The main objectives of this thesis are to:

1. Assess LAG3 expression on different cell types

2. Determine what branch of the TCR signaling pathway LAG3 inhibits

8.1 LAG3 expression across cell types

LAG3 is known to be expressed by NK cells, T cells, B cells, pDCs and neurons. However, no comparison of LAG3 expression between these or other cells has been published. Moreover, reports of LAG3 expression, which are mostly regarding T cells, show levels that vary considerably between studies. Knowing the distribution of LAG3 on cell types is important for understanding the complete scope of LAG3 function and to determine what cells may be impacted by therapeutic LAG3 blockade.

To determine LAG3 expression on different cell types, a flow cytometry panel was created that would differentiate several cell populations and evaluate LAG3 surface expression on each cell type. The panel created can classify 11 different cell populations based on the markers in

Table 4.

8.1.1 LAG3 expression varies, with monocytes exhibiting highest levels

LAG3 is most often studied on T cells, with very few studies examining LAG3 function or mechanism on other cell types. This neglect may be partially explained by the paucity of data on relative LAG3 expression across subsets. In this thesis, we designed a panel to assess LAG3 expression on multiple cell types.

LAG3 expression on lymphocytes was uniformly low, with a mean of 3.1% for CD8⁺ T cells and 1.7% for bulk T cells and <1% LAG3 expression for all other lymphocyte populations (Figure 3). This aligns well with the group of studies that show low levels of LAG3 expression on T cells, where LAG3 expression is typically less than 2%^{95,96,138,139}.

Unexpectedly, the studies performed in this thesis showed that LAG3 expression was highest in monocytes (Figure 3). Classical and intermediate monocytes were particularly high expressors, with an average of 25% and 32% of cells expressing LAG3, respectively. For one individual, 72% of each cell type were LAG3⁺. This is surprising since monocytes are thought not to express LAG3^{33,51,141}. However, recently LAG3 RNA expression was reported in monocytes and macrophages, while LAG3 protein has been reported on CD68 macrophages in tissue, although this study did not exclude pDCs, which may also express CD68^{38,142,160}. Interestingly, LAG3 RNA expression seems to be low in monocytes according to studies using transcriptomics and RNA-sequencing^{161–163}. However, these studies also show little or no LAG3 expression in pDCs, which are known to express LAG3 protein at high levels. It is unclear why this disconnect between RNA and protein exists for LAG3, but the fact that it exists for pDCs suggests that it may also exist for other cell types.

LAG3 expression on pDCs was discovered in 2014 by Camisaschi et al, where they reported that an average of 6% of pDCs express LAG3²⁹. This is supported by the results reported in Figure 3, where an average of 4.7% of pDCs expressed LAG3. Indeed, close inspection of the report by Camisaschi et al. shows that LAG3 expression on pDCs generally bifurcated individuals into high and low LAG3 expressing groups,

with most individuals clustering in the low LAG3 expressing group. This is corroborated by this thesis which shows a similar bifurcation where three out of seven individuals evaluated had undetectable levels of LAG3, whereas another three had between 5% and 16% of pDCs positive for LAG3 (Figure 3).

8.1.2 Discrepancies between LAG3 antibodies

When average *ex vivo* LAG3 expression levels are reported in studies, usually on T cells alone, they range from <1% to greater than 8% on healthy donor T cells^{56,95,96,134–139}. Most often, the studies reporting higher LAG3 expression levels use a goat polyclonal antibody (pAb) from R&D Systems Inc. (Cat #: FAB2319P), while those reporting lower LAG3 expression use a monoclonal antibody (mAb) (usually clones 17B4, 3DS223H or T47-530). While the multiple epitope binding ability of pAbs generally allows greater detection sensitivity, it can come at the compromise of specificity, where mAb may do better¹⁴⁰.

When the 17B4 mAb clone was compared to pAb across cell types, average pAb staining was significantly higher for all populations other than iNKT cells and intermediate monocytes (Figure 5a). Like the mAb, pAb exhibited the lowest %-positive staining amongst lymphocytes. The 8% of T cells staining LAG3⁺ by the pAb is very similar to expression determined by studies that use the pAb for LAG3 detection^{56,134–137}. Again, as with the mAb, pAb also detected LAG3 expression on monocytes, but to an even higher degree.

The general trend of LAG3 expression was similar by both antibodies, but fold differences of LAG3 expression between populations that express low levels and high levels of LAG3 were much larger with mAb staining due to the much lower baseline of LAG3 expression in lymphocytes. Interestingly, the “All negative” population, which is cells in the lymphocyte gate that stain negative for all markers in the panel, stain low for LAG3 by mAb, with similar levels to CD8⁺ T cells, but the pAb stains these cells highly. It is unclear what these cells are or why the difference in staining is so large for this population relative to others. It is possible that pAb is binding non-specifically to a protein that is highly expressed on these cells.

Polyclonal antibodies may show high lot-to-lot variability due to the way these antibodies are produced. For confirmation of experiment consistency, in addition to the similar staining level of the pAb to that of previous studies that use the pAb, three different lots of the pAb were compared and produced very consistent results. This indicates that lot inter-variability is low (Figure 5b).

While these results show that the pAb binds cells to a higher degree than the mAb, they do not answer the question of whether the pAb is non-specific or the mAb is less sensitive. To answer this and determine which antibody is preferable for LAG3 detection, each antibody was used to stain the LAG3-negative Jurkat cell line and transduced LAG3⁺ Jurkat cells. This comparison demonstrated that both mAb and pAb bind to LAG3, since binding was higher in the LAG3⁺ Jurkat cells (Figure 6). However, while the pAb exhibited non-specificity, interpreted from staining of LAG3⁻ Jurkat cells, the mAb showed high specificity and greater sensitivity, interpreted as greater staining

intensity of LAG3⁺ Jurkat cells. The increased specificity and sensitivity of the mAb suggests that it is preferable to the pAb for LAG3 detection.

This raises the question of what else is being detected by the pAb. One particular study characterized a group of CD4⁺ T cells that co-express LAG3 (as detected by the pAb) and CD49b, and classified these cells as T regulatory type 1 cells (Tr1)¹³⁵. This study has been cited at least 271 times, many of these using LAG3 and CD49b as co-markers of Tr1 cells. Since the original study used the pAb, detection of Tr1 cells using mAb may not be valid, due to the discrepancies in the binding of these antibodies. It is also likely that, at least in healthy individuals, since mAb detects LAG3 expression on far fewer CD4⁺ T cells, it would detect fewer Tr1 cells than the pAb.

8.1.3 Summary

Comparisons of LAG3 expression across cell types are needed, but are lacking, with most of the focus being dedicated to T cells. A flow cytometry panel distinguishing 11 cell types shows that LAG3 expression on lymphocytes is low compared to that on other immune cells. In particular, the panel detected high levels of LAG3 staining on monocytes, especially classical and intermediate monocytes, which were previously thought not to express LAG3.

Given the discrepant reports of LAG3 expression and the observation that studies detecting higher levels of LAG3 tend to use a pAb whereas those detecting lower levels of LAG3 typically use a mAb, flow cytometry was used to compare staining of pAb to that of mAb across cell populations. Staining of the pAb was higher than the

mAb across cell populations. Both antibodies exhibited a similar pattern of staining, with monocytes staining the highest.

To determine whether the mAb or pAb is more accurate, LAG3^{+/-} Jurkat cell lines were stained with each antibody and compared. The mAb exhibited higher specificity and higher sensitivity than the pAb, and therefore is more accurate than the pAb for LAG3 detection.

8.1.4 Limitations and opportunities for further study

This study is the first to compare LAG3 expression across lymphocytes, pDCs and monocytes. However, it focuses solely on PBMC from healthy donors. There remains the need to gauge LAG3 expression in different tissues and on different cells. Other future research would include the characterization of the LAG3⁺ cells in the “All negative” population. Comparison of LAG3 on these and other cells and tissues from people with diseases such as cancer and infection, where LAG3 is elevated on T cells, would be valuable as this is when LAG3 blockade is likely to be used as a therapeutic.

Further studies should be done to confirm LAG3 expression on monocytes, as indicated in Figure 3. Future confirmatory experiments should be completed and may be based on fluorescence-activated cell sorting of this population followed by western blots and RNA sequencing.

If monocytes do express LAG3, they would represent a new potential target of LAG3-targeted therapies. Future studies of LAG3 blockade or LAG3-depletion should then expand the scope to look at a breadth of cells, including monocytes. Further work

would also need to be done to characterize LAG3 function on these cells, and thereafter, a LAG3 mechanism.

The comparison between monoclonal and polyclonal antibodies provides an explanation for the discrepancies of LAG3 expression reported in the literature. A limitation of this study is that evaluation of this discrepancy was not done systematically for the following reasons:

1. Not all articles that detect LAG3 state which LAG3 antibody was used.
2. Not all articles that detect LAG3 have “LAG3” or any variant thereof in the abstract, so searching for these articles systematically is difficult.
3. Most articles do not report LAG3 expression raw data or the expression in numeric form, thus interpretation would need to be done by estimation based on graphs presented in the article.

Comparison of the specificity and sensitivity of the mAb to the pAb was performed on transduced Jurkat cells. A limitation associated with this is that specificity of the antibodies may differ based on the type of cell stained. Non-specific binding to a protein that is not expressed on Jurkat cells would not be detected by this methodology. Confirmation of non-specificity may be done by pull-down or by immunoblot. These techniques may also help determine what other proteins the pAb binds to.

8.2 Regulation of LAG3 expression

LAG3 is upregulated on T cells by activation and downregulated by mostly proteolytic cleavage via ADAM10 and ADAM17 metalloproteases and potentially partially by endocytosis. Despite knowledge of these mechanisms, surprisingly little is published on LAG3 expression kinetics or trafficking mechanisms.

8.2.1 LAG3 expression kinetics and regulation by TLR activation

LAG3 expression is increased on T cells after activation^{33,77,138,141}. To gain more insight into LAG3 expression kinetics, T cells were activated for up to 96 hours with daily LAG3 measurement (Figure 7). For the first 48 hours after activation, LAG3 expression increased substantially, then mostly plateaued, with only minor increase in expression thereafter. Plateauing LAG3 expression is likely driven by a steady-state equilibrium of new LAG3 expression and ADAM10/17 cleavage activity combined with dilution of LAG3 by proliferation.

To determine how this equilibrium was impacted after removing stimulation, T cells were activated for 24 or 48 hours before stimulation was removed and stained for LAG3 daily for the following 3 days (Figure 8). Surprisingly, after removing stimulus, T cells activated for 24 hours showed greater LAG3 expression following 24 hours, this was followed by a decline in LAG3 expression at a constant linear rate. This constant linear rate of declining LAG3 expression occurred throughout the 72 hours after removing stimulus from T cells activated for 48 hours (Figure 8). The fact that LAG3 expression on cells activated for 24 hours matched LAG3 expression on cells activated

for 48 hours one day after removing stimulus and thereafter indicates that the initial activation is most important for inducing LAG3 expression in an acute activation setting. This is the first study activating cells acutely followed by removal of stimulus for study of LAG3 expression.

TLR ligands have been used as adjuvants in cancer vaccines and as treatment of infectious diseases, including in attempts at a functional cure for HIV^{164,165}. The downregulation of immune checkpoints could be one mechanism for effective TLR-based treatment. Indeed, TLR activation has previously been shown to reduce expression of PD-1 on T cells¹⁴⁶, but the stronger immune response caused by downregulation of PD-1 can be compensated for by expression of LAG3.

Two studies show limited data in mice that TLR2, TLR3 and TLR4 stimulation reduce LAG3 expression while another study in mice shows no impact of TLR1/2, TLR3, TLR4, TLR7, TLR7/8, and TLR9 ligands on LAG3 expression^{144–146}. To shed new light on these inconsistencies, and test this hypothesis in human T cells, T cells were activated for 4 days with or without TLR4 (LPS) or TLR5 (flagellin) ligands and LAG3 expression was assessed (Figure 9). LPS had no impact on LAG3 expression, while flagellin reduced LAG3 expression in both CD4⁺ and CD8⁺ T cells. It is unclear why these different TLR ligands would have differential impact, but it may relate to TLR expression, since TLR5 is expressed at higher levels on T cells than TLR4¹⁶⁶. TLR ligand concentration or fundamental differences in the signaling of the TLRs may also explain the difference, although both signal through myeloid differentiation primary response 88¹.

Given that immune exhaustion likely evolved to limit the damage of autoimmunity or an overactive immune response and, that in being dependent on the presence of pathogens, TLR activation can serve as a signal that an immune response may be justified, TLR activation could serve as a mechanism to reverse exhaustion. This may occur by altering transcription factor activation (e.g., augmenting T-bet) or by blocking ICs or reducing their expression. Of the studies previously mentioned that detected reduced LAG3 expression after TLR activation, one proposed increased ADAM10/17 activity while the other implicated increased T-bet activity as the mechanism responsible for the effect^{62,145}. Indeed, TLR activation can increase both T-bet and ADAM10/17 activity, and therefore TLR activation could act on both levels to reduce LAG3 expression^{62,145,146}.

8.2.2 Summary

LAG3 is increased after activation, but the longer-term expression kinetics in the days following activation is understudied. Healthy donor PBMCs were used to study these dynamics. When stimulation is constantly applied, LAG3 expression increased rapidly for the first 48 hours, then mostly plateaued, implying a steady state equilibrium had been reached. When stimulus was removed from cells activated for 24 hours, LAG3 expression was higher 24 hours later followed by a constant decline, implying built up expression potential. In contrast, in cells activated for 48 hours, LAG3 expression was lower 24 hours later and experienced the same rate of decline as those cells activated for 24 hours for the next three days. This suggests that the initial activation, rather than the duration of activation, induces LAG3 expression.

One potential mechanism for this downregulation of LAG3 is proteolytic cleavage by ADAM10/17. This may also be a mechanism explaining the reduced LAG3 expression after addition of LPS and flagellin observed in Figure 9.

8.2.3 Limitations and further research opportunities

Limitations of the primary cell experiments on LAG3 kinetics are related to the specificity of conditions. The experiments presented herein activated cells with anti-CD3/anti-CD28 coated beads. While an effective activator of T cells, this stimulus does not engage co-receptors other than CD28, and therefore may not activate the cells with the same intensity or using the same signaling pathways as a typical antigen stimulation. The advantage of this stimulation is that it can activate a large fraction of T cells simultaneously. Other limitations of these experiments are the duration of stimulation and the frequency of LAG3 detection. When the stimulation was held constant, LAG3 was measured every day up to 4 days later, but LAG3 expression may change at later time points. Similarly, when the stimulation was removed, it is conceivable that shorter or longer durations of activation may have other effects that would not be predicted by the 24 and 48 hour stimulations done in these experiments. Likewise, if LAG3 was measured for longer than 72 hours after removal of stimulus, the constant declining expression may level off.

In testing the effects of TLR4 and TLR5 ligands on LAG3 expression, four days of stimulation was chosen to match previous studies that showed effects of TLR activation on PD-1 and LAG3, but it is likely that other durations would show different degrees of impact of TLR activation^{144,145}. 100ng/ml of LPS and flagellin were used in

these experiments. A limitation of this study is that higher concentrations of LPS were not tested, but may have an effect. Furthermore, since T cells were not isolated and were in the presence of other PBMCs, it cannot be certain that the effects seen are due to TLR activity on the T cell as opposed to TLR activity on bystander cells.

An overarching limitation of this study's evaluation of mechanisms governing LAG3 expression is that these experiments do not investigate what impact the activity of ADAM10/17 metalloproteases, T-bet or both have on the results. Therefore, future studies could measure T-bet activation, or use ADAM10/17 metalloprotease inhibitors or cells in which T-bet is deleted or otherwise inhibited. Furthermore, more TLR ligands should be tested to determine which are able to downregulate LAG3 or other IC expression.

8.3 LAG3 in primary T cell activation

A model of LAG3 function should be able to demonstrate the best characterized functions of LAG3 – inhibition of activation-induced cytokine production (particularly IL-2, IFN- γ and TNF- α) and proliferation^{86,92,113}. The superantigen SEB has previously been used to elicit LAG3 activity in human T cells^{92,113}, since it mimics peptide stimulation on a larger scale, allowing co-receptors to participate in signaling. When testing the ability of LAG3 blockade to increase cytokine production, these studies using the SEB model measured cytokine in the supernatant over two or three days of stimulation and found an augmentation effect of the blockade. However, this model is not conducive to evaluating LAG3's impact on TCR signaling because many signaling

events occur in the seconds or minutes following stimulation and LAG3 basal expression is low in the absence of previous activation. To characterize LAG3's impact on TCR signaling, LAG3 expression must be high while signaling levels are low, for subsequent induction. Therefore, an adapted model may pre-activate cells for a long duration to upregulate LAG3, rest the cells to lower signaling, then reactivate these cells for a short duration in the presence of LAG3 blockade to measure LAG3's impact on TCR signaling.

For this model to succeed LAG3 expression must remain high while signaling is low. Here, cytokine production was used as a proxy for signaling protein activation. Two days was determined to be the optimal rest period allowing relatively high LAG3 expression with a return to baseline in cytokine production (Figure 12). Cells readily produced cytokines when reactivated, but LAG3 blockade did not impact cytokine production (Figure 13).

This lack of an effect of LAG3 blockade was likely because LAG3 expression on these cells was not high, with 42% of CD8⁺ T cells and 9% of CD4⁺ T cells expressing LAG3 after the two-day rest period in the individual tested (Figure 12). The rapid downregulation of LAG3 after removal of stimulus and lack of an effect on cytokine production of the LAG3 blockade indicates that this model is not feasible for evaluation of LAG3's impact on TCR signaling.

To validate the inhibitory effect of LAG3 on proliferation and confirm the activity of the blocking antibody, T cell proliferation was measured. LAG3 blockade significantly enhanced proliferation of SEB-responsive T cells (Figure 10). This suggests that the

LAG3 blocking antibody is functional and confirms the inhibitory effect of LAG3 on proliferation.

8.3.1 Summary

To characterize a potential model for evaluation of LAG3 signaling, T cells were activated with SEB, rested, then reactivated in the presence of LAG3 blockade. LAG3 blockade did not augment cytokine production. This lack of effect is likely due to the rapid downregulation of LAG3 during the rest period, leading to fewer cells expressing LAG3 and therefore a lower signal:noise ratio. This suggests that this model is not conducive to evaluating LAG3's impact on TCR signaling.

While LAG3 blockade did not increase cytokine production, it did augment SEB-induced proliferation, confirming previous findings.

8.3.2 Limitations and further research opportunities

Only one duration and two concentrations of SEB stimulation were tested for the upregulation of LAG3, and this was only tested on one individual. It is possible that a longer duration would better sustain LAG3 expression, and therefore potentially be effective as a model evaluating TCR signaling. Other models may involve lentiviral transduction of primary T cells with LAG3.

Instead, to limit biological variability, a model using lentiviral transduced Jurkat cell lines was pursued to study the effect of LAG3 on TCR signaling.

8.4 A model of LAG3 activity and its use in assessing the impact of LAG3 on T cell receptor signal transduction

8.4.1 Creating a model for studying LAG3

A model of LAG3 activity for studying the LAG3 mechanism would ideally use primary human cells. However, as discussed in section 8.3, the low LAG3 expression at baseline, rapid downregulation of LAG3 after activation, biological variability and reliance on LAG3 blockade antibody limit the use of primary cells.

Another option to study the LAG3 mechanism would be to use animal models. Transgenic and LAG3^{-/-} mice are frequently used in LAG3 research to study LAG3's role in disease^{41,55,118,125,167}, so studying the LAG3 mechanism in mice would have the benefit of previous research characterizing the model. However, mice are genetically different to humans, with many differences in the LAG3 gene, and are thus an imperfect model. Furthermore, mouse cells have some of the same issues as human primary cells regarding the maintenance of LAG3 expression and there are ethical implications of using mice when other models are available.

For these reasons, the Jurkat human CD4⁺ T cell line was used to create the model. Much of the human T cell receptor pathway has been characterized using the Jurkat cell line, and thus its signaling mechanism is very well defined. Jurkat cells are also suitable for creating a LAG3 model because they do not express LAG3 at baseline or after activation^{58,149,168}. Because of this, the Jurkat cells must be made to express LAG3. This can be done by transfection with a plasmid or by transduction.

We chose to use lentiviral transduction to induce LAG3 expression in Jurkat cells. Lentiviral transduction takes advantage of the ability for lentiviruses to integrate their genetic material into the host genome to insert a desired genetic sequence. This method was chosen to create cell lines that would stably express LAG3 indefinitely, such that the cell lines could be frozen and used later.

The Jurkat E6-1 cell line was transduced with virus made from plasmids containing full-length LAG3, a truncated LAG3 possessing no cytoplasmic domain or a vector with no LAG3 insert. A puromycin tag allowed selection of successfully transduced cells.

Since we began creating this LAG3 model, two other groups have published similar models, using lentiviral transduction or transfection to overexpress LAG3 in Jurkat cells. Each of these other models differs from the one described in this thesis in four substantial ways:

- 1) After transfection/transduction, the cells were screened for high LAG3 expression.
- 2) The models are formed from single cell clones.
- 3) The models were co-transfected/co-transduced with NFAT luciferase reporter.
- 4) The models did not include controls transduced or transfected with the vector alone or with the truncated LAG3 lacking the cytoplasmic domain.

The caveats to these differences and the reason why the model in this thesis differs is that screening cells for high LAG3 expression and sorting on these cells may skew the cells, making them different from the untransduced or untransfected cells in ways other than LAG3 expression. However, it is the growing up from single cell clones that provides the greatest risk of this skew, especially since insertion into the genome will occur at different sites, potentially disrupting expression of other genes. Therefore to avoid this potential skew, which can introduce the doubt of whether the cell lines were similar in all other ways, the cell lines were not expanded from a single clone.

Use of the NFAT luciferase reporter should not alter the behaviour of the cells in any meaningful way.

The goal of these two studies was to make a model of LAG3 activity that would be sensitive to LAG3 blocking antibody. In contrast, characterizing the LAG3 signaling mechanism is the main objective of this model.

The cells used herein were shown to be largely uninfluenced by the lentiviral transduction other than LAG3 expression by the similar expression levels of CD3, CD2, CD28 and CD4 (Figure 15).

These models previously created by other groups and other studies have recently discovered that LAG3 inhibits NFAT activity following activation^{58,87,113}. However, one of these studies also showed that cyclosporin A, a calcineurin inhibitor that downregulates NFAT activation, did not impair the stimulatory effect of LAG3 blockade, suggesting LAG3 acts upstream. While NFAT activation is inhibited by LAG3,

what other transcription factors, signaling proteins or signaling pathways LAG3 is involved in is unknown.

These gaps in the knowledge of LAG3's mechanism hinders LAG3 research. A better understanding of LAG3's mechanism could help characterize LAG3 activity on other cells or help find new therapeutics, which are much needed for treatment of chronic diseases, such as autoimmune disorders.

8.4.2 Importance of LAG3's cytoplasmic domain in its translocation to the cell surface

Studies of LAG3 function and mechanism often use a truncated mutant of LAG3 lacking its cytoplasmic domain. In primary mouse cells, where this mutant is usually studied, when mutant LAG3 expression on these cells is assessed its expression is usually similar or slightly lower than that of wild-type LAG3 (personal communication with D. Vignali and C.J. Workman, May 20, 2017)⁸⁸. However, the only published study of this mutant in human cells was done by transfecting the Jurkat cell line with a LAG3 containing plasmid. In this model, the cytoplasmic domain deletion reduced surface expression of LAG3, while maintaining similar total cellular levels as Jurkats expressing full-length LAG3¹⁴⁹. Overall, clarity is needed for the role of the cytoplasmic domain in LAG3 trafficking.

To answer this question, Jurkat cell lines expressing full-length LAG3 (J.LAG3) or LAG3 lacking its cytoplasmic domain (J.ΔCY) were created by lentiviral transduction

and compared to LAG3-negative Jurkats that had been transduced with the vector alone (J.Vect) (Figure 15).

While J.LAG3 cells expressed high levels of LAG3, J.ΔCY cells expressed LAG3 at much lower levels than J.LAG3 cells. This supports research by Bae et al. in transfected Jurkat cells. A novel finding of this study was that when J.ΔCY cells were activated by superantigen, LAG3 expression was significantly increased to a level similar to J.LAG3 cells. These results suggest that trafficking of LAG3 to the cell surface when cells are resting may require the cytoplasmic domain, whereas when cells are activated, LAG3 is transported to the cell surface via a cytoplasmic domain-independent mechanism. Since LAG3 is known to reside intracellularly in endocytic vesicles and secretory lysosomes, the natural secretory pathways may be responsible for this cytoplasmic domain-independent mechanism^{54,149}. The mechanism governing baseline LAG3 trafficking to the cell surface is unclear, but previous research suggests it is not reliant on the EP motif, which would otherwise be the most likely candidate due to its interaction with LAP, which is 99% identical to the centrosomal P4.1-associated protein, part of the γ -tubulin complex^{89,149}.

These experiments also show that high density culture of the transduced cell lines reduces LAG3 expression by a consistent absolute of approximately 10%. This translates into a higher relative reduction in J.ΔCY cells since they have lower LAG3 expression. The large relatively lower LAG3 expression on J.ΔCY cells is a variable in future experiments regarding LAG3 function and signaling in these cells, but since J.ΔCY is meant to represent a non-functional LAG3 control, reduced LAG3 expression would not change the conclusions drawn from the results.

8.4.3 Cell line model reflects LAG3 activity

Many studies have described the ability of LAG3 to inhibit IL-2 production, making it one of the best characterized activities of the IC^{41,58,81,86}. Jurkat cells produce large quantities of IL-2 after activation, making IL-2 measurement an ideal outcome for measuring LAG3 activity.

For LAG3 to be active, it must bind to a ligand to facilitate cross-linking or colocalization with the TCR. Raji B cells coated with superantigen has been well characterized as a model for Jurkat activation. Since this model activates the cell in a peptide-like manner, it engages the relevant co-receptors through their respective ligands, including LAG3 (MHCII), CD4 (MHCII) and CD28 (CD80/CD86).

Therefore, after Jurkat cell lines were activated with SEE or SED coated Raji B cells for 24 hours, IL-2 was measured by ELISA as a surrogate for LAG3 activity (Figure 16). The two-fold reduction in IL-2 from the J.LAG3 culture compared to the J.Vector and J.ΔCY cultures indicated that LAG3 was indeed functional. This reduction was consistent in both SEE and SED stimulations and at several different concentrations of superantigen, suggesting that LAG3 activity is robust and independent of the type of superantigen.

Interestingly, the 17B4 antibody clone often used to block LAG3 activity in vitro did not restore IL-2 production in SEE activated cells. This is despite the ability for this antibody to bind to LAG3 specifically and sensitively. The other models of LAG3 activity

on Jurkat cells used clinical antibodies to block LAG3. Unfortunately, these antibodies are not commercially available. This difference may explain the differing results.

8.4.4 The impact of LAG3 on the TCR signaling pathway

With a model that allows comparison of cells with LAG3 activity to those without LAG3 activity, the impact of LAG3 on the TCR signaling pathway can be evaluated. Before focusing on the activation of individual signaling proteins, a kinomics based approach was used to obtain a broader picture of how LAG3 may disrupt TCR signaling. This approach, which is based on kinase activity from the cell lysates phosphorylating peptides printed on a slide, has previously been used to profile the immune response to viruses and other stressors⁸. Cells were activated for up to 90 minutes with SEE or SED, then flash frozen and later lysed. The lysates were added to slides printed with 1294 different peptides corresponding to regions phosphorylated by kinases, allowing the kinases in the lysates to phosphorylate these peptides. Then the slides were stained and scanned.

The resulting data was broken down by time point, then analyzed by filtering out peptides for which phosphorylation was different between the J.Vector and J.ΔCY in either SEE or SED stimulated conditions (Figure 17). Then, those peptides with increased or reduced phosphorylation in J.LAG3 compared to J.Vector and J.ΔCY in both SEE and SED stimulated conditions were carried forward and are presented Table 5 and Table 6.

Those proteins were then cross-referenced with signaling pathway databases to identify pathway involvement. Proteins in the TCR signaling pathway with differentiated phosphorylation of the peptides between the conditions were regarded due to the nature of the stimulation. The MAPK signaling pathway emerged from the data as a pathway with several of the proteins identified to be different between conditions. Of the proteins identified to have roles in these pathways, six were found in the list of proteins with phosphorylations induced by kinases downregulated in the J.LAG3 condition compared to three found in the upregulated list. Table 7 gives a brief description of the relevant function of each of these proteins. GRB2 is perhaps the most intriguing, since it plays a direct, important and somewhat early role in TCR signaling in forming the LAT signalosome¹⁶⁹. This early role would fit well with the potentially broad inhibitory nature of LAG3. Indeed, Grb2 inhibition is an inhibitory mechanism of the immune checkpoint TIGIT which uses Grb2 to recruit SHIP1 and thereby inhibit PI3K and MAPK signaling¹⁷⁰. Likewise, PP2A, of which PPP2R5D is a regulatory subunit, is involved in CTLA-4 mediated inhibition of T cell activation^{171,172}. In contrast, CDK-1 is among the least interesting since it is primarily involved in mitosis, with little known involvement in other activation¹⁷³. However, it is important to recognize that the kinomics array measures the activity of the kinases, not the phosphorylation of the proteins. Therefore, while knowing which protein's peptides are phosphorylated is important and relevant, the pathway and the kinase upstream of the peptide should be the focus. Unfortunately, many phosphorylation sites have multiple kinases and for many the kinase is unknown. Therefore, it is often the pathway or a sub-branch that should be considered.

For this reason, phospho-flow cytometry was used to confirm the LAG3-mediated inhibition of the MAPK pathway. ERK1/2 inhibition by LAG3 was observed after only five minutes of activation and maintained, at least in the SED activated condition for 15 minutes. The relatively minor inhibition of ERK1/2 is compared with IL-2 inhibition suggests that ERK1/2 and the MAPK pathway is only one branch that is inhibited by LAG3 and that LAG3 is acting on multiple pathways, and/or is acting upstream interrupting proximal signaling events. Indeed, previous demonstrations of LAG3 inhibition of calcium flux and NFAT suggests that LAG3 is acting at the proximal TCR signaling events^{58,83}.

Table 7 Roles of proteins from Tables 4 and 5

Downregulated phosphorylation in J.LAG3 compared to other conditions	
Protein name	Description
<i>B-cell lymphoma/leukemia 10</i>	TCR-mediated stimulation recruits Bcl-10 to the TCR complex via CARD-containing MAGUK protein-1. Bcl-10 is able to induce apoptosis through its interaction with caspase and activate NF-κB through its interaction with MALT lymphoma translocation protein-1 ¹⁷⁴ .
<i>Mitogen-activated protein kinase kinase kinase 5</i>	MAP3K5 is a crucial component of the MAP kinase pathway. MAP3K5 activates several other MAP kinase kinases ¹⁷⁵ .
<i>Cyclic AMP-responsive element-binding protein 1</i>	CREB-1 is a transcription factor that binds to the cAMP response element (CRE), a sequence present in the promoters of many genes ¹⁷⁶ .
<i>Growth factor receptor-bound protein 2</i>	Grb2 is a scaffold protein involved in the LAT signalosome, acting as an important amplifier of the TCR signaling pathway. Grb2 also encourages Ras signaling, upstream of the MAPK pathway ^{170,177} .
<i>Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit delta isoform</i>	PPP2R5D is a regulatory subunit of PP2A, which is a phosphatase that inactivates MAP kinases and Akt ^{171,178} .
<i>Glycogen synthase kinase-3 beta</i>	GSK-3 is a serine/threonine protein kinase with over 100 known substrates. GSK-3b regulates CREB-1, NFAT, NF-κB and many other transcription factors ¹⁷⁹ .
Upregulated phosphorylation in J.LAG3 compared to other conditions	
Protein name	Description
<i>Serine/threonine-protein kinase PAK 2</i>	PAK2 phosphorylates MAPK4 and MAPK6. PAK2 enhances mTORC1-mediated activation of S6 and enhances activation of PLCγ1 after TCR activation ¹⁸⁰ .
<i>Cyclic AMP-dependent transcription factor ATF-2</i>	ATF-2 is a transcription factor that can form complexes with other transcription factors, facilitating the transcription of many genes. ATF-2 can also act as an acetyltransferase. ATF-2 is phosphorylated by MAPK ¹⁸¹ .
<i>Cyclin-dependent kinase 1</i>	CDK1 is primarily involved in mitosis ¹⁷³ .

Red, blue and purple text indicates involvement in TCR signaling pathway, MAPK signaling pathway or both, respectively

8.4.5 Summary

A model of LAG3 activity was created using lentiviral transduction of the Jurkat cell line with full-length LAG3, a non-functional truncated mutant or the vector alone. These cells expressed similar levels of CD3, CD2, CD28 and CD4, indicating no significant change between cells induced by the transduction or selection process.

Jurkat cell lines transduced with full-length LAG3 (J.LAG3) or a truncated LAG3 mutant lacking its cytoplasmic domain (J. Δ CY) were used to study the necessity of the cytoplasmic domain for surface expression. These experiments showed that baseline surface expression of LAG3 in resting conditions is largely dependent on the cytoplasmic domain, whereas after activation, LAG3 surface expression of J. Δ CY was similar to that of J.LAG3. This implies that LAG3 uses a different trafficking mechanism after activation that is independent of the cytoplasmic domain. These results support a previous study in Jurkat cells, but the implication contradicts studies in murine T cells.

With LAG3 expression confirmed, LAG3 activity was confirmed in the cells by using IL-2 production as an indicator. IL-2 was reduced by two-fold in J.LAG3 cells compared to the other cell lines with no functional LAG3, suggesting robust LAG3 activity, although the LAG3 blockade did not restore IL-2 production.

Next, a kinomics approach was used to identify signaling proteins which may constitute LAG3 targets in the TCR signaling pathway. A list of proteins at 2 minutes, 15 minutes, 45 minutes and 90 minutes was generated with amino acids that are differentially phosphorylated in all conditions where J.LAG3 is being compared to

J.Vector or J.ΔCY and none of the conditions where J.Vector is being compared to J.ΔCY. This list of proteins was cross-referenced to proteins known to be involved in TCR and MAPK signaling pathways and candidates were identified from this list.

Finally, inhibition of the MAPK pathway was confirmed by showing ERK1/2 inhibition through use of phospho-flow cytometry, but the relatively minor impact on ERK1/2 along with previous literature showing inhibition of other pathways suggests that LAG3 acts at the proximal TCR signaling events.

8.4.6 Limitations and further research opportunities

8.4.6.1 Transduced Jurkat cells as a model for LAG3

While this thesis used a cell line, assessment of LAG3 activity, mechanism of action or the importance of the cytoplasmic domain for LAG3 expression would ideally be done in human primary cells. Unfortunately, primary cells have limitations, such as large biological variability, the need for a previous activation to induce LAG3 expression and the difficulty of maintaining high LAG3 expression in the absence of activation as shown in section 6. There is no perfect model for LAG3 activity.

Jurkat cells, although imperfect, are extensively characterized and have contributed much to the study of the TCR signaling pathway¹⁵¹. Jurkat cells have a higher level of basal activation and proliferate continuously, due in part to mutations in the genes for SH2-containing inositol 5-phosphatase (SHIP) and phosphatase and tensin homolog protein (PTEN), which are important regulators of signaling. This results

in abnormally high PI3K activity, which influences many aspects of the TCR signaling pathway.

Fortunately, while not insubstantial, these issues with the Jurkat cell line do not prevent these cells from being a useful model of LAG3 activity or signaling. The confirmation of LAG3 activity in Jurkat cell lines suggests that LAG3 is not dependent on SHIP or PTEN. Furthermore, so long as the only variable between cells is LAG3 expression, comparing differences in cell function or cell signaling between cells should be valid.

Jurkat cells are also not ideal for assessing the role of the cytoplasmic domain on trafficking to the surface. Again, this is because Jurkat cells have mutations, some of which increase signaling and proliferation. However, other models, such as mouse cells, are also imperfect due to genetic dissimilarity. When mouse cells and cell lines imply the same conclusions, one can be reasonably confident that the same is true of primary human cells, but when they differ, it can be unclear which is accurate. The cytoplasmic domain's involvement in LAG3 expression is such a case where these models are somewhat contrasting. Therefore, confirmation in primary human cells will need to be performed, perhaps by transfection or transduction with a fluorescently tagged LAG3.

8.4.6.2 Kinomics

Kinase activity, and phosphorylation events more broadly, are crucial in TCR signaling. The effects of TCR stimulation on kinase signaling in the T cell are broad and substantial.

The two main methods for assessing the activation of kinases are phosphoproteomics, which detects the degree of phosphorylation of specific residues on kinases, and kinomics, which measures the activity of kinases based on the occurrences of phospho-transfer events.

Phosphoproteomics is based on enrichment of phosphorylated proteins followed by analysis by mass spectrometry. Limitations of this procedure include issues with the selectivity of the enrichment process and the potential loss of proteins with low abundance or low stoichiometry of phosphorylation¹⁸².

The method used in this thesis is a kinomics approach involving the use of slides with spots of printed peptides. These arrays take advantage of the principles of kinase substrate specificity, which is determined by the four adjacent residues on either side of the phosphorylation site, and is not highly dependent on secondary or tertiary structure⁸. The main limitations of this method include the limited scope and the lack of phosphatase activity. For this method, specific peptides with known phosphorylation sites were chosen out of the ~230,000 total sites on human phosphoproteins. Therefore, this method suffers from a bias regarding which phosphosites are included and only encapsulates a small fraction of the total sites¹⁸². Phosphatase activity is very important in TCR signaling. Phosphatases are involved in the activation or inactivation of specific signaling proteins. This kinomics approach however uses phosphatase inhibitors, which allows a more stable and stronger kinase signal, but misses the entirety of the phosphatase signal, representing another limitation of this method.

Other limitations are to do with the lack of knowledge about these signaling pathways in general. Not all of the proteins involved in the TCR signaling pathway or associated pathways are known to be involved. Furthermore, the targets of kinases are not entirely characterized. This is important because one kinase can target many different proteins for phosphorylation and a given protein may be phosphorylated by several kinases to differing degrees. Also, the function or changes induced by some phosphorylation events are known, but many are not, making it difficult to interpret results.

8.4.6.3 Future research

Due to the limitations mentioned above and the fact that this kinomics array is a broad-based approach, other more focused methods should be used on this model to find specific signaling proteins that are differentially activated when LAG3 is active versus inactive. Examples include phospho-flow cytometry and western blot. This was performed with ERK1/2, but could be performed with several other proteins in other pathways.

While comparison between cell lines may be adequate, the ability to see a restoration of signaling activity when LAG3 blockade is applied would be ideal. If possible, the use of pre-clinical antibodies should be tested in this model, first to confirm the ability of blockade to restore IL-2 production, and eventually to compare activation of signaling proteins. Further research could use kinase inhibitors to determine which kinases are necessary for LAG3 function.

This section of the thesis focuses on LAG3 activity in CD4⁺ T cells, but one area of future research would be characterizing and comparing LAG3 activity and mechanism in several different cell types.

8.5 General discussion

In section 1.5.1, the two main themes of this thesis, *1. LAG3 expression profile, kinetics and mechanisms* and *2. LAG3 mechanism of action in T cells*, were presented. As theme 1 is related to expression and mechanisms of expression regulation, primary PBMC from healthy donors were used to answer related questions, since expression in an animal model or cell line would not necessarily replicate the situation in humans. In contrast, theme 2 relied on a cell line model for studying the LAG3 mechanism, since primary cells present challenges for this form of study and a cell line model presented some unique advantages as outlined in section 8.4.6.1.

8.5.1 LAG3 expression profile, kinetics and mechanisms

This thesis demonstrated that while LAG3 expression is low on lymphocytes, it may be expressed at high levels on monocytes. While more work is needed before this is confirmed, this study suggests that the scope of research on LAG3 is often too narrow. The discovery of LAG3 expression on monocytes prompts many important questions about LAG3 function that may be answered in future studies (Can LAG3 inhibit monocyte activation? If so, what types of activation?), mechanism (How does LAG3 inhibit activation of non-T cells? Does LAG3 inhibit monocytes through binding of MHCII on the same monocyte [i.e., in cis or in trans]) and potential therapeutic

importance on these cells (Does LAG3 blockade enhance monocyte activation? If so, in what contexts?).

In detecting LAG3, this study also compared a commonly used polyclonal antibody to a monoclonal antibody and in doing so, confirmed that the discrepant reports of LAG3 expression in the literature are due to the use of different antibodies. This study went further to compare specificity and sensitivity of each antibody on a T cell line and showed superior accuracy of the monoclonal antibody on both accounts. This comparison allows better interpretation of the literature, raises many questions regarding already published data and helps inform better design of future studies.

In addition to detecting *ex vivo* LAG3 expression, this thesis studied regulation of LAG3, in particular its expression kinetics after TCR-mediated activation and expression after cellular activation with TLR ligands. These studies showed that LAG3 expression is built up within the first 24 hours whereafter it maintains a steady state when stimulus is maintained. They also show that LAG3 expression can be downregulated in response to TLR activation, particularly TLR5 activation by flagellin. This has implications for TLR agonist use in vaccine studies and for treatment of disease, including for use in a functional cure for HIV.

8.5.2 LAG3 mechanism of action in T cells

The next main objective of this thesis was to build a model to study the LAG3 mechanism of action. A primary cell model was first attempted by upregulating LAG3 on T cells from PBMC of healthy donors by activation, then resting the cells to bring activation back to baseline while maintaining LAG3 expression. However, this model

was unsuitable because the required rest period allowed LAG3 expression to decline substantially, leading to no impact of LAG3 blockade on cytokine production.

The next attempt at creating a model for studying the LAG3 mechanism was based on overexpression of LAG3 on the Jurkat cell line. After lentiviral transduction, the Jurkat cells overexpressing wild-type LAG3 showed high LAG3 expression on the cell surface. However, Jurkat cells expressing LAG3 with the cytoplasmic domain deleted expressed far lower levels of LAG3, except after activation. This suggests that resting LAG3 trafficking to the cell surface is dependent on its cytoplasmic domain, confirming a previous study that suggested the same¹⁴⁹, but somewhat contradicting others that used mouse models⁸⁸.

Other than LAG3 expression differences, the cell lines had similar levels of other important proteins involved in TCR activation, namely CD3, CD2, CD28 and CD4. After activating these cell lines with superantigen, IL-2 production was reduced by half on cells expressing full-length LAG3, but was not rescued by LAG3 blockade. This suggests that comparison of Jurkat cells expressing full-length LAG3 to non-functional or no LAG3 is the optimal comparison rather than use of the 17B4 antibody as blockade. Other studies found in similar models that use of clinical LAG3 blockade antibodies inhibited LAG3 activity.

This model was then used to determine differences in TCR signaling due to the presence of functional LAG3. To this end, a kinome array was used and found differences in the phosphorylation of many proteins between conditions. After a stringent filtering process, some candidate molecules were identified for future analysis,

foremost among them GRB2 due to its wide and early impact in TCR signaling. Also, several proteins from the MAPK pathway were identified, and confirmed by phospho-flow cytometry, implicating this important pathway as one impacted by LAG3 activity. The MAPK pathway has several outcomes including proliferation and cell differentiation, which along with NFAT inhibition, could explain much of LAG3 activity^{58,157}. The fact that these two pathways are inhibited suggests that LAG3 acts very early in TCR signaling.

8.6 Significance

The identification of monocytes as potential LAG3-expressing cells provokes new questions about LAG3 function in monocytes and the effect of LAG3-targeting treatments currently in clinical trials on monocyte function.

The discrepant reports of LAG3 expression and the confirmation that differences in antibody choice explain this discrepancy should help interpret much of the LAG3 literature and help improve future study design.

The discovery that LAG3 expression is reduced by incubation with flagellin adds to the literature of TLR activation reducing IC expression, where discrepancies exist, and may help improve therapeutic vaccine efficacy or help in an HIV functional cure.

The model created herein for studying LAG3 activity and mechanism could continue being used by future studies on how LAG3 inhibits T cell activation. The discovery MAPK pathway inhibition by LAG3, along with other targets identified in the kinomics array serves as a starting point for greater understanding of the LAG3

mechanism, which may be important to improving LAG3-targeting treatments or creating LAG3-mimicking treatments or agonists.

Overall, this thesis sheds light on a number of areas of LAG3 research that may be used to better study LAG3's role in disease and potential for targeting therapeutics.

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9 Appendices

9.1 LAG3 plasmid insert full sequences

9.1.1 Full-length LAG3 for creation of J.LAG3

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ATGTGGGAGGCTCAGTTCCTGGGCTTGCTGTTTTCTGCAGCCGCTTTGGGTGGCTCCAGTGAAGCCTCTCC
AGCCAGGGGCTGAGGTCCCGGTGGTGTGGGCCAGGAGGGGGCTCCTGCCAGCTCCCCTGCAGCCCCAC
AATCCCCCTCCAGGATCTCAGCCTTCTGCGAAGAGCAGGGGTCACTTGGCAGCATCAGCCAGACAGTGGC
CCGCCCGCTGCCGCCCCGGCCATCCCCTGGCCCCGGCCCTCACCCGGCGGCGCCCTCCTCCTGGGGGG
CCAGGCCCGCCGCTACACGGTGTGAGCGTGGGTCCCGGAGGCTGCGCAGCGGGAGGCTGCCCTGCA
GCCCCGCTCCAGTGGATGAGCGCGGCCGCGCAGCGGGGACTTCTCGCTATGGCTGCGCCAGCCCGG
CGCGGGACGCCGGCAGTACCGCGCCGCGGTGCACCTCAGGGACCGCGCCCTCCTCCTGCCGCTCCGTC
TGCGCCTGGGCCAGGCCTCGATGACTGCCAGCCCCCAGGATCTCTCAGAGCCTCCGACTGGGTCATTTT
GAACTGCTCCTTCAGCCGCCCTGACCGCCAGCCTCTGTGCATTGGTTCCGGAACGGGGCCAGGGCCGA
GTCCCTGTCCGGGAGTCCCCCATCACCACCTTAGCGGAAAGCTTCCTCTTCCCTGCCCAAGTCAGCCCCA
TGGACTCTGGGCCCTGGGCTGCATCCTCACCTACAGAGATGGCTTCAACGTCTCCATCATGTATAACCT
CACTGTTCTGGGTCTGGAGCCCCAACTCCCTTGACAGTGTACGCTGGAGCAGGTTCCAGGTGGGGCTG
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GCCCTGACCTCCTGGTACTGGAGACAATGGCGACTTTACCCCTTCGACTAGAGGATGTGAGCCAGGCCCA
GGCTGGGACCTACACCTGCCATATCCATCTGCAGGAACAGCAGCTCAATGCCACTGTCACATTGGCAATC
ATCACAGTACTCCAAATCCTTTGGGTACCTGGATCCCTGGGGAAGCTGCTTTGTGAGGTACTCCAG
TATCTGGACAAGAACGCTTTGTGTGGAGCTCTCTGGACACCCATCCAGAGGAGTTTCTCAGGACCTTG
GCTGGAGGCACAGGAGGCCAGCTCCTTTCCAGCCTTGGAATGCCAGCTGTACCAGGGGGAGAGGCTT
CTTGGAGCAGCAGTGTACTTCACAGAGCTGTCTAGCCCAGGTGCCAACGCTCTGGGAGAGCCCCAGGTG
CCCTCCAGCAGGCCACCTCCTGCTGTTTCTCATCCTTGGTGTCTTTCTCTGCTCCTTTTGGTACTGG
AGCCTTTGGCTTTACCTTTGGAGAAGACAGTGGCGACCAAGACGATTTTCTGCCTTAGAGCAAGGGATT
CACCTCCGAGGCTCAGAGCAAGATAGAGGAGCTGGAGCAAGAACCGGAGCCGGAGCCGGAGCCGGAAC
CGGAGCCCGAGCCCGAGCCCGAGCCGGAGCAGCTC
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9.1.2 LAG3 with cytoplasmic domain deletion for creation of J.ΔCY

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ATGTGGGAGGCTCAGTTCCTGGGCTTGCTGTTTTCTGCAGCCGCTTTGGGTGGCTCCAGTGAAGCCTCTCC
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CCAGGCCCGCCGCTACACGGTGTGAGCGTGGGTCCCGGAGGCTGCGCAGCGGGAGGCTGCCCTGCA
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