Understanding the rapid expression of lymphocyte activation gene 3 (LAG-3) on healthy human T cells

by

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Abstract

LAG-3 is an immune inhibitory marker in the same category as PD-1, TIM-3, and CTLA-4. These immune inhibitory markers function to reduce the capability of immune cells to elicit a proper immune response and to help maintain tolerance. During an acute infection, these markers assist in controlling the immune system to prevent inflammatory damage, however, during a chronic infection these markers prevent the immune response from persisting to effectively fight the disease. Contrary to what has been observed with other immune inhibitory markers, LAG-3 is, relatively, not highly expressed on T cells during chronic viral infections, such as HIV.

LAG-3 is structurally similar to CD4 and also interacts with MHC class II molecules. Because of these similarities, LAG-3 is an interesting protein to study in regards to HIV infection. The majority of information available on LAG-3 has been gained from murine models and cell lines. This thesis uses primary human T cells in order to observe rapid expression of surface LAG-3 from a pre-formed intracellular store and cleavage of these surface molecules into soluble variants by matrix metalloprotease cleavage.

Early LAG-3 expression on human T cells coincides with the expression of activation marker CD69 on the surface of T cells. Soluble LAG-3 expression was not detected until later. Additionally, a greater proportion of CD8+ T cells were found to express LAG-3 than CD4+ T cells while CD69 expression was reversed. Soluble LAG-3 was also found to correlate with early CD8+ T cell IFN-γ and TNF-α production better than CD69. This observed early expression of LAG-3 may imply an important function in eliciting and controlling the early immune response.

Dedication

This thesis is dedicated to my mom. Your love, strength, and spirit is incomparable. Everything I am and have accomplished I owe to you. Thank you for supporting and encouraging me at every step of my journey.

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Table of Contents

Abstract i
Dedication ii
Acknowledgements iii
List of Figures: vii
List of Tables: viii
1. Introduction 1
1.1 The human immune system 1
1.1.1 The innate immune response 1
1.1.2 The Adaptive Immune Response 3
1.2 Human Immunodeficiency Virus 6
1.3 T cell exhaustion 6
1.3.1 Immune inhibitory markers 7
1.4 Impact of immune exhaustion on HIV/AIDS 8
1.4.1 PD-1 and HIV 8
1.4.2 TIM-3 and HIV 9
1.4.3 CTLA-4 and HIV 10
1.5 Lymphocyte Activation Gene-3 11
1.5.1 Cell Surface Expression of LAG-3 11
1.5.2 Soluble LAG-3 14
1.5.3 LAG-3 in HIV 15
1.5.4 LAG-3 in other chronic viral infections 17
1.5.5 LAG-3 as a target for intervention 17
Study Rationale, Hypothesis, and Objectives 18
Study Rationale 18
Hypothesis 19
Objectives 19
2. Materials and Methods 19
2.1 General Reagents 19
2.1.1 Solutions 19
2.1.2 Stimulants 21
2.1.3 Antibodies and Markers 21
2.2 Biological Samples 22
2.2.1 Source of human peripheral blood samples 22

2.2.2 Ethics 23
2.2.3 Processing of biological samples 23
2.3 General Methods 23
2.3.1 Flow Cytometry 23
2.3.1.1 Surface Staining 23
2.3.1.2 Intracellular cytokine staining 24
2.3.1.3 Surface staining for cell sorting 24
2.3.1.4 Compensation 25
2.3.1.5 Cell sorting 26
2.3.1.6 Acquiring and analyzing data 26
2.3.2 sLAG-3 ELISA 26
2.5 Impact of cycloheximide on surface LAG-3 expression 28
2.6 Matrix Metalloprotease Mediated Cleavage of Surface LAG-3 29
2.6.1 Impact on surface LAG-3 29
2.6.2 Contribution of matrix metalloprotease cleavage to soluble LAG-3 29
2.7 LAG-3 Expression Time Course 30
2.8 LAG-3 expression by T cell subsets 30
2.9 Statistics 32
3. Results 33
3.1 Contribution of *de novo* protein synthesis to surface LAG-3 expression 33
3.2 Impact of matrix metalloprotease inhibitor GM6001 on surface LAG-3 expression 36
3.3 Ionomycin stimulation induces surface LAG-3 expression 38
3.4 Kinetics of LAG-3 expression 38
3.5 LAG-3 and sLAG-3 expression by CD4+ and CD8+ T cell subsets 46
4. Discussion 55
4.1 LAG-3 is present in an intracellular store within T cells 55
4.1.1 Cycloheximide reduces cytokine, but not LAG-3. expression 56
4.1.2 Limitations and Opportunities 57
4.2 Human surface LAG-3 is cleaved by matrix metalloproteases 58
4.2.1 Inhibition of matrix metalloprotease cleavage by GM6001 increases surface LAG-3 59
4.2.2 Limitations and Opportunities 59
4.3 LAG-3 is expressed following stimulation with ionomycin alone 60
4.3.1 Ionomycin stimulation increases LAG-3 expression 61
4.3.2 Opportunities and Limitations 63

4.4 Kinetics of LAG-3 expression 64
4.4.1 Expression of LAG-3 and CD69 65
4.4.2 LAG-3 expression kinetics of CD4+ and CD8+ T cells 66
4.4.3 Expression of sLAG-3 67
4.4.4 Limitations and Opportunities 68
4.5 CD4+ and CD8+ T Cell Expression Profiles 68
4.5.1 LAG-3 and CD69 surface expression 68
4.5.2 sLAG-3 and cytokine production 69
4.5.3 Limitations and Opportunities 71
4.6 General Discussion – Major Findings 72
4.6.1 Future Directions 73
4.7 Hypothesis and Conclusion 74
4.7.1 Significance 75
References 76

List of Figures:

Figure 1. Depiction of LAG-3 and CD4 and their downstream effects………………………...12

Figure 2. Representative staining of PBMCs follow 6 hour stimulation with PMA and Io. 34

Figure 3. LAG-3 and cytokine expression following PMA/Io and cycloheximide treatment..... 35

Figure 4. Impact of Matrix Metalloprotease Inhibitor GM6001 on LAG-3 expression. 37

Figure 5. LAG-3 expression following 6 hour stimulation with PMA/Io or Io alone. 39

Figure 6. Representative staining of PBMCs following 16 hour stimulation with CD3/CD28 beads. 41

Figure 7. Early LAG-3 and CD69 expression time course. 42

Figure 8. Early LAG-3 and CD69 expression on CD3+, CD4+, and CD8+ T cells................... 44

Figure 9. Expression and co-expression of LAG-3 and CD69 time course................................ 45

Figure 10. sLAG-3 time course.. 47

Figure 11. Representative staining and gating of ex vivo PBMCs for sorting. 48

Figure 12. Expression of LAG-3 and CD69 on sorted CD4+ and CD8+ T cell subsets. 50

Figure 13. sLAG-3 and cytokine concentrations in supernatants of CD3/CD28 bead stimulated, flow sorted CD4+ or CD8+ T cells. 52

Figure 14. Stimulated CD8+ T cell supernatant correlations... 53

List of Tables:

Table 1. Surface staining panel used for flow cytometry analysis of PBMCs............................ 21

Table 2. Intracellular staining panel used for flow cytometry analysis of PBMCs 22

Table 3. Surface staining panel used for cell sorting of PBMCs 22

Table 4. Antibodies used for sLAG-3 ELISA.. 22

Table 5. CD4+ and CD8+ sorted sample purity.. 49

1. Introduction

1.1 The human immune system

The human immune system is constantly challenged by, and must react to, pathogens encountered in the environment. In order to keep the host healthy, the immune system must be quick to respond to, and able to recognize, many different types of pathogen, yet remain tightly regulated as to not damage the host itself. The human immune system is divided into two arms: the innate immune arm and the adaptive immune arm. These two branches work in concert to efficiently respond to the wide array of pathogens the body may encounter. This section will introduce the different cell types, receptors, and functions that are integral to the innate and/or adaptive responses. The regulation of the immune response through the regulatory protein LAG-3 is the specific focus of this thesis.

1.1.1 The innate immune response

The innate immune response is the first line of defence against invading pathogens and must, therefore, be rapid and robust in order to respond to many different types of pathogens. Activation of innate cells primarily relies on common pathogen-associated molecular patterns (PAMPs) being recognized by pattern recognition receptors (PRRs). PRRs initiate a signaling pathway resulting in the production of interferons (IFN) and cytokines. Common human PRRs include toll-like receptors (TLRs), nod-like receptors (NLRs), and RIG-I-like receptors (RLRs). TLRs were the first PRR to be identified and are capable of responding to different PAMPs presented by bacteria, viruses, fungi, and parasites including DNA, RNA, lipopolysaccharide (LPS), mannan, and flagellin. At least 10 different functional TLRs have been characterized to date and may be found on the cell surface or intracellularly (**reviewed in** [1]). NLRs and RLRs

are found within the cytoplasm and recognize intracellular bacterial cell products and viral RNA respectively (**reviewed in** [2]). These broadly reactive PRRs are what allow the innate immune cell subsets to react quickly to a diverse array of pathogens.

The immune cells involved in innate immunity include natural killer (NK) cells, granulocytes, and professional antigen presenting cells (APCs). Cytotoxic NK cells, defined as CD3- $CD56^{dim}CD16^{+}$, are able to recognize and lyse pathogen infected cells [3, 4]. NK cell activation is based on interactions between various killer immunoglobin-like receptors (KIRs) and their inhibitory or activating ligands. One such inhibitory ligand is the major histocompatability complex (MHC) class I molecule which may be down-regulated on virally infected or cancerous cells (**reviewed in** [5]). Some NK cells are able to play an immunoregulatory role and secrete large quantities of cytokines. These NK cells are defined as $CD56^{hi}CD16^{-}$ and are a smaller proportion of circulating peripheral NK cells than cytotoxic NK cells [3].

Granulocytes include neutrophils, eosinophils, and basophils and are also referred to as polymorphonuclear cells (PMNs). Granulocytes, named as such for the granules found within the cell, are phagocytic cells. Pathogens phagocytosed by these cells may be killed by the digestive and hydrolytic enzymes stored within the granules which generate reactive oxygen species and hypochlorous acid. This process is also known as the respiratory burst because of increased oxygen use during phagocytosis (**reviewed in** [6]). Granule proteins may also be secreted by PMNs to enhance the function of other innate immune cells such as macrophages. Heparin-binding protein and human neutrophil peptides 1-3 have been shown to induce macrophage production of cytokines and to enhance phagocytosis [7].

Macrophages, dendritic cells (DCs), and B cells are all professional APCs, responsible for pathogen recognition, uptake, and presentation to the adaptive immune system. APCs express various PRRs in order to recognize invading pathogens. Upon uptake of the pathogen, APCs produce cytokines and chemokines in order to recruit and help direct the immune response. Presentation of antigens (Ags) to the adaptive immune response occurs in the lymph nodes via MHC class I and II molecules [6, 8].

1.1.2 The Adaptive Immune Response

The adaptive immune system can itself be divided into 2 arms: the humoral and cell-mediated responses, which are orchestrated by the B and T lymphocytes respectively. The adaptive immune response involves antigen-specific recognition of pathogens, requires a longer period of time to evolve compared to the innate immune response, and invokes a long lasting memory response facilitating rapid clearance of invading pathogens upon a secondary encounter.

The humoral response is characterized by the production of antibodies by B lymphocytes. B lymphocytes are able to recognize antigens through the B cell receptor (BCR), which is a membrane-bound antibody (Ab) made of covalently linked immunoglobulin (Ig) chains, the stimulation of which induces B lymphocyte proliferation into Ab-producing plasma cells and memory B cells [9]. The specificity of the BCR is generated in pre-B-cell development by genetic rearrangements; however, after recognition of a pathogen the B cell undergoes affinity maturation which further increases the antibody-antigen affinity. Abs produced by the B cell may also undergo class switching which allows for the production of the most suitable Ab given the characteristics of the infection. Abs have various functions including: enhancing phagocytosis, neutralization, opsonization, and antibody-dependant cellular cytotoxicity (ADCC)

[10]. B lymphocytes may also assist in antigen presentation and activation of CD4+ T lymphocytes [9].

The cell-mediated immune response is predominantly composed of CD4+ and CD8+ T lymphocytes. CD4+ T lymphocytes are commonly referred to as T helper (Th) cells and are responsible for coordinating the other cells involved in immune responses. This coordination is primarily accomplished through the secretion of chemokines and cytokines to attract immune cells and induce specific responses. The type of Th cell response elicited is largely a result of the cytokine/chemokine micro-environment during activation. Th cells are activated by the interaction of the TCR and CD4 molecule with antigen-bound MHC II [11]. The first Th cell functions and phenotypes described were the Th1 and Th2 responses which promote cell-mediated and humoral responses respectively (**reviewed in** [12]). Today there are many different Th responses which have been characterized including: Th9, Th17, Th22, T follicular helper cells (Tfh), and regulatory T cells (Tregs) [13]. As the name implies, Tregs play a role in the negative regulation of the immune response in order to maintain peripheral tolerance, prevent autoimmune disease, and limit chronic inflammatory disease. Immune suppression is achieved through the production of inhibitory cytokines (IL-10, TGFβ), cytolysis, metabolic disruption, and suppression of DC maturation via inhibitory receptors (CTLA4 and LAG-3) [14].

CD8+ T lymphocyte activation is restricted by MHC I antigen presentation [11] and results in proliferation and differentiation into effector T cells (also known as cytotoxic T lymphocytes (CTLs)), effector memory cells (T_{EM}), or central memory cells. CTLs are short lived cells that produce Granzyne B and Perforin (molecules involved in cytolysis) and/or secrete IFN-γ. Effector memory cells have similar function to CTLs, however they are able to persist within the host even when the antigen has been successfully cleared. Effector memory cells are essentially

long-lived CTLs. Central memory cells are long-lived cells with minimal functional activity until they re-encounter their specific antigen, undergo proliferation, and differentiate into cells with cytotoxic capabilities. Memory cells allow for a rapid response from the adaptive immune system upon secondary exposure to a specific antigen [15].

T lymphocytes that do not express CD4 or CD8 are referred to as double negative T (DNT) cells. This $CD3^+CD4^-CD8^-$ population is comprised of various cell types including: αβ T cells, γδ T cells, and NKT cells. Each of these cell types elicits different functions and responds to different antigens. DN αβ T cells express the standard αβTCR found on both CD4+ and CD8+ T lymphocytes and can elicit many different functions such as: Treg-like immunosuppression, cytotoxicity, and Th-like function (production of IL-17, IFN-γ, and TNF-α) [16]. The γδ T cells are capable of recognizing phosphoantigens. γδ T cell functions are numerous, including: killing of infected cells, antiviral and pro-inflammatory cytokine production, antigen presentation, and regulatory activity **(reviewed in**[17]**)**. NKT cells express markers of both T cells (TCR, and sometimes CD4 or CD8) and NK cells (CD161). Two types of NKT cells have been described; type I or invariant NKT cells express an invariant TCR (Vα24 Jα18 Vβ11) which recognise lipid antigens (including the marine sponge-derived α-galactosylceramide, or αGalCer) and are restricted to CD1d antigen presentation, while type II NKT cells have diverse TCRs and respond to other lipid antigens but cannot be uniformly activated by a single lipid antigen **(reviewed in**[18]**)**. The primary role of NKT cells is to act as a link between the innate and adaptive immune responses and respond with robust Th1 and Th2 cytokine production [19].

The many diverse and complementary components of the immune system need to work together to amount a properly directed and balanced immune response to the many different pathogens

the host may encounter. If any of these cell subsets become disregulated or destroyed, the immune response may not be capable of successfully clearing an infection.

1.2 Human Immunodeficiency Virus

Human immunodeficiency virus (HIV) is a chronic infection that primarily targets and kills CD4+ T cells resulting in an ineffective or absent ability to amount an immune response [20]. Shortly following infection, during the acute phase of illness, patients may present with non-specific symptoms (such as fever, rash, or swollen lymph nodes). Immunologically, there is rapid viral replication, and a decline of CD4+ T cells during acute infection [21]. As the host immune response reduces or controls HIV replication, infection enters the asymptomatic, chronic phase. Throughout the chronic infection, virus continues to be produced, and targets and destroys immune cells. CD4+ T cell count is used as a measurement of disease progression. In a healthy individual, a count of ~1000 CD4+ T cells/μL is normal; a count of <200 CD4+ T cells/μL defines progression to acquired immune deficiency syndrome (AIDS) [22]. The loss of CD4+ T cells results in immune dysfunction and an increase in viral load. There are many factors which can affect the rate of progression to AIDS and disease severity, including: viral load set point [23], treatment with anti-retroviral (ARV) therapy (ART) [24], and host factors [25-27].

1.3 T cell exhaustion

Under conditions of chronic inflammation, expression of inhibitory markers can result in a phenotype known as immune exhaustion. T cell exhaustion is described as the dysfunction and subsequent deletion of T cells in a chronically activated immune system [28]. Exhaustion has been observed in a multitude of animal models and humans in response to chronic viral, bacterial, and parasitic infections and also in cancer patients [29]. Exhaustion can result from the

expression and accumulation of immune inhibitory markers (such as LAG-3, PD-1, TIM3) on T cells which will result in progressive loss of function and, eventually, the physical deletion of cells. While these markers assist in controlling immune responses, their expression during chronic infection can result in the inability for the immune system to continue to fight the infecting pathogen [30]. Exhaustion has also been attributed to Treg and other regulatory cell subsets, as well as soluble inhibitory factors such as IL-10 and TGFβ (**reviewed in** [31]). The severity of T cell exhaustion has been shown to correlate with HIV viral load and declined CD4 T cell help in humans [30].

Exhaustion of CD8+ effector T cells has been well characterized; loss of effector function generally begins with the loss of proliferative capacity, IL-12 production, and *ex vivo* killing capabilities followed by the loss of TNFα production, and eventually, in severe cases of exhaustion, an inability to degranulate, and effectively produce IFN-γ and chemokines [32-35]. As exhaustion progresses, the rate of apoptosis also increases resulting in the removal of T cells from the immune environment [32]. Exhaustion during a chronic infection can result in an inability of memory CD8+ T cells to develop [36]. Less is known about the effects of exhaustion on antigen specific CD4+ T cells, however, dysregulation of CD4+ T cell functions, such as effector cytokine secretion, have been observed during chronic viral infections [37, 38].

1.3.1 Immune inhibitory markers

The activation of CD4+ and CD8+ T cells by APCs requires precise control, and the regulation of that activation can be elicited in part by the expression of immune inhibitory markers. Immune inhibitory markers are a group of cell surface and soluble proteins that elicit an inhibitory effect on T cells when bound by their respective ligands. Examples of these markers

include lymphocyte activation gene-3 (LAG-3), programmed cell death 1 (PD1) **(reviewed in** [39, 40]), T cell Ig mucin domain-containing molecule 3 (TIM-3) (**reviewed in** [41, 42]), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (**reviewed in** [43]), CD244 (2B4) (**reviewed in** [44]), and CD160. These markers play an important role in self-tolerance, preventing autoimmunity, and regulating immune responses [45].

1.4 Impact of immune exhaustion on HIV/AIDS

As HIV infection progresses towards AIDS, a continual increase in viral load and immune activation is observed. In addition to HIV infection killing various immune cells, exhausted immune cells also contribute to the immune system's inability to control HIV replication [46]. Several studies have shown that HIV-positive individuals with lower numbers of Tregs have higher levels of immune activation [47, 48] and that complete removal of Tregs results in increased HIV-specific T-cell responses [49, 50], indicating that Tregs contribute to exhaustion within HIV-positive individuals. A multitude of immune inhibitory markers related to immune exhaustion have been characterized within HIV-positive individuals (**reviewed in** [31]).

1.4.1 PD-1 and HIV

PD-1 has been found to be expressed on a large proportion of both CD4+ and CD8+ HIV-specific T cells [51]. Expression of PD-1 positively correlates with plasma viral load and CD38 expression (a marker of CD8+ T cell activation in HIV-positive individuals [52]) and inversely correlates with CD4+ T cell count [53]. Upregulation of PD-1 has been shown in response to HIV Nef protein [54]. All of these observations together show that PD-1 expression increases as HIV infection progresses and worsens.

Blocking the engagement of PD-1 to one of its ligands, PD-L1, was shown to enhance proliferation and production of both cytokines and cytotoxic molecules by HIV specific CD8+ T cells [55]. Exhausted CD4+ T cell proliferation and cytokine secretion has also been observed to be partially restored and enhanced following PD-1 ligand blockade in HIV-positive individuals; this intervention was, however, less impactful on individuals with controlled viremia (ART-induced or spontaneous), possibly due to their reduced PD-1 expression [51, 56]. By preventing PD-1's contribution to exhaustion the immune response, it is able to continue to fight against the virus for longer and may delay disease progression. However, targeting PD-1 alone is insufficient to prevent exhaustion ([57]).

HIV-positive individuals also express PD-1 on non-T cell subsets, including DCs [58], B cells, NKs, and non-differentiated monocytes (**reviewed in** [59]). HIV-pulsed DCs have been shown to expand T cell populations expressing immune inhibitory molecules [60]. Engagement of PD-1 on monocytes was shown to induce IL-10 production in response to lipopolysaccharide (LPS) from bacteria that may be present from HIV-induced microbial translocation in the gut [61].

1.4.2 TIM-3 and HIV

TIM-3 has also been observed to be upregulated on CD4+ and CD8+ T cells in HIV-positive individuals. Similar to PD-1, TIM-3 expression positively correlates with viral load and CD38 expression, and inversely correlates with CD4+ T cell count. Although both TIM-3 and PD-1 contribute to an exhausted phenotype, it was observed that these markers are not co-expressed on the majority of cells [57]. Different immune inhibitory markers are expressed through different pathways and, therefore, an intervention to prevent exhaustion must be multi-pronged [62].

TIM-3 expression on T cells correlated with decreased CTL responses, cytokine production, and proliferation. Highly active anti-retroviral therapy (HAART) was observed to reduce TIM-3 expression in a majority of patients tested [57]. Interestingly, individuals whose CD4+ T cell count does not rebound following HAART treatment were found to have a higher proportion of NKs expressing TIM-3 compared with those whose count did rebound [63].

A soluble TIM-3 protein has also been observed in plasma of HIV-positive individuals, with its expression correlating with disease progression. Soluble TIM-3 was not found to be upregulated in individuals on HAART or those who can control viral replication on their own [64]. Shedding of TIM-3 was not observed to improve CD8+ T cell IFN-γ secretion or reduce rates of apoptosis [64], this may be due to expression of other inhibitory markers on these cells with redundant or overlapping function. However, it has been theorized that soluble TIM-3 may provide competition for the TIM-3 ligand, reducing the amount of surface TIM-3 being stimulated and thereby reducing exhaustion [57].

A recent study observed that TIM-family proteins (including TIM-1, TIM-3, and TIM-4) can enhance HIV entry into cells but also prevents the release of the virion, reducing virus production [65].

1.4.3 CTLA-4 and HIV

CTLA-4 was observed to be highly expressed on HIV specific CD4+ T cells [66] and over-expressed on bulk CD4+ T cells during the course of HIV disease progression [67], but not CD8+ T cells [68]. Expression of CTLA-4 correlates with viral load and loss of CD4+ T cells [69]. Unlike PD-1 and TIM-3, individuals on HAART do not appear to have reduced CTLA-4 expression compared to untreated individuals. Interestingly, individuals who can control the

virus without treatment do have reduced CTLA-4 expression [68]. HIV specific CD4+ T cells producing IFN-γ but not IL-2 were observed to express more CTLA-4 than those capable of producing both. The majority of cells expressing CTLA-4 also co-expressed PD-1 [69]. Additionally, signalling through CTLA-4 was shown to induce high CCR5 expression and greater viral susceptibility [70].

1.5 Lymphocyte Activation Gene-3

1.5.1 Cell Surface Expression of LAG-3

LAG-3, also known as CD223, is a cell surface receptor that is highly homologous to CD4 and may be derived from a common evolutionary ancestor. The *LAG-3* gene is located on the distal portion of the short arm of chromosome 12 (12p13.32) [71], in close proximity to the *CD4* gene (12p13.31) [72], and is composed of 8 exons [73]. Transcription of *LAG-3* is believed to be controlled by the same regulatory elements that interact with the *CD4* promoter [74]. Structurally, the LAG-3 protein, like CD4, contains a single transmembrane domain and is a member of the Ig superfamily with 4 Ig domains. Unlike CD4, LAG-3 contains an additional 30 amino acid loop in the V domain (domain 1) that does not disrupt the Ig fold [73, 75] (Figure 1).

It has been reported that LAG-3 is expressed as a weak dimer on the surface of T and NK cells, although expression levels are negligible on resting cells [76]. LAG-3 on T cells will co-localize with CD3 and CD4/CD8 after cellular activation [77]. LAG-3 is capable of binding MHC class II molecules; in fact, LAG-3 binds MHC II with 100-fold greater affinity than CD4 [73, 76]. However, despite the ability for LAG-3 to compete with CD4 for MHC class II engagement, this does not contribute significantly to the inhibitory function of LAG-3, as observed *in vitro* systems [78]. Other LAG-3 ligands, LSECtin [79] and galectin-3 [80], have been found recently,

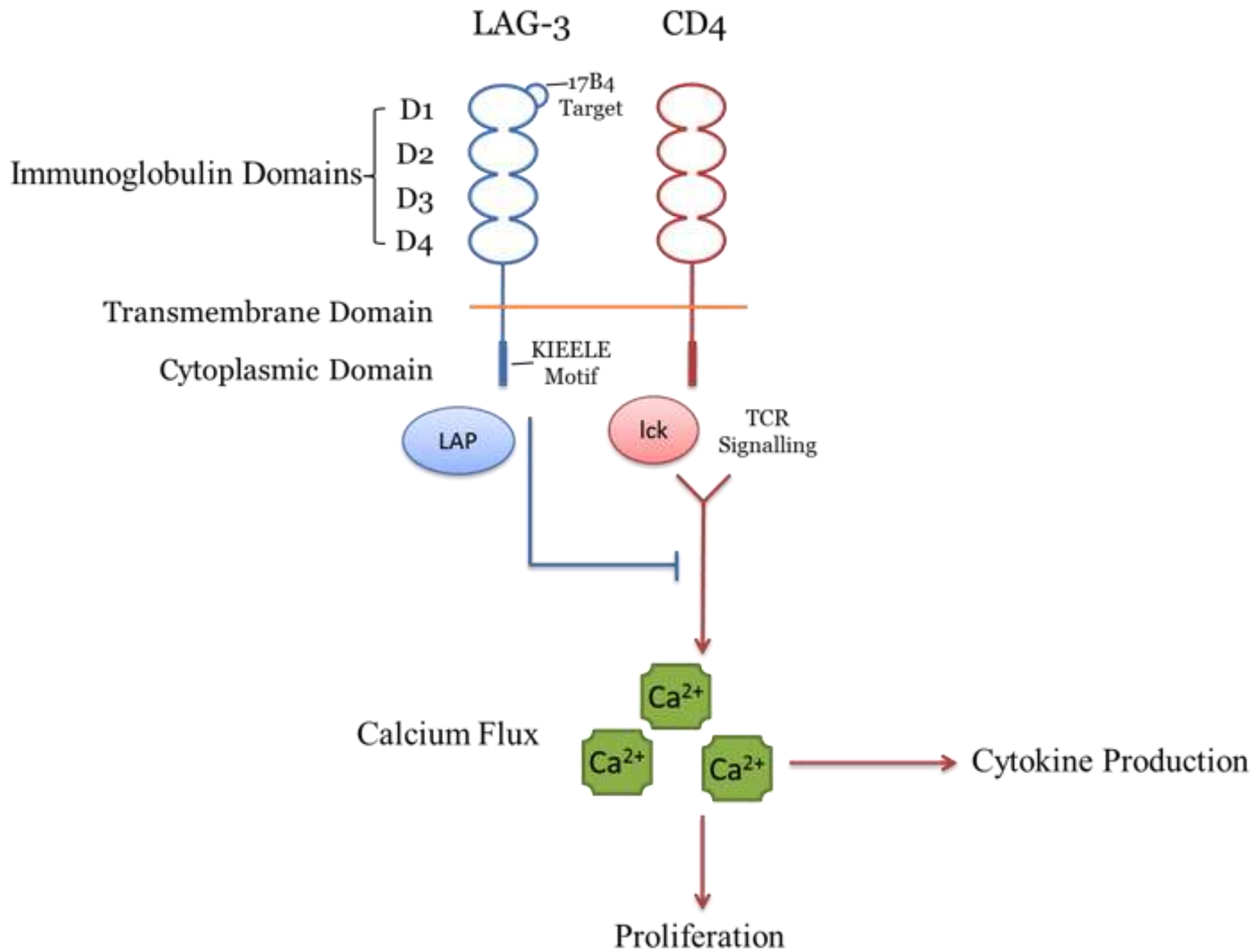


Figure 1. Depiction of LAG-3 and CD4 and their downstream effects. LAG-3 and CD4 are both composed of 4 immunoglobulin domains however LAG-3 contains as additional 30 amino acid loop in domain 1. CD4 is responsible for generating an active immune response while LAG-3 acts to diminish immune activation by impeding the calcium flux.

however these observations have not yet been replicated. LAG-3 does not interact with gp120 [73], the HIV envelope protein that binds CD4 and is responsible for cell attachment and entry [81]. The cytoplasmic tail of LAG-3 contains a KIEELE motif as well as an EP repeat motif that binds LAG-3 associated protein (LAP), an intracellular protein responsible for signal transduction [82].

Engagement of LAG-3 on T cells contributes to homeostasis, or the regulation of T cell numbers within an individual [83, 84]. Expression of LAG-3 on the surface of T cells is induced by IL-12 in an IFN-γ-dependent manner [85]. Cross-linking of LAG-3 on both CD4 and CD8 T cells results in multiple regulatory mechanisms being engaged, including inhibition of the CD3-induced calcium flux [86], cell proliferation and activation (based on CD69 expression), and Th1 (but not Th2) cytokine production [77].

Within T cells, LAG-3 has been shown to be stored in endosome compartments in both mice [87] and human cell lines (Jurkat) [88]. These preformed stores of LAG-3 can account for up to half of the LAG-3 molecules generated by a cell and facilitate rapid trafficking and restoration of LAG-3 to the cell surface. Storage in recycling endosomes suggests that LAG-3 may be continuously recycled back to the surface. Alternatively, these recycling endosomes may help to tightly regulate the amount of LAG-3 expressed at the cell surface [87].

In addition to T and NK cells, LAG-3 has also been found on the surface of some plasmacytoid DCs (pDCs) [89], iNKTs [90], and a subset of Tregs [91] in humans. In mice, however, LAG-3 has been found to be expressed by pDCs [92], some B cells [93], NKT cells [94], and γδ T cells [95]. Interestingly, LAG-3 function varies between cell types and species. In mice, LAG-3 has been shown to impact the ability of Treg cell to regulate conventional T cells [96, 97], however,

the involvement of LAG-3 on Treg function in humans is not well understood **(reviewed in [98])**. Murine LAG-3 has also been shown to limit NK cytotoxic responses [99] while human LAG-3 fails to impact NK cytotoxicity [100].

1.5.2 Soluble LAG-3

LAG-3 can also be produced as a truncated protein, consisting of the extracellular Ig domains, known as soluble LAG-3 (sLAG-3). Using murine cell lines it was observed that 70 kDa surface bound LAG-3 was cleaved into a 54 kDa sLAG-3 fragment, found in the medium, and a 16 kDa fragment containing the transmembrane and intracellular domains [101]. Murine LAG-3 cleavage was shown to be mediated by transmembrane metalloproteases ADAM10 and ADAM17 [102]. sLAG-3 has also been detected in human serum, however human sLAG-3 has been shown to be a product of splicing [103, 104] and the impact of metalloproteaes on surface LAG-3 is unknown.

In addition to having an effect on the cell expressing LAG-3, sLAG-3 can also induce a signalling pathway in APCs through its ligand, MHC class II. It has been observed that cross-linking of MHC class II by sLAG-3 can induce DC maturation and production of Th1-driving cytokines [104-106]. Interestingly, cross-linking MHC class II using antibodies will not elicit the same signals or signal strength induced by sLAG-3 cross-linking [105, 106]. Monocytes have also been shown to produce IL-2, IFN-γ, and TNF-α in response to MHC class II cross-linking by LAG-3 [107]. However, LAG-3 has also been shown to impair the differentiation of monocytes into DCs and macrophages. This may serve as an immune suppressive mechanism to limit the recruitment of APCs into an activated T cell enriched environment [108].

Levels of sLAG-3 in human serum have been shown to correlate with severity and prognosis of mycobacterium tuberculosis infection. Individuals who remained healthy or had a favorable outcome were observed to have higher levels of sLAG-3 [109]. sLAG-3 has also been shown to induce an enhanced anti-tumor responses in mice [110] and has been used successfully as an adjuvant to improve antibody production, Th1 but not Th2 cytokine productions, and CTL responses [111]. Beyond these studies, the significance of plasma sLAG-3 concentration in health or disease is not well appreciated in humans.

1.5.3 LAG-3 in HIV

In contrast to the other immune inhibitory markers, the majority of studies report that LAG-3 has not been upregulated on bulk T cells within HIV-positive individuals [112]. In fact, the majority of exhausted HIV-specific CD4+ and CD8+ T cells are also observed to lack LAG-3 expression [51, 113]. Within in the same HIV-positive individuals, PD-1, CD160, and 2B4 were all up-regulated on CD8+ T cells and were differentially expressed between CD4+ and CD8+ T cell subsets, whereas LAG-3 was not [51]. Although LAG-3 is grouped together with these other exhaustion markers, it has been observed to follow different expression patterns and may therefore be playing additional or alternate roles during HIV infection.

A single, recent study found that LAG-3 expression was significantly increased in both the lymph nodes and peripheral blood following HIV infection. In this study, LAG-3 expression was observed to correlate with disease progression and was diminished following extended antiretroviral therapy. Additionally, blockade of LAG-3 was found to restore function of HIV specific T cells [114]. This study is contradictory to the other, aforementioned, studies that

found LAG-3 to be relatively unaltered during HIV infection when compared to other immune inhibitory markers.

In a study based out of our lab, LAG-3 expression was observed to be increased in HIV-positive ART treated Kenyan individuals on all cell subsets examined when compared to HIV-negative individuals [115]. This is again in contrast to observations with other immune inhibitory markers where HIV-treated individuals had reduced expression of PD-1[56] and TIM-3 [63] or unchanged expression of CTLA-4 [68]. Another study compares expression of LAG-3 at the female genital tract (FGT) with systemic expression in HIV-positive individuals and found that LAG-3 expression was elevated in the FGT, however, interestingly LAG-3 was most enriched on the DNT cell subsets in both compartments [116].

Transcription of LAG-3 has also been examined within both HIV-infected individuals and simian immunodeficiency virus (SIV) infected non-human primates which observe enhanced LAG-3 expression by microarray study [117, 118]. Transcription levels were shown to positively correlate with viral load set point and were higher within rapid progressors when compared to non-progressors [119]. Despite this increase in LAG-3 transcription, it is unknown why we do not see an increase in bulk T cells or HIV-specific T cells expressing LAG-3 during HIV infection. Some theories to explain this phenomenon include: LAG-3 expressing cells may be a preferred target for HIV infection resulting in loss of these cells, HIV infection or proteins may prevent LAG-3 expression, or LAG-3 may be cleaved from the surface of these cells in order to reduce its impact on exhaustion.

1.5.4 LAG-3 in other chronic viral infections

LAG-3 has been examined during other chronic viral infections such as hepatitis C and B virus (HCV, HBV). Mirroring what we see with LAG-3 expression during HIV infection, T cells also express low levels of LAG-3 during HCV and HBV infection as well [120, 121]. A study that examined T cells from the liver of individuals who successfully cleared HCV found a small population of CD45-RA, LAG-3 expressing effector memory T cells that have persisted without continual antigen exposure [122]. This observation suggests that LAG-3 may be playing a role in maintaining a transient population in addition to its exhaustion function.

1.5.5 LAG-3 as a target for intervention

Blockade of immune inhibitory markers has been used very successfully in treatment for various cancers (**reviewed in** [123, 124]). In a recent study, mice with multiple myelomas were treated with a low dose of whole body irradiation and combinations of antibodies to block immune inhibitory markers. These blockades provided increased survival rates which were further increased from 30% to 80% by multiple blocking antibodies (PD-1 + LAG-3, TIM-3, or CTLA-4) [62]. This study highlights the importance of maintaining the host's immune response as well as targeting exhaustion from multiple avenues.

Several other studies have examined the effects of using recombinant sLAG-3 in order to help activate the immune response (**reviewed in** [123]). Briefly, recombinant sLAG-3 (IMP321) was shown to bind MHC class II expressing cells causing TNF-α and CCL4 production and induction of Tc1 cytokine production (such as IFN-γ and/or TNF-α) by CD8+ T cells in healthy and metastatic breast cancer patient blood samples [125]. IMP321 was given to patients with metastatic breast cancer as an injection and the observations were consistent with the previous *in*

vitro experiments: increased APC activation, and more T_{EM} and NK cells. The LAG-3 treatment provided clinical benefits for 90% of patients [126]. IMP321 has also been given to patients with advanced renal cell carcinoma, inducing sustained CD8+ T cell activation and increased levels of long-lived T_{EM} cells. Additionally, patients who received a higher dose of IMP321 had reduced tumor growth and better progression-free survival [127].

LAG-3 has also been shown to play a role in autoimmune disease and organ transplant rejection [128]. A study in non-human primates observed that by depleting LAG-3 expressing T cells in the lymph nodes, they were able to prevent Th1-driven skin inflammation in a tuberculin-induced delayed type-hypersensitivity model. This approach is useful for dampening the immune response, preventing a response against a transplanted tissue , however resting T cells would be spared [129]. This could also be an interesting intervention against HIV, perhaps as a mucosal microbicide, allowing the depletion of T cells that have become activated and serve as a target for HIV infection.

Study Rationale, Hypothesis, and Objectives

<u>Study Rationale</u>

LAG-3 has been well described as an immune inhibitory marker, potentially contributing to T cell exhaustion in chronic disease. However, contrary to other markers, LAG-3 is not observed to be highly expressed on T cells during chronic viral infections, including HIV, HCV, and HBV. An additional difference of LAG-3 compared to other immune inhibitory markers is the notable early expression observed post stimulation. Early expression of LAG-3 may allude to an important function outside of inhibition. Murine studies have shown an intracellular store of preformed LAG-3 facilitating rapid expression [87]. A human cell line (Jurkat) has also been

observed to generate a preformed storage of LAG-3. The energy and resource expenditure of the cell to generate this store further depicts the importance of the early expression of LAG-3. Also in murine studies it has been observed that surface expressed LAG-3 can be cleaved by matrix metalloproteases to generate soluble LAG-3. Several studies have examined the ability for sLAG-3 to activate APCs and enhance T cell responses. We wondered if human cells would also have a preformed store of LAG-3 to facilitate rapid expression and if matrix metalloproteases may cleave this surface expressed LAG-3 in order to reduce the inhibitory capacity and increase sLAG-3 expression. We were also interested in observing a time line of when the initial burst of LAG-3 would be observed on the cell and when *de novo* protein synthesis would start to enhance or supplement this.

Hypothesis

We hypothesize that soluble and membrane LAG-3 will be rapidly expressed from an intracellular store following stimulation. Membrane LAG-3 and will decline over time due to metalloprotease cleavage which will concurrently increase soluble LAG-3.

Objectives

1. To determine if human T cells possess an intracellular store of LAG-3 protein.
2. To determine the role of metalloproteases in surface LAG-3 regulation.
3. To assess the kinetics of early LAG-3 and sLAG-3 expression.

2. Materials and Methods

2.1 General Reagents

2.1.1 Solutions

- Phosphate-buffered saline (PBS)
  - 9.55g PBS Powder (137.93mM NaCl, 2.67mM KCl, 8.1mM Na_2HPO_4; Gibco) dissolved in double distilled H_2O (ddH_2O). Final volume of 1L. pH adjusted to 7.0.

- FACS Wash
  - PBS + 2% heat inactivated fetal bovine serum (FBS; inactivated at 56ºC for 1 hour; Hyclone)

- R-10 Cell Culture Media
  - RPMI-1640 (Hyclone)+ 10% FBS + 1% Penicillin/Streptomycin/Fungizone (PSF; Gibco)

- R-10 Media
  - RPMI-1640 (Hyclone)+ 10% FBS

- R-30 Cell Culture Media
  - RPMI-1640 (Hyclone)+ 30% FBS + 1% PSF

- Freezing Media
  - FBS + 10% dimethyl sulfide (DMSO, tissue culture grade; Sigma)

- ELISA Coating buffer
  - 1.59g Na_2CO3 + 2.93g $NaHCO_3$ dissolved in ddH_2O. Final volume 1L

- ELISA Blocking buffer
  - PBS + 10% Bovine Serum Albumin (BSA; Sigma)

- ELISA Dilution buffer
  - PBS + 2% BSA + 0.1% Tween 20

- ELISA Wash buffer
  - 4.38g NaH_2PO_4 (monobasic; Fisher) + Na_2HPO_4 (bibasic; Fisher) + 80g NaCl dissolved in 500mL ddH_2O. Add 10ml Tween 20. Bring final volume to 1L

using ddH_2O (10x wash buffer). Filter with 0.2μm filter. Dilute 1:10 with ddH_2O for use (1x wash buffer).

2.1.2 Stimulants

- Phorbol 12-myristate 13-acetate (PMA; Sigma)
  - Activator of protein kinase C used to stimulate production of cytokines
- Ionomycin (Io; Sigma)
  - Used to study the effects of calcium flux and stimulate production of cytokines
- Dynabeads Human T-Activator CD3/CD28 for T cell Expansion and Activation (Gibco)
  - Provides a primary and co-stimulation for CD3+ T cells. Stimulated cells exhibit *in vivo*-like function and properties.

2.1.3 Antibodies and Markers

Table 1. Surface staining panel used for flow cytometry analysis of PBMCs

Flurochrome	Target	Antibody Clone	Source	Volume Used (μl per sample)
FITC	LAG-3	17B4	Enzo Life Science	2
PE-Cy5	CD3	UCHT1	BD	10
APC-H7	CD4	RPA-T4	BD	3
V500	CD8	RPA-T8	BD	3
APC	CD69	FN50	BD	10
PE-Texas Red	Live/Dead (Amine Reactive)		Life Technologies	1

Table 2. Intracellular staining panel used for flow cytometry analysis of PBMCs

Flurochrome	Target	Antibody Clone	Source	Volume Used (µl per sample)
PE-Cy7	TNF-α	MAb11	BD	5
Alexa Fluor 700	IFN-γ	B27	BD	5

Table 3. Surface staining panel used for cell sorting of PBMCs

Flurochrome	Target	Antibody Clone	Source	Volume Used (µl in 1mL master mix)
PE-Cy5	CD3	UCHT1	BD	100
APC-H7	CD4	RPA-T4	BD	30
V500	CD8	RPA-T8	BD	30

Table 4. Antibodies used for sLAG-3 ELISA

Function	Target	Antibody Clone	Source
Capture Antibody	LAG-3	11E3	Enzo Life Science
Detection Antibody (Biotinylated)	LAG-3	17B4	Enzo Life Science

2.2 Biological Samples

2.2.1 Source of human peripheral blood samples

All samples used in this project were collected from local donors at the University of Manitoba. Donors are HIV negative with low risk of infection.

2.2.2 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.

2.2.3 Processing of biological samples

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 at 600xg for 7 minutes and plasma was removed and discarded. The remaining whole blood samples were diluted in PBS +2% FBS, layered on Ficoll (Lymphoprep; MJS BioLynx Inc.) and centrifuged at 400xg for 25 minutes with the break turned off. The PBMC layer was collected and washed in PBS +2% FCS, centrifuged at 600xg for 10 minutes. PBMCs were then resuspended and washed in R-10 cell culture media, and centrifuged at 400xg for 10 minutes. Finally, PBMCs were resuspended in R-10 cell culture media and counted using trypan blue (HyClone) dye exclusion. PBMCs were allowed to rest overnight in R-10 cell culture media before being assayed. Any unused cells were cryopreserved in freezing media.

2.3 General Methods

2.3.1 Flow Cytometry

2.3.1.1 Surface Staining

PBMCs were transferred to 96 well V bottom plates for staining. Either $1x10^6$ or $2x10^6$ cells were stained per well. Cells were pelleted by centrifugation at 514xg for 10 minutes. The

supernatant was discarded and cells were resuspended and washed in FACS wash (centrifuged at 514xg for 10minutes). Each well of cells was then resuspended in a final volume of 100µL of surface staining antibody master mix, with 30 minutes of incubation at 4ºC in the dark. Cells were then washed twice in FACS wash before being resuspended in 300µL of 1% paraformaldehyde fixation buffer (BD Bioscience) or permeabilized for intracellular staining (as per section 2.3.1.2). The fixed cells were transferred to 5mL FACS tubes for acquisition by the LSRII flow cytometer.

2.3.1.2 Intracellular cytokine staining

Surface stained cells were resuspended in fixation/permeabilization buffer (CytoFix/CytoPerm; BD Bioscience) for 20 minutes at 4ºC in the dark. Cells were then washed in 200ul Fixation/Permeabilization wash buffer (1:10 diluted in ddH2O; BD Bioscience) and centrifuged at 600xg for 10 minutes. Each well of cells was then resuspended in a final volume of 50µL intracellular cytokine staining antibody mastermix, allowing 30 minutes of incubation at 4ºC in the dark. Cell were then washed with Fixation/Permeabilization wash buffer, resuspended in FACS wash, and transferred to 5mL FACS tubes for acquisition by the LSRII flow cytometer.

2.3.1.3 Surface staining for cell sorting

PBMCs were transferred to 5mL FACS tubes for staining. Ten million cells were stained per tube. Cells were pelleted by centrifugation at 514xg for 10 minutes. The supernatant was discarded and cells were resuspended and washed in FACS wash (centrifuged at 514xg for 10minutes). Each well of cells was then resuspended in a final volume of 1mL surface staining antibody master mix, allowing 30 minutes of incubation at 4ºC in the dark. Cells were then washed twice in FACS wash before being resuspended in 1mL FACS wash. The cells were

filtered through 35µm cell strainer caps (Falcon) and sorted by the FACS Aria cell sorter into R-30 cell culture media.

2.3.1.4 Compensation

The use of multiple fluorochromes in flow cytometry results in some of the fluorochromes having overlapping emission spectra. Compensations allow for 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 controlCompBeads was added to 400µL of FACS wash and mixed. One hundred µL of this bead mixture was transferred to a 5mL FACS tube for each marker in the panel. Each tube had 1µL 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 300µL with FACS wash and acquired on the LSRII flow cytometer or FACS Aria cell sorter.

Compensations for the amine reactive live/dead viability dye were prepared using ArC Beads (Invitrogen). One drop of ArC amine beads and 1µL of Live/Dead Red stain was mixed together in a 5mL FACS tube and incubated for 30 minutes at room temperature in the dark. The bead mixture was then washed twice in 3mL FACS wash (centrifuged at 514xg for 5 minutes) before being resuspended in a final volume of 300µL of FACS wash. One drop of ArC negative beads was added to the bead mixture before being acquired on the LSRII flow cytometer.

2.3.1.5 Cell sorting

Samples were acquired and sorted using the FACSAria (BD Bioscience) cell sorter. Samples were sorted using 85µm nozzels at a flow rate of 2.0 in purity mode. Compensations and gating was done using FACS Diva software (v6; BD Bioscience). Sorted cells were collected in R-30 cell culture media and assessed for purity. Cells were washed and resuspended in R-10 cell culture media overnight; an aliquot was then counted by trypan blue (HyClone) dye exclusion before the sorted samples were used for stimulations.

2.3.1.6 Acquiring and analyzing data

Final acquisition was done on the LSRII flow cytometer (BD Bioscience). For adequate T cell analysis, 100,000 lymphocyte gate events were collected. This was altered for the LAG-3 time course experiment (Section 5) where 60,000 CD3+ gate events were collected to accommodate red blood cell contamination of several samples. 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.).

2.3.2 sLAG-3 ELISA

In order to quantify soluble LAG-3 concentrations in cell supernatant samples, a sandwich ELISA was optimized and used. 96-well, flat bottom, polystyrene ELISA plates (Corning) were coated with 100µL of 5µg/mL anti-human LAG-3 antibody, 11E3, diluted in ELISA coating buffer. Plates were incubated overnight at 4ºC, in a wet box, in the dark.

Plates were washed 6 times with 300µL ELISA wash buffer by a microplate washer (ELx405; Biotek). Three hundred µL of ELISA blocking buffer was then added to each well to block non-specific binding. Plates were incubated for 2 hours, at room temperature, in a wet box, in the dark.

Plates were again washed 6 times before samples, standards, and background controls were added to the plate. Duplicate 100µL standard curves were generated by a 10-point serial dilution of recombinant human LAG-3-Fc (Enzo Life Science) from 8ng/mL to 15.6pg/mL in R-10 cell culture media. One hundred µL of cell stimulation supernatant was added to each well, in duplicate. A 100 µL background control of R-10 cell culture media only was also added to each plate, in duplicate, as well. Plates were incubated overnight at 4ºC, in a wet box, in the dark

Plates were washed 6 times. One hundred µL of 0.5µg/mL LAG-3 detection antibody, biotynilated 17B4, diluted in ELISA buffer was added to each well. Plates were incubated for 1 hour at room temperature, in a wet box, in the dark. Plates were again washed 6 times before adding 100µL of streptavidin-horseradish peroxidase (HRP; Invitrogen), diluted 1:5000 in ELISA buffer, to each well and incubated for 30 minutes at room temperature, in the dark.

Plates were washed a final 6 times. One hundred µL of room temperature super sensitive tetramethylbenzidine (TMB; Sigma) was added to each well. The resulting colour change was monitored by a measurement of optical density (OD) using a SpectraMax Plus spectrophotometer (Molecular Devices) at 650nm at 5, 10, 15, 20, and 30 minutes. Colour development was arrested using 50µL of 3% HCl in each well, typically at the 30 minute time point. The OD was examined again at 450nm.

Data was collected and analyzed using SoftMax Pro software (v3.1.2; Molecular Devices). Measured ODs were background subtracted based on the average OD of the background control wells. The measured concentrations of sLAG-3 in the samples were based on interpolating values from the standard curve. The standard curve is calculated using a four point logistic regression. The fit of the regression curve to the standard data points must have an r value of >0.995 to be acceptable.

<u>2.5 Impact of cycloheximide on surface LAG-3 expression</u>

PBMC samples were isolated from local donors. PBMC samples were transferred into 12-well tissue culture plates as 1mL/well at a concentration of $2x10^6$cells/mL. To observe LAG-3 expression from an intracellular store, *de novo* protein synthesis was stopped using cycloheximide. Samples were treated with 100µM of cycloheximide 30 minutes prior to stimulation and an additional 100µM of cycloheximide 3 hours post stimulation. PBMCs were stimulated with PMA (25ng/mL) and Io (500ng/mL) for 6 hours. Cytokine production was used as a control to ensure *do novo* protein synthesis has been stopped. In order to detect cytokine production, GolgiStop (containing momensin; BD Bioscience) and GolgiPlug (containing brefeldin A; BD Bioscience) were added to the PBMC culture at 0.66µL/mL and 1µL/mL respectively, 2 hours post simulation, to prevent transport though the Golgi.

Surface LAG-3 and intracellular cytokine expression was measured by flow cytometry. $2x10^6$ PBMCs were surface and intracellular stained as per Table 1 and Table 2 respectively.

Flow cytometry data was analyzed using the Wilcoxon Rank Sum test (non-parametric, paired, continuous variables). GraphPad Prism (v 6.0; GraphPad Software) was used for all statistical analysis. A p value of <0.05 was considered to be statistically significant.

2.6 Matrix Metalloprotease Mediated Cleavage of Surface LAG-3

2.6.1 Impact on surface LAG-3

PBMC samples were isolated from local donors. PBMC samples were transferred into 12-well tissue culture plates as 1mL/well at a concentration of $2x10^6$cells/mL. Matrix metalloprotease mediated cleavage was prevented using a broad-spectrum matrix metalloprotease inhibitor, GM6001 (Enzo Life Science). 0, 12.5, 25, 50, or 100μM of GM6001 was added to PBMC culture 30 minutes prior to stimulation. PBMCs were stimulated with PMA (25ng/mL) and Io (500ng/mL) for 6 hours.

Surface LAG-3 expression was measured by flow cytometry. $2x10^6$ PBMCs were surface stained as per Table 1.

Flow cytometry data was analyzed using the Wilcoxon Rank Sum test and Friedman test (non-parametric, paired, continuous variables). Dunn's post-test was also performed to determine inter-group differences when the Friedman test was significant. GraphPad Prism was used for all statistical analysis. A p value of <0.05 was considered to be statistically significant.

2.6.2 Contribution of matrix metalloprotease cleavage to soluble LAG-3

PBMC samples were isolated from local donors. PBMC samples were transferred into 24-well tissue culture plates as 500μL/well at a concentration of $4x10^6$cells/mL. Fifty μM of GM6001 was added to PBMC culture 30 minutes prior to stimulation. PBMCs were stimulated with PMA (50ng/mL) and Io (1μg/mL) or Io (1μg/mL) alone for 6 hours. PBMC samples were centrifuged at 514xg for 10 minutes and supernatants were collected.

Surface LAG-3 expression was measured by flow cytometry. $2x10^6$ PBMCs were surface stained as per Table 1. Supernatants were used for sLAG-3 ELISA as per 2.3.2.

Flow cytometry data was analyzed using the Wilcoxon Rank Sum test (non-parametric, paired, continuous variables). GraphPad Prism was used for all statistical analysis. A p value of <0.05 was considered to be statistically significant. ELISA data was below limit of detection and unable to be analyzed.

2.7 LAG-3 Expression Time Course

PBMC samples were isolated from local donors. PBMC samples were transferred into 12-well tissue culture plates as 1mL/well at a concentration of $2x10^6$cells/mL. PBMCs were stimulated with CD3/CD28 beads at a 1:1 ratio of cells to beads for 2, 4, 6, 10, 16, or 24 hours. PBMC samples were centrifuged at 514xg for 10 minutes and supernatants were collected.

Surface LAG-3 expression was measured by flow cytometry. $2x10^6$ PBMCs were surface stained as per Table 1. Supernatants were used for sLAG-3 ELISA.

Flow cytometry and ELISA data was analyzed using the Wilcoxon Rank Sum test, Friedman test (non-parametric, paired, continuous variables), and Tukey's multiple comparisons test. Dunn's post-test was also performed to determine inter-group differences when the Friedman test was significant. GraphPad Prism was used for all statistical analysis. A p value of <0.05 was considered to be statistically significant.

2.8 LAG-3 expression by T cell subsets

PBMC samples were isolated from local donors. PBMC samples were stained as per Table 3 and sorted into CD3+CD4+ and CD3+CD8+ populations. Sorted samples were then transferred

into 24-well tissue culture plates as 500µL/well at a concentration of $2x10^6$ cell/mL. Samples were stimulated with CD3/CD28 beads at a 1:1 ratio of cells to beads for 16 hours. Sorted samples were centrifuged at 514xg for 10 minutes and supernatants were collected.

Surface LAG-3 expression was measured by flow cytometry. One million PBMCs were surface stained as per Table 1. Supernatants were used for sLAG-3 ELISA and MilliplexMAP bead array assay (Millipore). Milliplex assay was prepared and run according to manufacturer's instruction. A six point standard curve was generated for each analyte, ranging from 10,000pg/mL to 3.2 pg/mL, using 5-fold dilutions of provided human cytokine standard in ddH_2O. High and low concentration quality control samples were resuspended in 250µL ddH_2O, vortexed, and incubated for 10 minutes. For each analyte, 60µL of antibody-immobilized beads were combined and brought to a total volume of 3mL using bead diluent. Analytes (with given limits of detection) examined include: IFN-γ (0.8pg/mL), IL-4 (4.5pg/mL), MIP-1β (3.0pg/mL), and TNF-α (0.7pg/mL).

Plates were incubated with 200µL of 1X wash buffer per well for 10 minutes at room temperature on a plate shaker. Wash buffer was removed and 25µL of either standard, quality control, or assay buffer were added to their appropriate wells. Twenty-five µL of R10 cell-culture media was also added to these wells. Twenty-five µL of sample was added to the remaining wells and as well as 25µL of assay buffer. Lastly, 25µL of mixed beads was added to each well. The plate was incubated for 2 hours at room temperature on a plate shaker.

The plate was washed twice with supplied wash buffer using the ELx405 (Biotek) plate washer. 25µL of detection antibodies were added to each well and the plate was incubated for 1 hour at room temperature on a plate shaker. Twenty-five µL of streptavidin-phycoerythrin was added to

each well and incubated for 30 minutes at room temperature on a plate shaker. The plate was again washed twice before adding 150µL sheath fluid to and placed on a plate shaker for 5 minutes to resuspend the wells.

Data was acquired on a Bio-Plex 200 (Bio-Rad) and analyzed with Bioplex Manager software (v5.0, Bio-Rad). Standard curves for each analyte were generated using 5 parameter logistic regression.

Flow cytometry, ELISA, and Milliplex data was analyzed using the Wilcoxon Rank Sum test and Friedman test. Dunn's post-test was also performed to determine inter-group differences when the Friedman test was significant. GraphPad Prism was used for all statistical analysis. A p value of <0.05 was considered to be statistically significant.

2.9 Statistics

All statistical analysis was performed using GraphPad Prism (v 6.0; GraphPad Software). In order to compare LAG-3, CD69, or cytokine expression between 2 different conditions, the Wilcoxon Rank Sum test (non-parametric, paired, continuous variables) was used. The Friedman test (non-parametric, paired, continuous variables) was used to compare protein expression in response to a single changing variable (such as dose response or time course). Dunn's post-test was also performed to determine inter-group differences when the Friedman test was significant. A 2way ANOVA (non-parametric, paired, continuous variables) and Tukey's multiple comparisons test was used to examine protein expression with multiple changing variables (such as time and cell type). Surface and soluble proteins were correlated using Spearman's test (non-parametric) and a line of best fit was calculated using linear regression. A p-value of <0.05 was considered to be statistically significant.

3. Results

3.1 Contribution of *de novo* protein synthesis to surface LAG-3 expression

To determine whether LAG-3 expression on the surface of T cells following stimulation is a result of *de novo* protein synthesis or trafficking of a pre-formed cellular store of LAG-3 protein, *de novo* protein synthesis was inhibited by the addition of cyclohexamide to cell cultures during stimulation. We hypothesized that LAG-3 would be rapidly expressed from an intracellular store following stimulation.

LAG-3 and cytokine expression were measured in CD3+ T cells by flow cytometry. The gating strategy is shown in Figure 2. LAG-3, IFN-γ, and TNF-α were expressed at low levels ex vivo from healthy donors, with median expression of 2.99%, 0.300%, and 0.185% of CD3+ T cells respectively. Following stimulation with PMA and Io, LAG-3, IFN-γ, and TNF-α expression were significantly up-regulated by all donors (n = 6, p-value = 0.0318, 0.0313, and 0.0364 respectively) with median expression of 12.44%, 16.95%, and 14.98% of CD3+ T cells, respectively.

Prevention of *de novo* protein synthesis through treatment with cycloheximide significantly reduced IFN-γ and TNF-α expression by all donors (p-value = 0.0313 for both) with median expression of 6.16% and 2.41% of CD3+ T cells respectively, as seen in Figure 3B. LAG-3 expression was not significantly altered following cycloheximide treatment (p-value = 0.8438) as seen in Figure 3B, with median expression of 12.61% of CD3+ T cells. The reduction of IFN-γ and TNF-α expression was significantly greater than the reduction of LAG-3 (p-value < 0.05 and 0.001 respectively) by cycloheximide treatment as seen in Figure 3C. Although not a significant

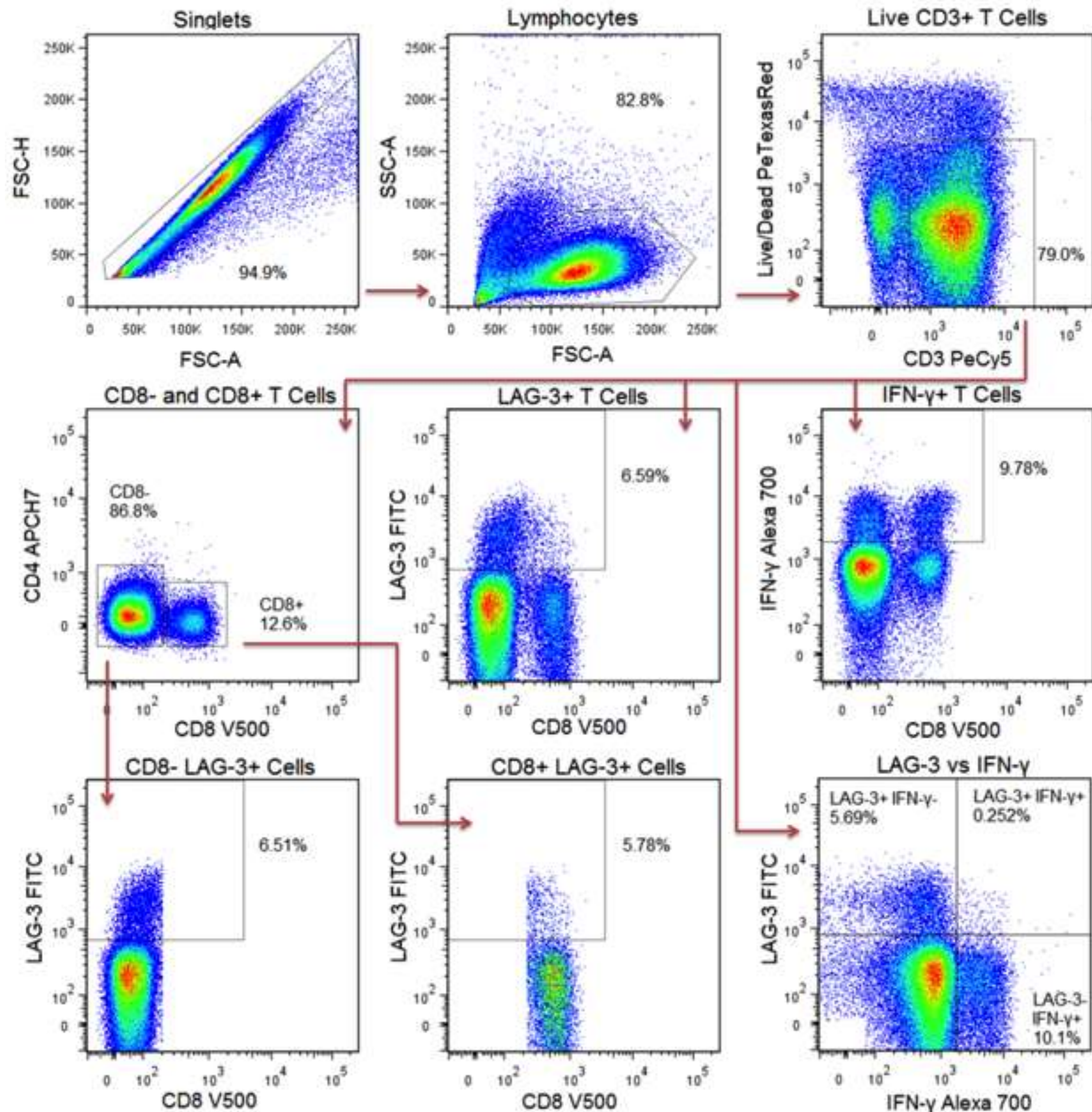


Figure 2. Representative staining of PBMCs follow 6 hour stimulation with PMA and Io. Singlets were first identified using forward scatter-area (FSC-A) versus forward scatter-height (FSC-H) gating. Lymphocyte gating was based on FSC-A and side scatter-area (SSC-A). Live CD3+ T cells were identified as Live/Dead amine reactive dye negative or dim and CD3+. LAG-3 and cytokine or CD69 expression gating was based on fluorescence minus one (FMO) staining. CD3+ T cell subsets were identified as CD3+CD8+ or CD3+CD8- as stimulation with PMA down-regulates CD4 expression. LAG-3 and IFN-γ are observed to be expressed independently.

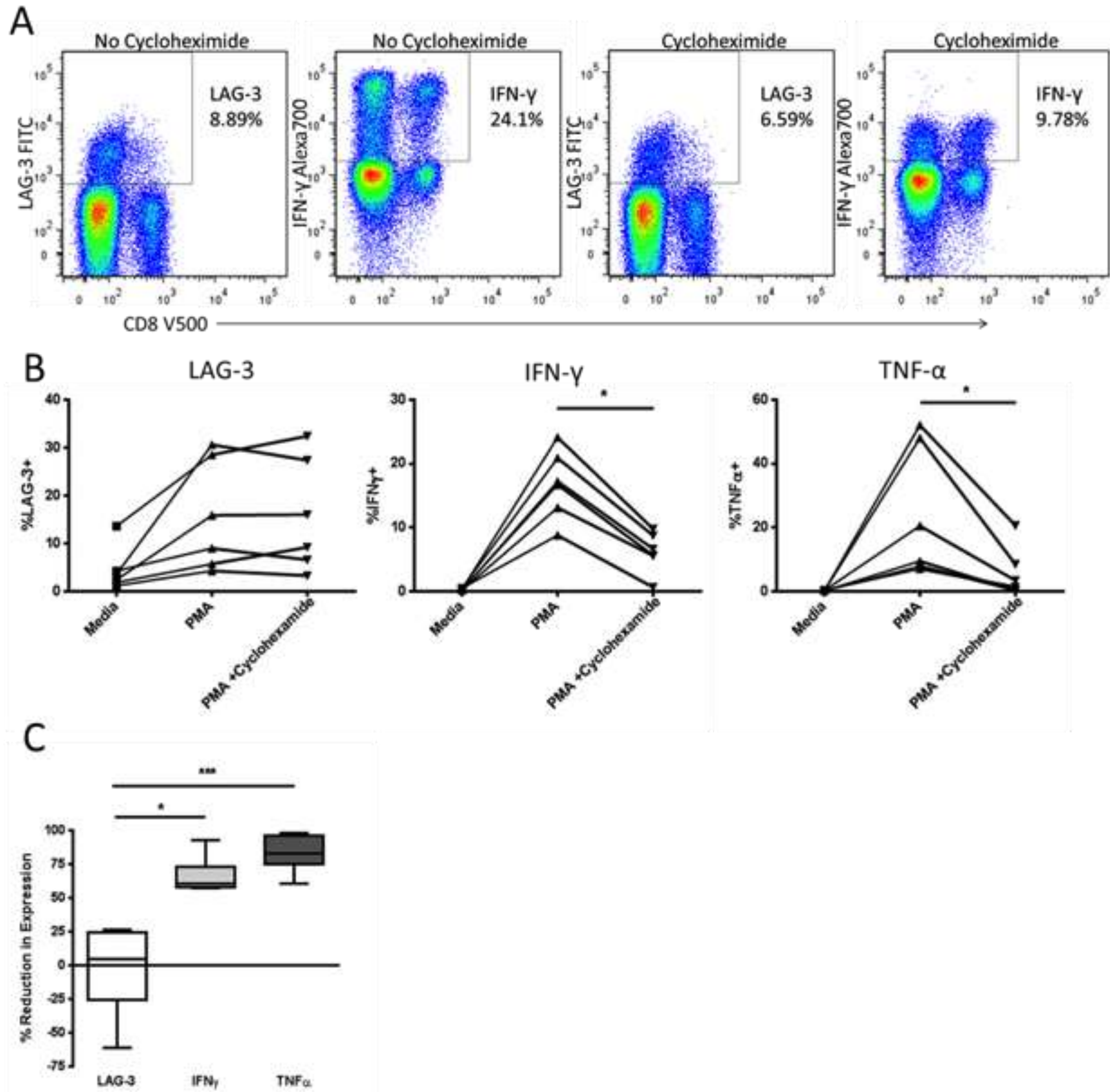


Figure 3. LAG-3 and cytokine expression following PMA/Io and cycloheximide treatment. **(A)** Representative staining of LAG-3 and IFN-γ on CD3+ T cells (D170). **(B)** LAG-3 expression remains unchanged following cycloheximide treatment while cytokine production is significantly decreased. Differences between conditions were assessed by Wilcoxon Rank Sum test. **(C)** LAG-3 expression is not significantly reduced and is enhanced by 3 donors following cycloheximide treatment while cytokine expression was significantly reduced by comparison using 1way ANOVA and Dunn's multiple comparison test. *$p<0.05$, ***$p<0.001$

difference, 3 donors showed increased LAG-3 expression following cycloheximide treatment compared to PMA/Io alone as seen in Figures 2B and C.

3.2 Impact of matrix metalloprotease inhibitor GM6001 on surface LAG-3 expression

Previously, cleavage of membrane bound LAG-3 by matrix metalloproteases has been observed to generate sLAG-3 in mice [87]. To determine the impact of metalloprotease mediated cleavage on human LAG-3, broad spectrum matrix metalloprotease inhibitor, GM6001, was added to cell cultures during stimulation to measure increased retention of surface LAG-3. We hypothesized that membrane LAG-3 would decline over time due to matrix metalloprotease mediated cleavage.

LAG-3 expression was measured on CD3+ T cells following stimulation with PMA/Io and varying treatment concentrations with GM6001 by flow cytometry. The gating strategy is the same as above (Figure 2). LAG-3 expression was significantly increased when treated with either 50 or 100µM GM6001 (p-value <0.01 and <0.05 respectively) (Figure 4), but was highest at the concentration of 50uM (Figure 4-B). The increase in LAG-3 expression was also consistent on both CD8- (50µM p -value<0.01 and 100µM p-value<0.001) and CD8+ (50µM p -value<0.01 and 100µM p-value<0.05) T cell subsets (Figure 4-B). Because 50µM GM6001 treatment was observed to be most effective, additional PBMC samples were treated and their supernatants collected to assess the impact of GM6001 treatment on sLAG-3 secretion. Unfortunately, the LAG-3 ELISA was not sensitive enough to detect sLAG-3 in these samples; however, these samples were stained for flow cytometry and added to the analysis to boost the sample size, and demonstrated increased surface LAG-3 expression on CD3+ T cells following GM6001 treatment (p-value = 0.0017) (Figure 4-C).

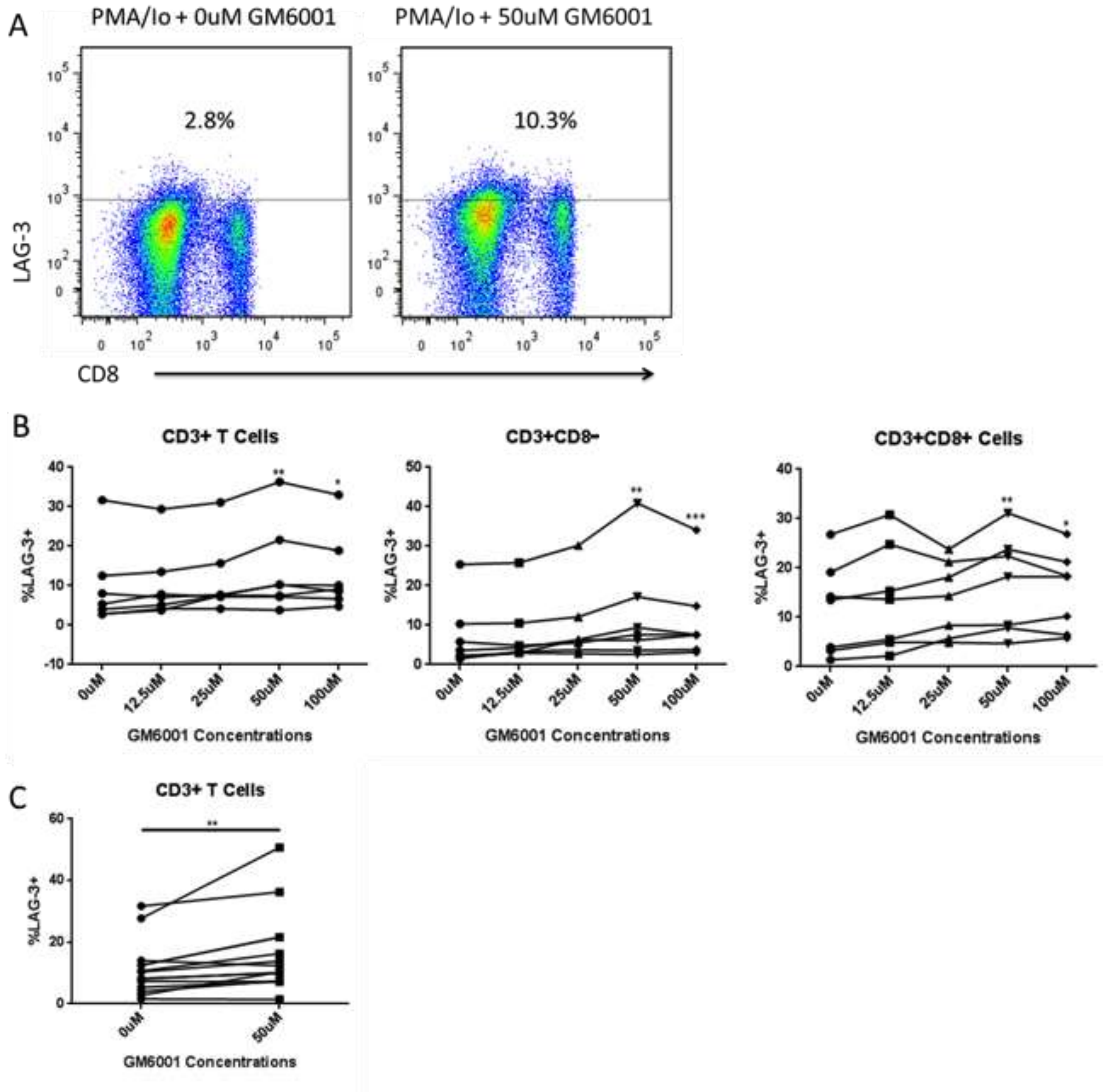


Figure 4. Impact of Matrix Metalloprotease Inhibitor GM6001 on LAG-3 expression. (**A**) Representative staining of LAG-3 on PMA/Io stimulated CD3+ T cells with and without GM6001 treatment. (**B**) GM6001 dose response of LAG-3 expression on PMA/Io stimulated CD3+ T cells, and CD8- and CD8+ T cell subsets (n = 7). LAG-3 expression is significantly enhanced by 50 and 100µM GM6001 treatment. Differences assessed by the Friedman test and Dunn's multiple comparison test. (**C**) LAG-3 expression on untreated and 50µM GM001 treated, PMA/Io stimulated CD3+ T cells (n = 14). Difference assessed by Wilcoxon Rank Sum test. *p<0.05 **p<0.01 ***p<0.001

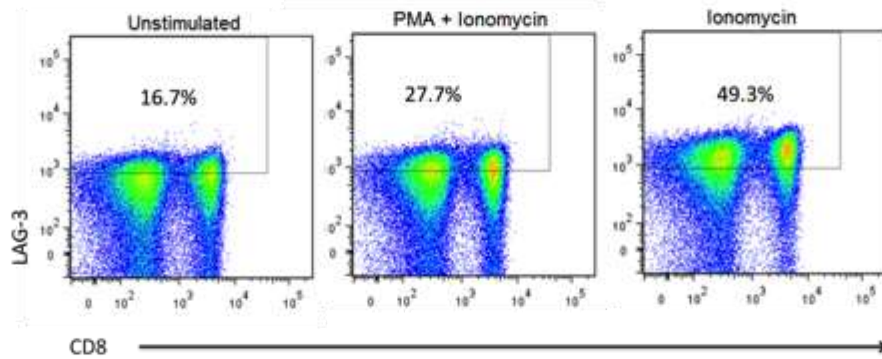
3.3 Ionomycin stimulation induces surface LAG-3 expression

We have repeatedly observed an increase in LAG-3 expression on T cells after 6 hours of PMA/Io stimulation compared with unstimlated cells (p-value = 0.0156) (Figure 5). PMA as a stimulant bypasses the TCR and initiates downstream calcium signalling events. PMA and Io work together to enhance phosphorylation of PKC [130]. Io mobilizes intracellular calcium [130], and the unopposed calcium signalling leads to cellular anergy [131]. Genes expressed during anergy are distinct from genes induced in an activated immune response and are largely NFAT dependent [131]. In order to determine whether LAG-3 was upregulated by a stimulus that leads to T cell anergy, LAG-3 was measured by flow cytometry following Io stimulation. We hypothesized that LAG-3 and sLAG-3 would be expressed following stimulation with Io and the induction of anergy.

Io stimulation alone was also able to increase LAG-3 expression on T cells after 6 hours compared with unstimulated cells (p-value = 0.0078) (Figure 5). Additionally, Io stimulation alone led to greater increase in LAG-3 expression than PMA/Io combination stimulation (p-value = 0.0391) (Figure 5).

3.4 Kinetics of LAG-3 expression

Literature suggests that LAG-3 is not upregulated until 24-48 hours post-T cell stimulation. Because LAG-3 can be detected on the surface of T cells by strong PMA/Io stimulation, and because we demonstrated evidence of a pre-formed cellular store of LAG-3 protein, we monitored LAG-3 expression from 0 – 24 hours post-stimulation with a more physiologically



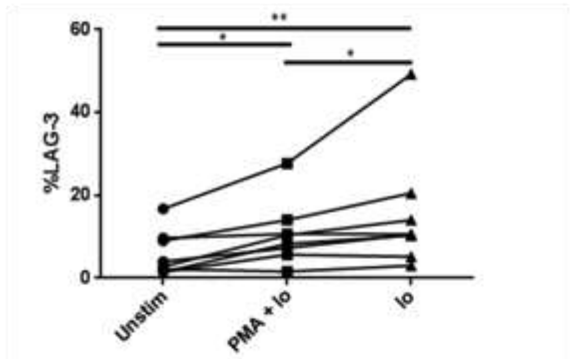


Figure 5. LAG-3 expression following 6 hour stimulation with PMA/Io or Io alone.
(A) Representative staining of LAG-3 on unstimulated, PMA/Io stimulated, and Io alone stimulated CD3+ T cells. **(B)** Percentage of CD3+ T cells expressing LAG-3 is significantly increased when stimulated with PMA/Io or Io alone. Io alone increases LAG-3 expression significantly more than PMA/Io. All differences assessed by Wilcoxon Rank Sum test. *$p<0.05$ **$p<0.01$

relevant TCR-dependent stimulus. We hypothesized that LAG-3 would be expressed within a few hours post stimulation from an intracellular and decline over time due to matrix metalloprotease mediated cleavage before being expressed by *de novo* protein synthesis at a later time point.

To determine when LAG-3 expression would peak on T cells and T cell subsets, CD3+ T cells were activated using CD3/CD28 beads and LAG-3 expression was followed alongside early activation marker CD69. LAG-3 and CD69 were measured by flow cytometry on CD3+ T cells as well as CD4+ and CD8+ subsets following stimulation with CD3/CD28 beads for 2, 4, 6, 10, 16 or 24 hours. The gating strategy is shown in Figure 6. Compared with unstimulated cells cultured for 24 hours, LAG-3 was significantly upregulated on all T cell subsets at the 4 hour time point (p-value < 0.05 for all subsets) (Figure 7-B). The 4 hour time point was also when CD69 was first significantly upregulated compared to the unstimulated control (CD3 p-value < 0.01, CD4 p-value < 0.01, CD8 p-value < 0.05) (Figure 7-C). LAG-3 expression proceeded to decrease at the 6 and 10 hour time point and was no long significantly higher than the unstimulated control until the 16 hour time point when LAG-3 expression peaked (CD3 p-value < 0.001, CD4 p-value < 0.01, CD8 p-value < 0.0001) (Figure 7-B). Following the peak, a non-significant decrease in LAG-3 expression was observed in 5 of the 6 donors at the 24 hour time point, however, expression was still increased compared to the unstimulated control (CD3 p-value < 0.001, CD4 p-value < 0.05, CD8 p-value < 0.001) (Figure 7-B). CD69 expression continued to increase from the 4 hour time point to the 16 hour time point. Consistent with LAG-3, CD69 expression was decreased at the 24 hour time point, although not significantly, by all donors except one (same donor with increased LAG-3 expression).

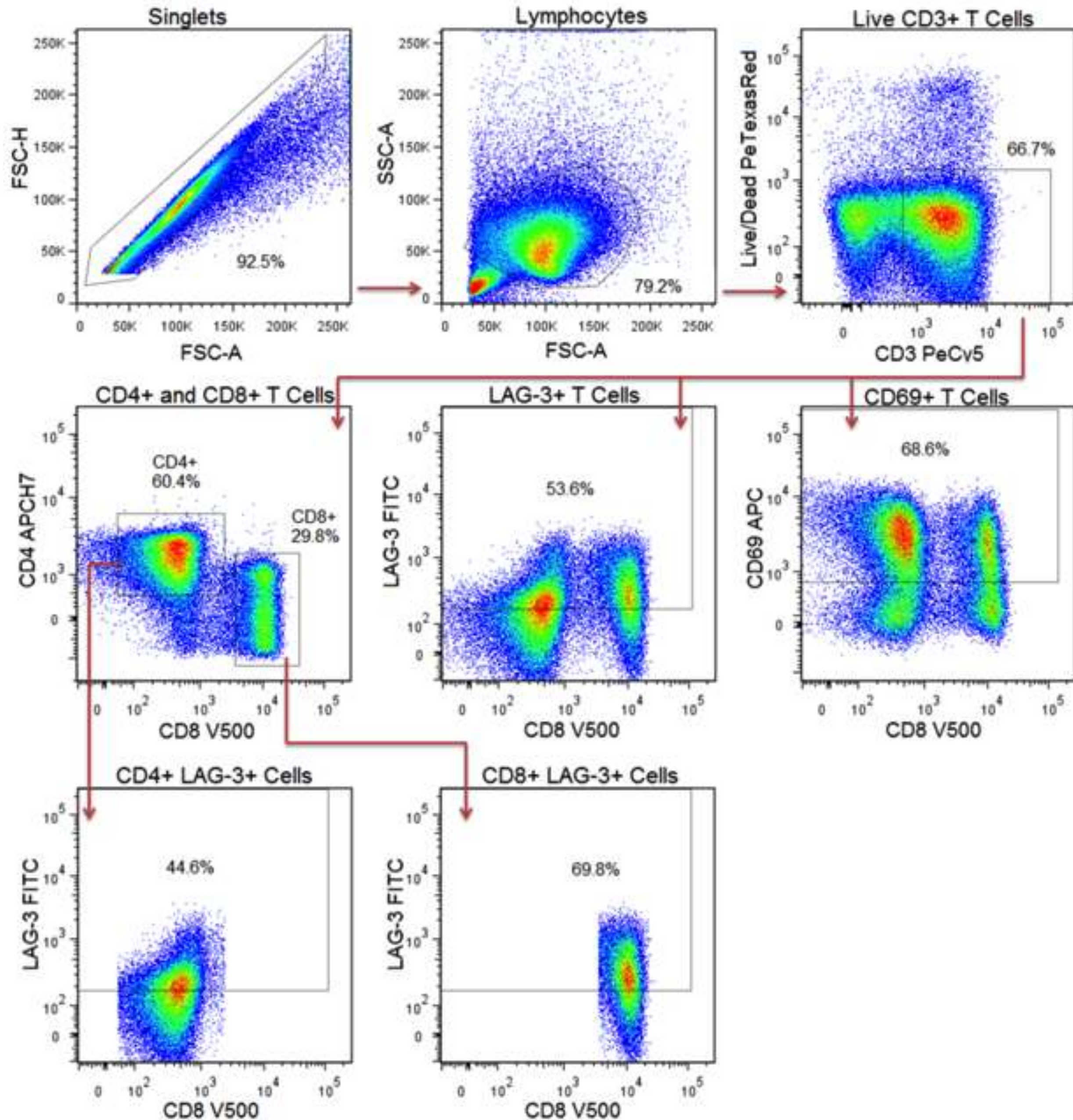


Figure 6. Representative staining of PBMCs following 16 hour stimulation with CD3/CD28 beads. Singlets were first identified using FSC-A versus FSC gating. Lymphocyte gating was based on FSC-A and SSC-A. Live CD3+ T cells were identified as Live/Dead amine reactive dye negative or dim and CD3+. LAG-3 and CD69 expression gating was based on FMO staining. CD3+ T cell subsets were identified as CD3+CD4+ or CD3+CD8+ allowing subset differences to be examined.

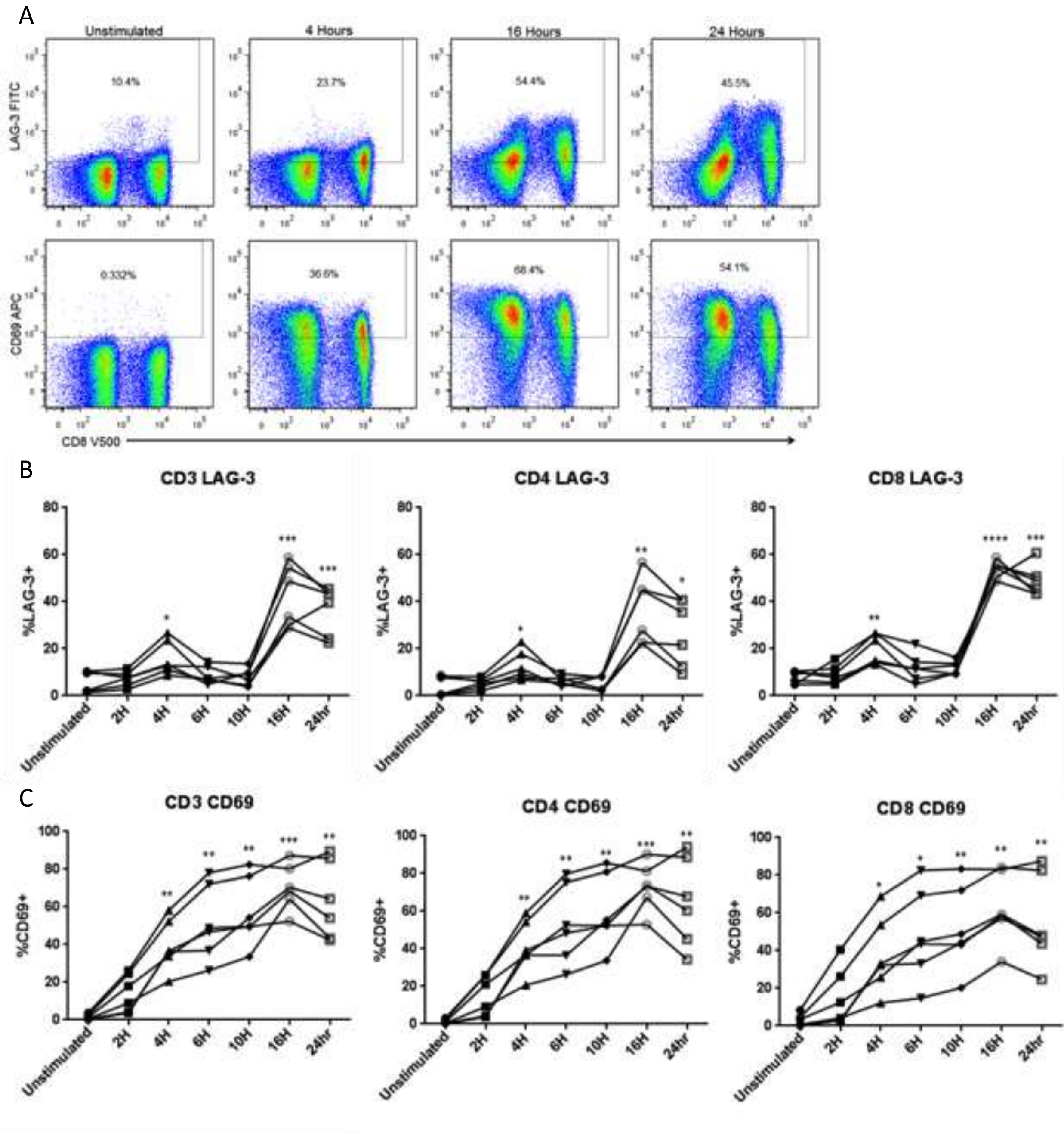


Figure 7. Early LAG-3 and CD69 expression time course. **(A)** Representative staining plots of LAG-3 and CD69 expressing CD3+ T cells. **(B)** LAG-3 expression on CD3+ T cells and CD4+ and CD8+ subsets. LAG-3 is significantly upregulated at 4, 16, and 24 hours post stimulation in all subsets.**(C)** CD69 expression on CD3+ T cells and CD4+ and CD8+ subsets. CD69 is significantly upregulated at 4, 6, 10, 16, and 24 hours post stimulation in all subsets. Differences assessed by the Friedman test and Dunn's multiple comparison test. n = 6 *$p<0.05$ **$p<0.01$ ***$p<0.001$

T cell subsets were also compared to one another and bulk T cells at each time point (Figure 8). CD8+ T cells expressed significantly more LAG-3 than CD4+ T cells at the 4, 6, 10, 16, and 24 hour time points (p-value < 0.01, 0.05, 0.01, 0.0001, and 0.0001 respectively). CD8+ T cells also expressed significantly more LAG-3 than bulk CD3+ T cells at the 16 and 24 hour time point (p-value < 0.0001 for both). CD4+ T cells expressed significantly less LAG-3 than bulk CD3+ T cells at the 16 and 24 hour time point (p-value < 0.01 and 0.0001 respectively). CD69 expression was significantly higher on CD3+ and CD4+ T cells than CD8+ at 10, 16, and 24 hours (CD3 vs CD8 p-value < 0.05, 0.01, and 0.01 respectively, CD4 vs CD8 p-value < 0.01, 0.0001, and 0.001 respectively).

T cells were also examined for the co-expression of LAG-3 and activation marker CD69 over the time course (Figure 9). The frequency of the CD69+LAG-3- population was significantly greater than both the CD69+LAG-3+ and CD69-LAG-3+ populations at 2, 4, 6, and 10 hours post stimulation (CD69+LAG-3- vs CD69+LAG-3+ p-value < 0.01, 0.0001, 0.0001, 0.0001 respectively, CD69+LAG-3- vs CD69-LAG-3+ p-value < 0.05, 0.0001, 0.0001, 0.0001 respectively). At 16 hours post stimulation, both the CD69+LAG-3- and CD69+LAG-3+ population frequencies were greater than the CD69-LAG-3+ population (p-value < 0.0001 for both). At 24 hours post stimulation, the frequency of the CD69+LAG-3+ population was significantly greater than both the CD69+LAG-3- and CD69-LAG-3+ populations (p-value < 0.05 and 0.0001 respectively). The CD69+LAG-3- population remained greater than the CD69-LAG-3+ population at 24 hours (p-value < 0.01) Although LAG-3 expression was significantly upregulated 4 hours post stimulation (Figure 7B), there is no significant difference between the frequencies of the CD69+LAG-3+ and CD69-LAG-3+ populations at this

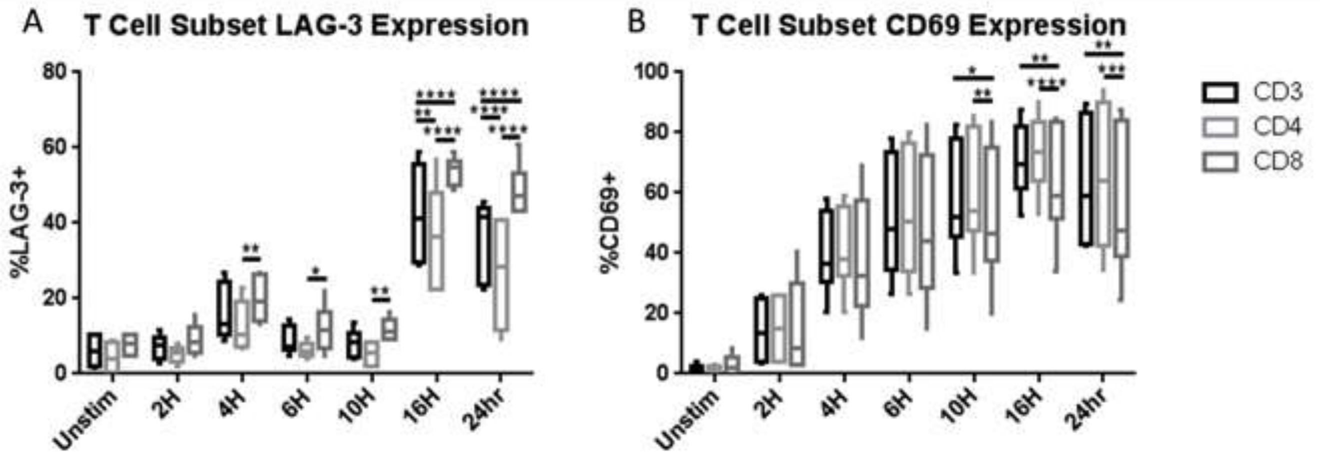


Figure 8. Early LAG-3 and CD69 expression on CD3+, CD4+, and CD8+ T cells. (A)LAG-3 and CD69 expression were compared between bulk CD3+ T cells, and CD4+ and CD8+ T cell subsets by 2way ANOVA and Tukey's multiple comparisons test. LAG-3 expression was significantly higher on CD8+ than CD4+ cells at 4, 6, 10, 16, and 24 hours. LAG-3 expression was significantly higher on CD8+ than bulk CD3+ cells at 16 and 24 hours. LAG-3 expression was significantly lower on CD4+ than bulk CD3+ cells at 16 and 24 hours. A significant interaction of time and subset was found ($p < 0.0001$). **(B)** CD69 expression was significantly higher on bulk CD3+ and CD4+ cells than CD8+ cells at 10, 16, and 24 hours. A significant interaction of time and subset was found ($p < 0.0074$). Subset differences assessed by 2way ANOVA and Tukey's multiple comparisons test. n = 6 *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001

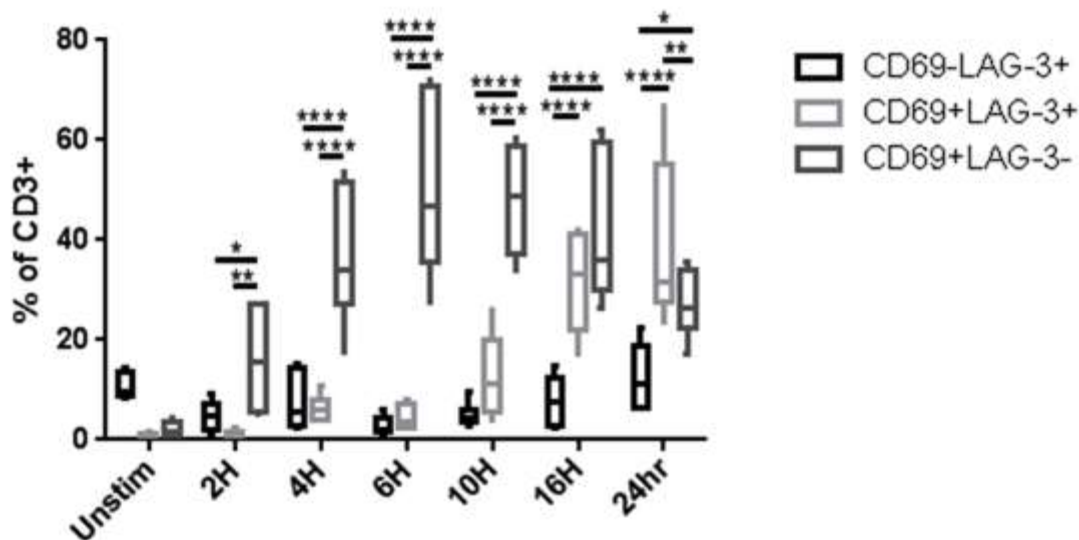


Figure 9. Expression and co-expression of LAG-3 and CD69 time course. LAG-3 and CD69 expression were examined as a proportion of CD3+ T cells following CD3/CD28 bead stimulation. CD69+LAG-3- cells are significantly greater than both CD69+LAG-3+ and CD69-LAG-3+ cells at 2, 4, 6, and 10 hours post stimulation. CD69+LAG-3- and CD69+LAG-3+ cells are significantly greater than CD69-LAG-3+ cell at 16 hours post stimulation. CD69+LAG-3+ cells are significantly greater than CD69+LAG-3- and CD69-LAG-3+ cells 24 hours post stimulation. Differences were assessed by 2way ANOVA and Tukey's multiple comparisons post test. A significant interaction of time and cell subset was found ($p < 0.0001$). $n = 6$
*$p<0.05$ **$p<0.01$ ****$p<0.0001$

time point. When LAG-3 expression peaks at 16 hours post stimulation, the major population expressing LAG-3 is also co-expressing CD69.

Release of sLAG-3 from stimulated, bulk PBMCs was measured at 2, 4, 6, 10, 16, and 24 hour time points (Figure 10). Concentration of sLAG-3 was significantly increased compared to unstimulated cells at 16 and 24 hours (p-value < 0.05 and 0.01 respectively). 1 donor produced detectible levels of sLAG-3 at all time points. An additional 4 donors produced detectible levels of sLAG-3 10 hours post stimulation. All donors (n = 9) reached detectible levels by 16 hours post stimulation.

3.5 LAG-3 and sLAG-3 expression by CD4+ and CD8+ T cell subsets

In order to assess the expression profiles of CD4+ and CD8+ T cells without the influence of other cell signals, CD4+ and CD8+ cells were sorted into pure populations. Based on the data from Figure 8, we hypothesized that CD8+ T cell would express more LAG-3 and have a more inhibited phenotype than CD4+ T cell which would express more activation marker, CD69. CD4+ and CD8+ T cell subsets were sorted out of bulk PBMC samples as shown in Figure 11. Sorted samples were assessed for purity (Table 5). CD4+ and CD8+ samples were stimulated with CD3/CD28 beads for 16 hours. LAG-3 and CD69 expression were measured by flow cytometry (Figure 12). LAG-3 expression was higher on CD8+ T cells than CD4+ T cells both *ex vivo* (p-value = 0.0469) and post stimulation (p-value = 0.0156). CD69 expression was higher on CD4+ T cells than CD8+ T cells both *ex vivo* (p-value = 0.0156) and post stimulation (p-value = 0.0156). These results were consistent with the stimulated bulk PBMCs, 16 hours post stimulation, examined above (Figure 8); LAG-3 expression was higher on the CD8+ T cells and CD69 expression was higher on the CD4+ T cells.

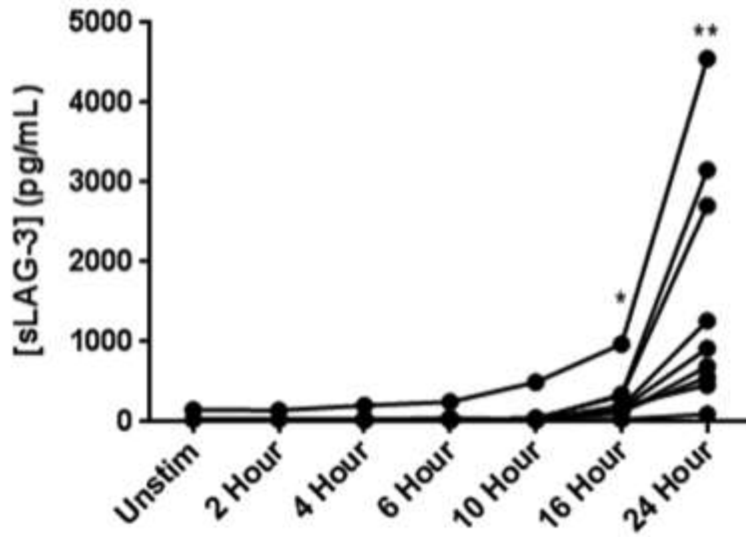


Figure 10. sLAG-3 time course. sLAG-3 concentrations of supernatants from CD3/CD28 stimulated PBMCs measured by ELISA. sLAG-3 concentration significantly upregulated compared to unstimulated control at 16 and 24 hours. Undetectable concentrations adjusted to minimum level of detection, 300pg/mL. Differences assessed by Friedman's test and Dunn's multiple comparisons test. n = 9 *p<0.05 **p<0.01

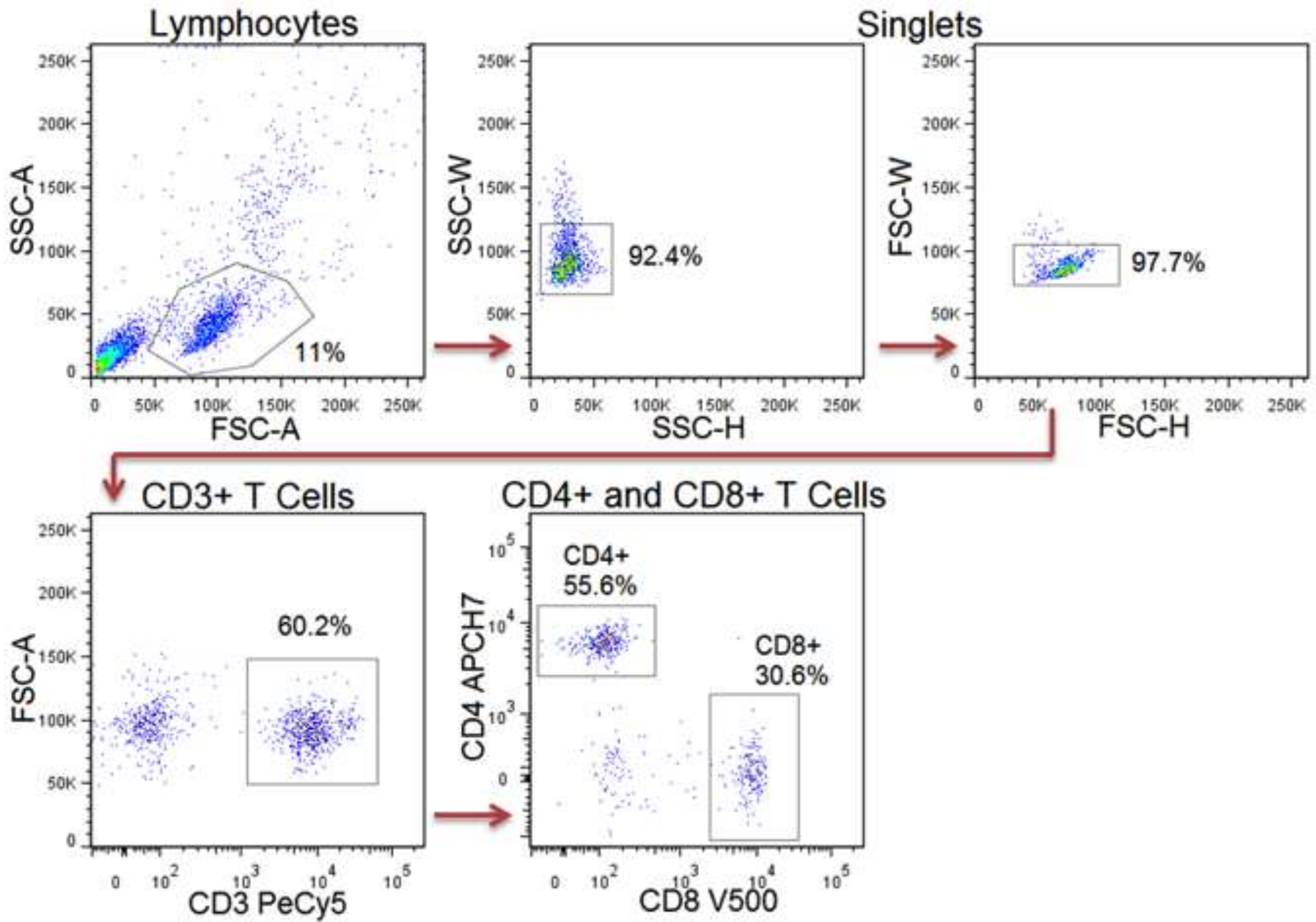


Figure 11. Representative staining and gating of ex vivo PBMCs for sorting. Lymphocyte gating was based on FSC-A and SSC-A. Singlets were then identified based on SSC-H and side scatter-width (SSC-W), and FSC-H and forward scatter-width (FSC-W). T cells were identified as CD3+. CD3+ T cells were sorted into pure CD4+ and CD8+ populations. Sorting was performed on the FACS Aria.

Table 5. CD4+ and CD8+ sorted sample purity. Purity reported as %CD4+ or %CD8+ of CD3+ T cells

<table>
<tr><th>Donor</th><th>CD4 Purity (%)</th><th>CD4 Purity Mean ±Std. Dev.</th><th>CD8 Purity (%)</th><th>CD8 Purity Mean ±Std. Dev.</th></tr>
<tr><td>D39</td><td>99.1</td><td rowspan="8">98.9 ± 1.04</td><td>n/a</td><td rowspan="8">98.9 ± 1.40</td></tr>
<tr><td>D82</td><td>99.4</td><td>99.9</td></tr>
<tr><td>D177</td><td>99.6</td><td>99.7</td></tr>
<tr><td>D179</td><td>98.9</td><td>97.6</td></tr>
<tr><td>D229</td><td>98.8</td><td>99.9</td></tr>
<tr><td>D232</td><td>96.5</td><td>96.3</td></tr>
<tr><td>D237</td><td>99.6</td><td>99.6</td></tr>
<tr><td>D238</td><td>99.6</td><td>99.3</td></tr>
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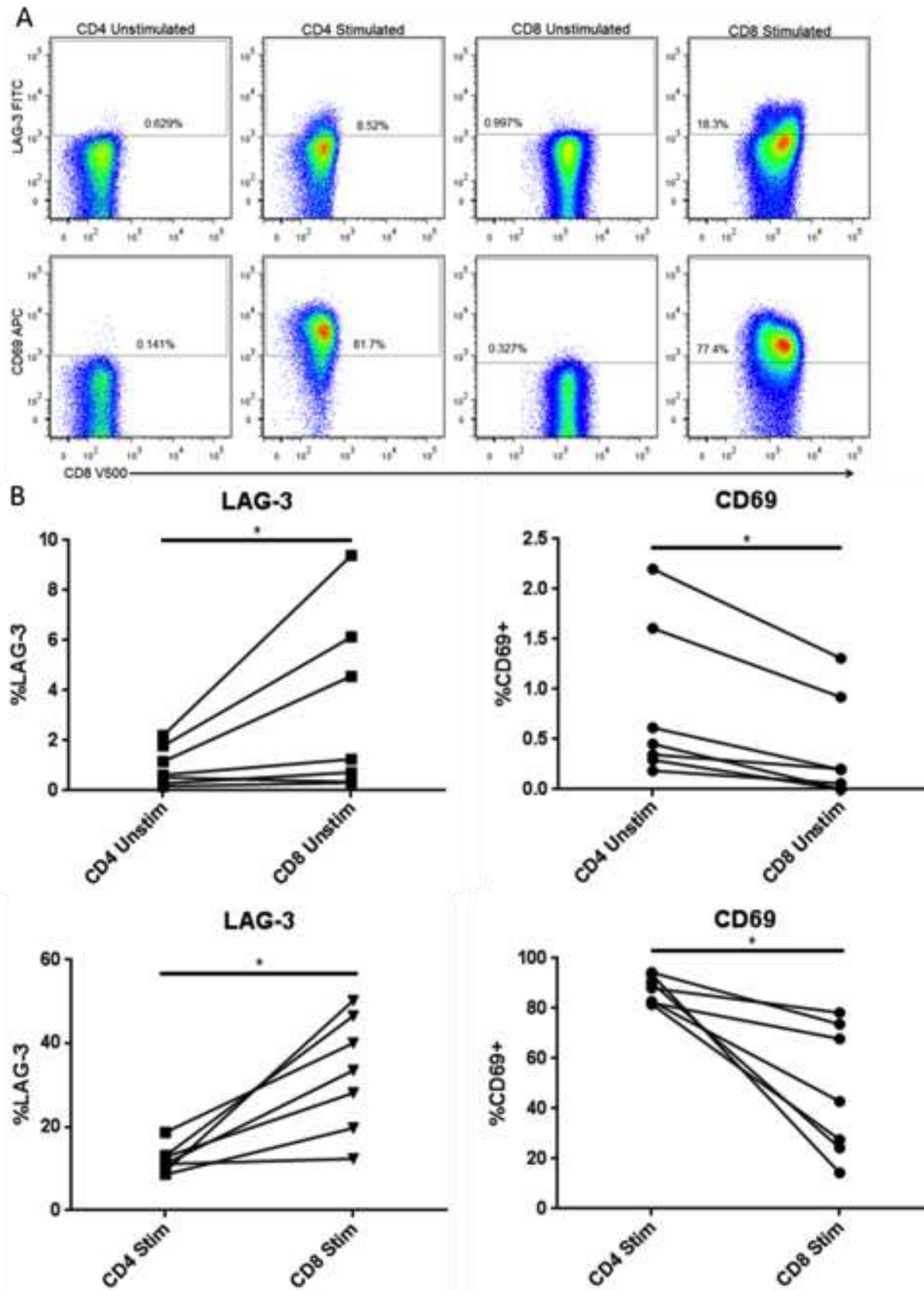

Figure 12. Expression of LAG-3 and CD69 on sorted CD4+ and CD8+ T cell subsets. (**A**)Representative staining plots of LAG-3 and CD69 expression on sorted CD4+ or CD8+ cells.(**B**)CD8+ T cells express significantly more LAG-3 than CD4+ T cells both *ex vivo* and post 16 hour CD3/CD28 bead stimulation. CD4+ T cells express significantly more CD69 than CD8+ T cells both *ex vivo* and post 16 hour CD3/CD28 bead stimulation. Matched CD4 and CD8 data points originated from the same donor. Differences between subsets assessed by Wilcoxon Rank Sum test. n = 7 *p<0.05

sLAG-3 was measured from supernatants of stimulated CD4+ and CD8+ T cells by ELISA (Figure 13-A). sLAG-3 concentrations were not detectible for unstimulated samples. sLAG-3 concentrations were not significantly different between stimulated CD4+ and CD8+ T cells, however there is a trend towards CD8+ T cells producing more sLAG-3 than CD4+ (p-value = 0.0781)

Supernatants were also used to measure IFN-γ, TNF-α, MIP-1β, and IL-4 concentrations by milliplex (Figure 13-B). IFN-γ concentrations were not significantly different between CD4+ and CD8+ cell supernatants (p-value = 0.469). TNF-α and IL-4 concentrations were significantly higher in the CD4+ T cell supernatants than the CD8+ (p-value = 0.0156 for both). MIP-1β concentration was significantly higher in the CD8+ T cell supernatants than the CD4+ (p-value = 0.0156). A significant correlation was observed between sLAG-3 and both IFN-γ and TNF-α in CD8+ T cell supernatants (p-value = 0.0123 and 0.048 respectively) (Figure 14). No significant correlations were observed between CD4+ T cell supernatant sLAG-3 and cytokines. No other significant correlations were observed between any markers tested, however, a trend was observed between CD4+ T cell LAG-3 and IFN-γ (p-value = 0.0694) (Table 6).

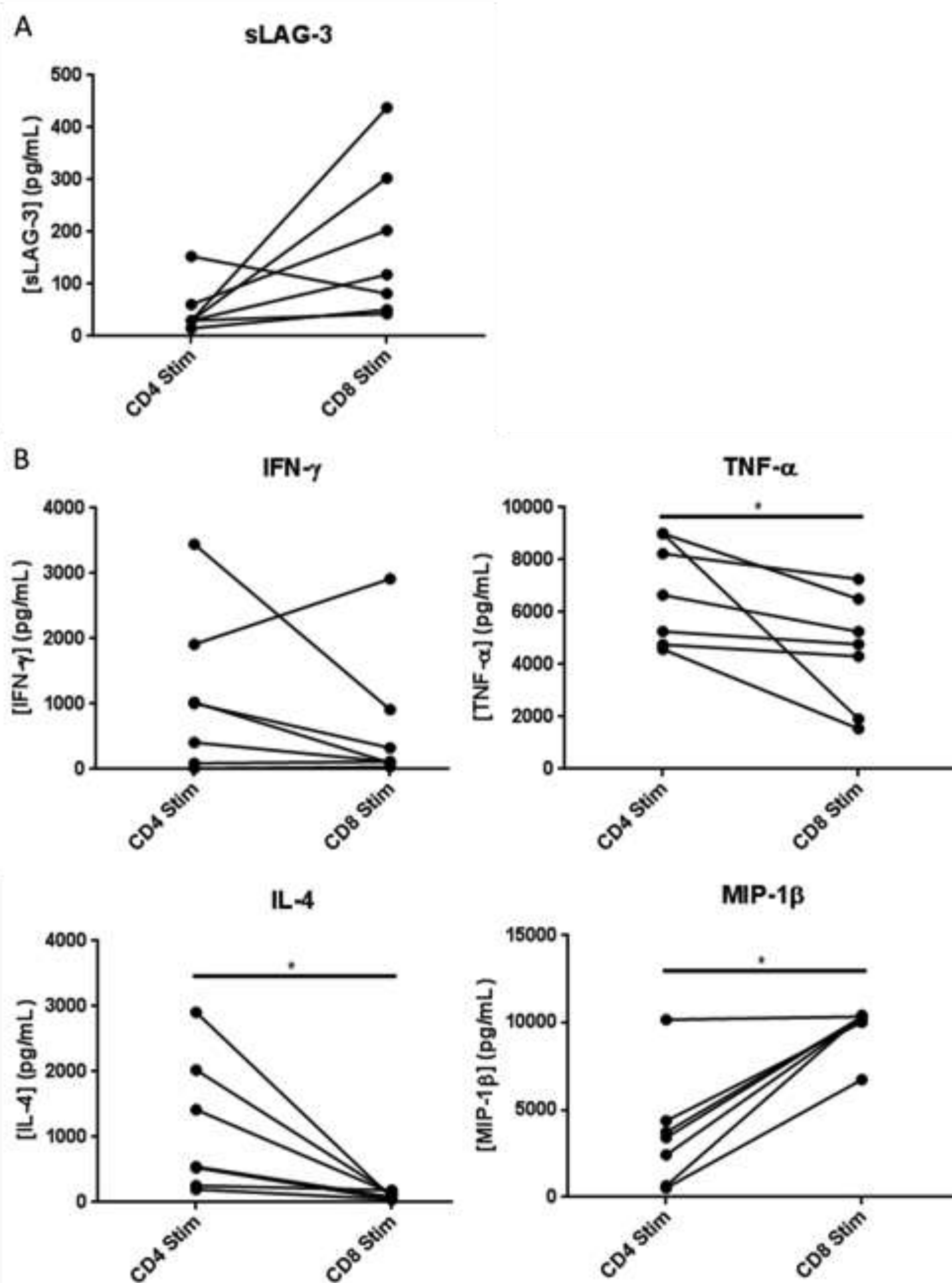

Figure 13. sLAG-3 and cytokine concentrations in supernatants of CD3/CD28 bead stimulated, flow sorted CD4+ or CD8+ T cells. **(A)** sLAG-3 production measured by ELISA is not significantly different between CD4+ and CD8+ T cell subsets. **(B)** Cytokine concentration of supernatants measured by Milliplex. IFN-γ concentration were not significantly different between CD4+ and CD8+ T cell subsets. TNF-α and IL-4 concentration significantly higher in CD4+ T cell supernatants. MIP-1β concentration significantly higher in CD8+ T cell supernatants.Differences between subsets assessed by Wilcoxon Rank Sum test. n = 7 *$p<0.05$

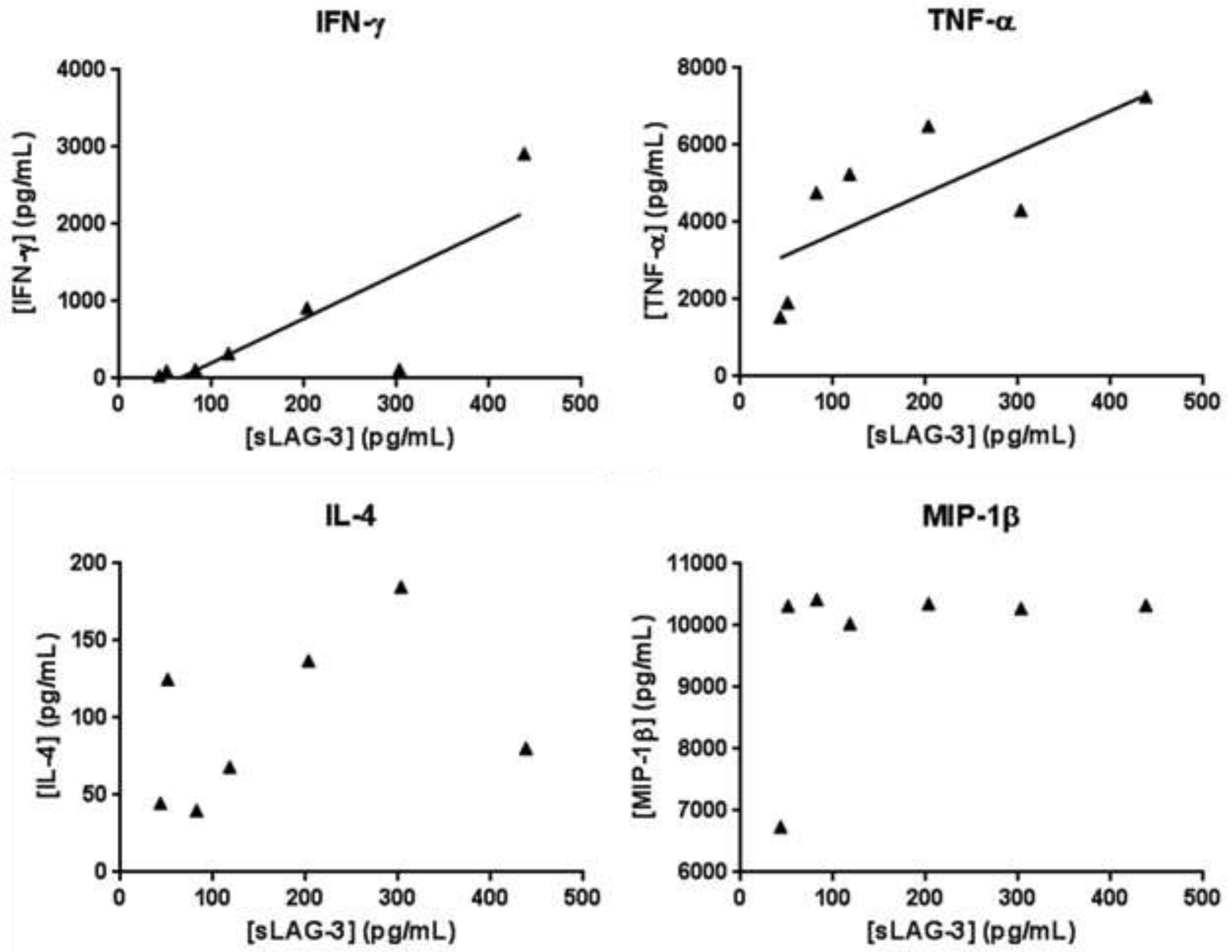

Figure 14. Stimulated CD8+ T cell supernatant correlations. Stimulated CD8+ T cell supernatant's concentration of sLAG-3 significantly correlates with both IFN-γ ($p = 0.0123$, $r = 0.8929$) and TNF-α ($p = 0.0480$, $r = 0.7857$) concentrations but not IL-4 or MIP-1β.

Table 6. Correlations of LAG-3, CD69, sLAG-3, and cytokines on CD4 and CD8+ T cells. sLAG-3 was significantly correlated with IFN- and TNF- in CD8+ T cell supernatant (Dark grey). A trend towards significance was observed between CD4+ surface LAG-3 and IFN- in CD4+ supernatants (light grey).

		CD4		**CD8**	
		p-value	**r**	**p-value**	**r**
LAG-3	**CD69**	0.3268	0.4048	0.4444	0.3571
	sLAG-3	0.1071	-0.4910	0.6615	-0.2143
	IFN-γ	***0.0694***	***0.6905***	0.9063	0.07143
	TNF-α	0.1333	0.8000	0.7825	0.1429
	IL-4	0.1150	0.6190	0.1389	-0.6429
	MIP-1β	0.1511	0.5714	0.4976	-0.3214
CD69	**sLAG-3**	0.5714	-0.1091	>0.9999	0
	IFN-γ	0.9349	-0.04762	0.7825	-0.1429
	TNF-α	0.1333	0.8000	0.8397	-0.1071
	IL-4	0.7930	0.1190	0.5948	-0.2500
	MIP-1β	0.3268	-0.4048	0.5560	-0.2857
sLAG-3	**IFN-γ**	0.6310	0.2182	**0.0123**	**0.8929**
	TNF-α	0.8000	0.3536	**0.0480**	**0.7857**
	IL-4	0.6786	0.1909	0.2357	0.5357
	MIP-1β	0.3214	0.4364	0.4976	0.3214

4. Discussion

LAG-3 is a protein with complex function, involved in both immune activation and suppression; by improving our understanding of the regulation and expression of LAG-3 we will gain insights into the relative importance of these contradicting effects. Engagement of LAG-3 on the surface of T cells results in the inhibition of the CD3-induced calcium flux, cell proliferation and activation, and Th1 cytokine production [77, 86]. Engagement of MHC class II by LAG-3 results in maturation of DCs and production of Th1-driving cytokines [104-106]. sLAG-3 has also been shown to enhance anti-tumor responses, antibody production, Th1 cytokine production, and CTL responses [111].

This thesis examines surface-expressed and soluble LAG-3 regulatory mechanisms in human T cells that have previously only been observed in murine and non-human primate studies. Although LAG-3 is an immune inhibitory marker known to contribute to the T cell exhaustion phenotype, expression of LAG-3 during several chronic viral infections, including HIV, is uniquely low when compared to other immune inhibitory markers [51].

The hypothesis of this thesis is that soluble and membrane LAG-3 will be rapidly expressed from an intracellular store following stimulation, and that membrane LAG-3 will decline over time due to metalloprotease cleavage, thereby concurrently increasing release of soluble LAG-3.

4.1 LAG-3 is present in an intracellular store within T cells

We hypothesized that LAG-3 would be rapidly expressed from an intracellular store following stimulation. Although previous studies have observed the presence of an intracellular store, these studies were performed either in mice [87], which do not perfectly mimic the human immune system or LAG-3 function, or Jurkat cells which require expression vectors in order to

express LAG-3 [88]. For our studies, primary human PBMCs were treated with cycloheximide in order to inhibit *de novo* protein synthesis.

4.1.1 Cycloheximide reduces cytokine, but not LAG-3. expression

Consistent with the previous studies and our hypothesis, we observed that despite a significant downregulation of both IFN-γ and TNF-α, LAG-3 expression was not changed upon cyclohexamide treatment (Figure 3). This implies that the early LAG-3 expression observed 6 hours post PMA/Io stimulation is predominantly due to surface expression of pre-formed LAG-3 protein and not *de novo* protein synthesis. The production and storage of LAG-3 prior to activation of the T cell requires the commitment of cellular energy and resources, which implies an importance for the early expression or release of LAG-3 proteins. Previous studies have observed LAG-3-induced protection from apoptosis [132] which may be important for strongly activated cells at the start of an infection. Additionally, rapid expression of LAG-3 followed by downregulation may act as a point of control for the immune response in order to prevent or reduce damage from inflammation. LAG-3 and sLAG-3 also play a role in immune activation and recruitment through their interaction with MHC class II [104-107]. This interaction may also explain the importance of a rapidly available store and rapid trafficking of LAG-3 to the surface.

Another immune regulating protein that is present in pre-formed stores within T cells is CD40L [133, 134]. Similar to LAG-3, CD40L plays a role in controlling CD4+ and CD8+ T cell responses, and prevention of autoimmunity. CD40L also plays an important role in CD4+ T cell and B cell interactions [135]. Interestingly, the presence of a pre-formed store of CD40L was found within most CD4+ T cell subsets, including: Th1, Th2, Th17, follicular helper cells, and

iNKTs, but was not found within Tregs [136]. In their study, Koguchi *et al.,* suggest that the lack of expression of CD40L from pre-formed stores in Tregs implies that the primary role of preformed CD40L to activation of APCs, a function also observed with LAG-3 [136]. It is possible that early LAG-3 expression is also predominantly intended to provide immune activation and recruitment as opposed to inhibition. Other pre-formed protein stores found in human immune cells include IL-12 in macrophages/DCs [137], and IL-16 in eosinophils [138].

In some of the individuals tested, LAG-3 expression was observed to be higher following treatment with cycloheximide (Figure 3C). This observation may be attributed to cycloheximide preventing the synthesis of proteins which function to cleave or internalize membrane LAG-3.

4.1.2 Limitations and Opportunities

Performing assays on primary human T cells is the best way to make accurate observations about the human immune system. Our observations are, however, limited by the use of PMA/Io as a stimulant. PMA/Io is a strong stimulant that bypasses the TCR in order to stimulate the cells. Unfortunately, this stimulation does not mimic a typical viral infection. PMA/Io was selected because cycloheximide mediated inhibition of *de novo* protein synthesis is readily reversible and PMA/Io allows for a shorter stimulation time. Cycloheximide treatment was optimized for a 6 hour stimulation and required adding cycloheximide 30 minutes before stimulation and 3 hours after in order to maintain inhibition.

The use of PMA as a stimulant causes the downregulation of membrane bound CD4. This effect can make detection of different cell subsets difficult. Through the use of different stimulants or more markers, one is able to detect which cell subsets are expessing LAG-3 from the

intracellular store. It may be possible to detect CD4+ T cells stimulated with PMA by flow cytometry through intracellular staining using an appropriately optimized antibody.

Additionally, we are unable to differentiate between sLAG-3 and full length LAG-3 protein within the intracellular store via flow cytometry. The LAG-3 antibody used in this study was found not to be suitable for intracellular staining (data not shown) and, therefore, *ex vivo* intracellular LAG-3 or sLAG-3 could not be measured. Fluorescent microscopy was used to observe intracellular LAG-3 in mice, however, mice do not produce sLAG-3 splice variants and rely on matrix metalloprotease mediated cleavage to produce sLAG-3 [87]. It may be possible to differentiate between LAG-3 and the truncated sLAG-3 proteins using a Western blot, however, this was beyond the scope of this project. sLAG-3 proteins lack up to 3 immunoglobulin domains (starting at D4 to D2) but would all be detectible using the 17B4 antibody, specific for the 30 AA loop in D1 (Figure 1).

4.2 Human surface LAG-3 is cleaved by matrix metalloproteases

Murine LAG-3 is known to be cleaved by matrix metalloproteases ADAM10 and ADAM17 in order to generate sLAG-3 [102]. Mice do not have LAG-3 splice variants that produce sLAG-3, however, humans do [103, 104]. Because of this known difference in sLAG-3 production, we sought to test the impact of matrix metalloprotease cleavage on human LAG-3 to assess if this process was consistent between humans and mice. Previous studies have also examined the impact of ADAM10 and ADAM17 on another immune inhibitory marker, TIM-3 [139]. Ionomycin stimulation has been shown to activate ADAM10 mediated cleavage while PMA stimulation will activate ADAM17 [140]. In our study, GM6001 was used to inhibit matrix metalloprotease mediated cleavage on primary human PBMCs following PMA/Io stimulation.

We hypothesized that membrane LAG-3 would decline over time due to metalloprotease cleavage and the use of GM6001 will prevent this decline.

4.2.1 Inhibition of matrix metalloprotease cleavage by GM6001 increases surface LAG-3

Our hypothesis was observed to be correct; surface LAG-3 was significantly upregulated on PMA/Io stimulated human T cells treated with GM6001 compared with untreated, stimulated cells (Figure 4). The cleavage of membrane LAG-3 may act to regulate the inhibitory capacity by preventing downstream effects of LAG-3 engagement by its ligands. Increased concentrations of sLAG-3 would, therefore, increase the relative immune activating capacity of LAG-3 generated by downstream signalling through the MHC class II on APCs. Unfortunately, the sLAG-3 concentration in the supernatants of 6 hour PMA/Io stimulated human PBMCs was below the limit of detection (30pg/mL) of the sLAG-3 ELISA and we were unable to measure any change in sLAG-3 concentration after GM6001 treatment.

Matrix metalloprotease ADAM10 has been observed to be constitutively expressed on the surface of human T cells, however, PKC activation and calcium mobilization are both required for activation of the protein. ADAM17 is only found at low levels on the surface of unstimulated cells but is rapidly expressed following stimulation, likely from a preformed store within the cell [141]. These observations would facilitate the rapid cleavage of membrane bound LAG-3 which, as described above, is also rapidly trafficked to the surface following stimulation from an intracellular store.

4.2.2 Limitations and Opportunities

Although ADAM10 and ADAM17 have been observed to cleave LAG-3 in murine studies [87] and TIM-3 [139], we were not able to attribute the cleavage of LAG-3 to specific matrix

metalloproteases in our experiments as GM6001 is a broad-spectrum inhibitor of matrix metalloproteases. In the TIM-3 cleavage study, specific ADAM10 and ADAM17 inhibitors were developed and used in order to target the inhibitory effects and pinpoint the cause of membrane bound TIM-3 retention [139].

Through the use of a cell line, it would be possible to generate a LAG-3 protein construct that includes a green fluorescing protein in the immunoglobulin domains of the protein. This would facilitate the measurement of both LAG-3 and sLAG-3within the cell. Additionally, it would be possible to determine where the matrix metalloproteases cleave the surface LAG-3 protein through combined detection of D1 using the 17B4 antibody and the GFP within another domain. Using fluorescent intensity of the inserted GFP, it would also be possible to quantify the relative abundances of sLAG-3 splice variants within the cell.

4.3 LAG-3 is expressed following stimulation with ionomycin alone

Ionomycin mobilizes and increases the level of intracellular calcium. Ionomycin stimulation is often paired with PMA in order to generate a calcium flux and activate cells through phosphorylation of PKC [130]. This combined stimulation is commonly used in order to generate the production of various cytokines. Stimulation with Io alone, however, has been implicated in the induction of anergy, a form of immune tolerance [131, 142, 143]. An anergic T cell does not produce IL-2, will not proliferate following antigen stimulation [144], and is known to express anergy-associated genes which are not the same as those expressed during activation [142]. The anergic phenotype arises from a strong calcium signal (as seen with Io stimulation or TCR engagement) without a secondary, activating, signal (such as PMA or CD28 engagment).

The anergic phenotype may also require nuclear factor of activated T-cells (NFAT) activation and the resulting interaction with transcripton factors [142].

In order to help elucidate the signals required to induce LAG-3 expression or the trafficking of pre-formed intracellular LAG-3 to the surface, we stimulated primary human PBMCs with Io alone in parallel with PMA/Io stimulation. We hypothesized that the calcium flux from Io stimulation would be sufficient to upregulate LAG-3 and sLAG-3.

4.3.1 Ionomycin stimulation increases LAG-3 expression

Our hypothesis was correct that LAG-3 was upregulated on human T cells following stimulation with Io for 6 hours (Figure 6). Interestingly, LAG-3 expression was significantly greater following Io stimulation compared with PMA/Io combination stimulation (Figure 6). This observation may be a result of LAG-3 expression being upregulated more strongly in an anergic environment or, alternatively, the cytokines expressed from the use of PMA have a suppressive effect on LAG-3 expression or the PMA-mediated activation of ADAM17 would increase the matrix metalloprotease mediated cleavage of LAG-3, further reducing the measurable surface LAG-3. A previous study that looked at LAG-3 gene regulation found possible NFAT binging sites [74] which may associate LAG-3 regulation with the anergic phenotype. LAG-3 expression has been shown to be induced by IL-12 in an IFN-γ dependent manner [85].

LAG-3 has also been used to define Treg populations associated with tolerance, CD4+CD25-LAG-3+ cells. This population was first identified in mice and were found to express early growth response gene 2 (Egr-2), a molecule associated with anergy induction, and produce large amounts of IL-10 [145]. These CD4+CD25-LAG-3+ cells have also been found in humans and have similar gene expression profiles to their murine counterpart [146]. CD4+CD25+FOXP3+

are thymus derived Tregs which are critical in early life to prevent autoimmune disease. Tr1 cells are LAG-3+CD49b+ Tregs, found in both humans and mice, which produce high levels of IL-10 rapidly following stimulation and TGF-β. These cytokines are responsible for the majority of Tr1-mediated regulation, however, human Tr1 cells are also capable of lysing myeloid cells using Granzyme B [147].

Immune inhibitory markers, such as LAG-3, contribute to an exhaustion phenotype but have also been observed to induce lymphocyte anergy [148]. It is therefore possible that LAG-3 is not only expressed in response to an anergic environment but also contributes to the formation or maintenance of the anergic phenotype. The classic definition of an anergy associated gene is the regulation by NFAT and calcium signaling [144]. Although we have shown expression of LAG-3 in response to ionomycin stimulation, we do not know if the observed expression is from the intracellular store or *de novo* protein synthesis. Additionally, it has not been confirmed whether NFAT does regulate LAG-3, although NFAT target sites have been observed around the LAG-3 gene [74].

The anergic phenotype has recently been examined in human CD4+ T cells in response to TLR7 engagement. TLR7 is capable of recognizing single stranded RNA within endosomes which is a common feature of viral infections such as HIV. In this study, it was observed that silencing of TLR7 decreased the frequency of HIV infected CD4+ T cells in addition to restoring function to HIV infected CD4+ T cells. It is possible that this anergic state contributes to viral persistence within infected cells [149]. LAG-3 may be an early marker of anergy during HIV and other infections and may, therefore, help to provide a target for potential HIV reservoirs. Additionally, LAG-3 expression may be reduced during chronic HIV infection due to the host attempting to

revert and activate these anergic T cells. It was not, however, observed if TLR7-mediated anergy is sufficient to increase LAG-3 expression.

Unfortunately, sLAG-3 concentrations were below the limit of detection for our ELISA in the supernatants of human PBMCs following 6 hour stimulation with Io or PMA/Io. We are, therefore, unable to assess if sLAG-3 is also upregulated in the anergic environment.

4.3.2 Opportunities and Limitations

The expression of LAG-3 in response to ionomycin stimulation alone may allow for the considerable upregulation of membrane LAG-3 without strongly activating T cells. This may allow for longer stimulations than what is feasible with PMA/Io stimulation, and will permit the accurate identification and analysis of CD4+ T cells by flow cytometry. Unfortunately, the induction of anergy would change the expression patterns of cells which may make them unsuitable for some downstream analysis.

In order to study a more physiologically relevant anergic state, it is possible to induce anergy by stimulating cell through the TCR without a costimulatory signal [144]. Alternatively, a recent study has observed induction of anergy through engagement of TLR7 within CD4+ T cells. The engagement of TLR7 by intracellular viral RNA resulted in an intracellular calcium flux and use of the NFATc2 transcription factor to produce the anergic phenotype [149]. By inducing anergy through a natural mechanism, it would be possible to study LAG-3 expression at both the RNA and protein level during a normal immune response or specifically a viral infection.

LAG-3 expression following Io stimulation may be limited to the trafficking of the pre-formed intracellular store of LAG-3 to the surface and may not be sufficient to induce LAG-3 gene expression. In order to assess if LAG-3 is in fact an anergy associated protein, increased

understanding of the genetic regulation of the LAG-3 gene in an anergic environment would be necessary. Through the use of real time PCR or gene expression chips it would be feasible to measure rates of gene expression.

In order to measure sLAG-3 concentrations in response to Io or PMA/Io stimulations, it may be possible to increase the stimulation time in order to increase sLAG-3 concentration to detectible levels. Alternatively, further reduction in the volume of supernatant or increase the number of cells could be attempted in order to increase the concentration of sLAG-3 produced.

4.4 Kinetics of LAG-3 expression

Previous studies of LAG-3 focused predominantly on LAG-3 expression in a chronic activation environment and did not examine the expression at early time points. Based on our observation of an intracellular store of pre-formed LAG-3 that is rapidly trafficked to the surface following PMA/Io stimulation, we aimed to examine LAG-3 expression over 24 hours following a more physiologically relevant stimulus to better understand the kinetics of LAG-3 expression. CD3/CD28 beads were selected because activation by these beads required engagement and signalling through the TCR, which would be required for antigen presentation during a real infection, but is also broadly activating allowing for us to examine many activated cell within each donor. We hypothesized that LAG-3 expression would be increased quickly, from the intracellular store, following stimulation and would decline over time, from matrix metalloprotease mediated cleavage, before peaking at a later time point following the initiation of *de novo* protein synthesis.

4.4.1 Expression of LAG-3 and CD69

Consistent with our hypothesis, LAG-3 expression was first observed to be significantly upregulated 4 hours post stimulation (Figure 7). This early increase in LAG-3 is likely due to therapid expression from the intracellular store. Additionally, the 4 hour time point coincides with the significant upregulation of activation marker CD69 (Figure 7). Increased CD69 expression is well documented as an early marker of T cell activation [150]. While CD69 expression continues to increase over the 24 hour time course, LAG-3 expression had declined by the 6 hour time point (Figure 7). We believe this reduction is due to matrix metalloprotease mediate cleavage as well as internalization of existing membrane bound LAG-3 which is not yet replenished by *de novo* protein synthesis. By the 16 hour time point LAG-3 and CD69 expression have peaked (Figure 7). LAG-3 was not significantly upregulated at the 10 hour time point therefore *de novo* protein synthesis of LAG-3 required between 10-16 hours to begin following TCR stimulation.

Interestingly, at the 4 hour time point, LAG-3 was not preferentially expressed on the activated, CD69+, cells but was equally expressed by both the CD69+ and CD69- populations (Figure 9). This implies that early LAG-3 does not require the same signals as CD69 to be expressed, likely because LAG-3 is being trafficked to the surface from intracellular stores and CD69 is being expressed via *de novo* protein synthesis. The CD69-LAG-3+ population remained consistently low at all time points. CD69+LAG-3- cells predominated until the 16 hour time point when the CD69+LAG-3+ population was increased. By the 24 hour time point CD69+LAG-3+ was the predominant population (Figure 9). This implies that at the later time points, LAG-3 is preferentially expressed on activated T cells, which is consistent with literature reports of LAG-3 expression and T cell activation after 24 – 48 hours of stimulation.

4.4.2 LAG-3 expression kinetics of CD4+ and CD8+ T cells

LAG-3 expression kinetics were consistent between the bulk CD3+, CD4+ and CD8+ T cell subsets (Figure 7). However, we did observe an increased proportion of CD8+ T cells expressing LAG-3 compared to CD4+ T cells starting at 4 hours post stimulation and continuing for the entire 24 hour time course (Figure 8). Although CD8+ T cells do not traditionally interact with MHC class II APCs, LAG-3 may be sufficient to establish an interaction between these cell types, although direct evidence for this has not been examined. Alternatively, the study that found galectin-3 to be a LAG-3 ligand, proposed this interaction to contribute to LAG-3-mediated regulation of CD8+ T cells [80]. Galectin-3 has previously been shown to inhibit CD8+ T cell function by disrupting the interaction between the TCR and the MHC class I [151]. In the study by Kouo *et al*, they found that suppression of CD8+ T cells by galectin-3 required the presence of LAG-3 *in vitro*. The interaction between LAG-3 and galectin-3 may be possible *in vivo* as well based on the capability of LAG-3 to be extensively glycosylated [80].

Although LAG-3 is not increased during chronic HIV infection, other immune exhaustion markers, such asCD160 and 2B4, are increased on CD8+ HIV specific T cells during chronic HIV infection; this increase in expression is, however, not observed on CD4+ HIV-specific T cells [51]. This expression profile resembles what we observed in healthy donors with LAG-3: CD8+ T cells express more LAG-3 than CD4+, although, CD4+ T cells do have significantly increased expression when stimulated compared to unstimlated cells in healthy donors. The tendency for CD8+ T cells to express immune inhibitory markers may be a result of requiring tighter regulation or perhaps reach exhaustion more quickly than CD4+ T cells. A parallel comparison of T cell subsets under chronic activation conditions would need to be performed in order to assess their relative rates of exhaustion.

Conversely, a greater proportion of CD4+ than CD8+ T cells expressed CD69 at later time points (10-24 hours post stimulation) (Figure 8). Although these time points are towards the end of our time course, 24 hours is still relatively early in the course of an infection. CD4+ T cells may be required to be more active during the early portion of the immune response in order to adequately recruit and organize other immune cells. Tighter regulation of CD8+ T cells may be beneficial in order to ensure the CD8+ T cell response is appropriate to address a new infection. Additionally, the expression of LAG-3 on the surface of CD8+ T cells may contribute to the activation of APCs through interactions with the MHC class II, a function not classically associated with CD8+ T cells.

4.4.3 Expression of sLAG-3

Concentrations of sLAG-3 were significantly upregulated by 16 hours and substantially increased further by 24 hours post stimulation. We did not observe a detectible increase of sLAG-3 at 4 hours post stimulation in the supernatants to compliment the observed increase in surface LAG-3 expression (Figure 10). It is possible that the pre-formed intracellular store of LAG-3 consists predominantly or entirely of membrane LAG-3. More likely, it is possible that our ELISA’s limit of detection is not sufficient to observe the increase in sLAG-3 at 4 hours. The detectible increase of sLAG-3 expression does, however, correlate with the observed peak in LAG-3 expression 16 hours post stimulation.

In humans, it is known that sLAG-3 may be generated from splice variants [103, 104] which is consistent with our notion that LAG-3 *de novo* protein synthesis would be activated between 10-16 hour post stimulation as this is also when we see a peak in sLAG-3 expression.

4.4.4 Limitations and Opportunities

The use of CD3/CD28 beads allows us to examine T cell responses through TCR-mediated activation, however, this stimulation is considerably stronger than antigen presentation because the beads will stimulate all T cells with a functional TCR. Stimulations with peptides require that the peptide be recognized by specific memory cells and presented by APCs to T cells through MHC/TCR interactions. The use of CEF peptides was attempted however because we are limited by our local donor pool it was we were unable to find HLA matched donors for proper CEF response comparisons.

4.5 CD4+ and CD8+ T Cell Expression Profiles

Based on our observations that LAG-3 expression was significantly higher on CD8+ T cells than CD4+ T cells following stimulation of PBMCs with CD3/CD28 beads (Figure 8), we sought to examine the expression of LAG-3, sLAG-3, CD69, and several Th1 and Th2 cytokines in pure populations of CD4+ and CD8+ T cells. The expression of various cytokines by immune cells facilitates cross-talk and induction of different functions or roles. In order to assess the expression of cell subsets without the signals from other cells, we sorted bulk PBMCs into pure populations. We hypothesized that CD8+ T cells would express more LAG-3 and sLAG-3 while CD4+ T cells would express more CD69 and Th1 cytokines and Th2 cytokines would remain unchanged between subsets following stimulation with CD3/CD28 beads.

4.5.1 LAG-3 and CD69 surface expression

Consistent with our hypothesis, LAG-3 was more highly expressed by CD8+ T cells and CD69 was more highly expressed by CD4+ T cells (Figure 12). Interestingly, these differences were observed both *ex vivo* and following CD3/CD28 bead stimulation. The increased expression of

LAG-3 on CD8+ T cell *ex vivo* may contribute to the regulation of the cell-mediated versus humoral immune response balance. By expressing LAG-3 on resting CD8+ T cells, it may be possible to quickly shut down cytotoxic capabilities when the cell-mediated response is not appropriate. The lack of correlation between LAG-3 and CD69 expression implies that their expression is controlled by separate mechanisms. LAG-3 is believed to share some regulatory elements with CD4, based on their proximity, structural similarity, and the resulting belief that CD4 and LAG-3 may have resulted from gene duplication. One study reports that the LAG-3 gene has a promoter that lacks TATA and expression may be controlled by GATA, NF-κB, and/or NFAT transcription factors [74]. The CD69 gene has a TATA box promoter and putative binding sites for transcription factors NF-κB, Egr-1, and AP-1[152]. These differences in gene control may explain not only the cell-specific expression differences, but also the differences in expression kinetics.

4.5.2 sLAG-3 and cytokine production

Although the sLAG-3 production in CD8+ T cell supernatants is not significantly higher than that of CD4+ T cells we observe a convincing trend that is consistent with the hypothesis (Figure 13). With a larger samples size, it is possible this would become a significant difference. sLAG-3 has been used to activate APCs through the MHC class II. As discussed previously, sLAG-3 production in humans is a result of splice variants [103, 104] and matrix metalloprotease mediated cleavage. The observed greater production of sLAG-3 in CD8+ T cell supernatants may be a by-product of the greater surface LAG-3 on CD8+ T cells providing more targets for metalloprotease cleavage or if CD8+ T cells have higher transcription rates of the LAG-3 gene which may produce more splice variants. Likely, it is a combination of both these methods resulting in the observed increased sLAG-3 production.

Our hypothesis was not correct regarding the expression of cytokines. We expected CD4+ T cells to produce more Th1 cytokine due to the higher percentage of activated CD69+ cells, however, only TNF-α was observed to be produced in larger quantities by CD4+ T cells. MIP-1β was observed to be produced in greater quantities by CD8+ T cells and IFN-γ was produced in equal amounts by both subsets. IL-4, our only Th2 cytokine examined, was expected to be produced equally by both subsets but was found to be more highly produced by CD4+ T cells (Figure 13). Our observations are consistent with the known functions of CD4+ and CD8+ T cells; CD4+ T helper cells are responsible for coordinating the immune response through the secretion of various Th1 and Th2 cytokines [12] while CD8+ T cells are primarily involved in the Th1 response and are known to secrete levels of IFN-γ [15]. MIP-1 is a chemoattractant for multiple immune cells [153] and has been shown to be secreted by CD8+ T cells as a viral suppressor during HIV infection [154].

It is interesting to note that although not as many CD8+ T cells expressed the CD69 activation marker, the production of IFN-γ was not different between the CD8+ and CD4+ subsets (Figure 13). Unfortunately, we were unable to measure co-expression of surface markers and cytokines due to the methods of detection. In order to measure cytokine production by flow cytometry, we require the use of Golgi stop and/or Golgi plug; the use of Golgi stop and Golgi plug will interfere with surface and soluble LAG-3 expression pathways such as trafficking and release of *de novo* matrix metalloproteases proteins. In order to observe LAG-3 expression in a physiologically relevant manner, we chose to forego the use of Golgi stop and Golgi plug and measure cytokine expression in the supernatant instead. We are, therefore, unable to conclude if the majority of secreted IFN-γ was produced by the CD69 expressing cells or both CD69+ and CD69- subsets equally. We did not observe a significant correlation between CD69 expression

and IFN-γ concentration therefore it is likely that it is not solely the CD69+ cells producing IFN-γ. We did, however, observe positive correlations between sLAG-3 with IFN-γ and TNF-α in our CD8+ T cell supernatants (Figure 14). It is possible that sLAG-3 production may be a better marker for CD8+ T cell activation than CD69 expression.

No other significant correlations were observed between the markers measured on either T cell subset (Table 6). This observation is likely due to the diversity of both regulation/induction mechanisms and importance of early expression following activation. Measurements of these markers at later time points following stimulation or during chronic activation may show more correlations due to the interactions of their expression and signalling pathways.

4.5.3 Limitations and Opportunities

By sorting CD4+ and CD8+ T cells into pure populations we were able to assess the expression of various markers and cytokines without the cross-talk between cells. Although this approach is not physiologically relevant, it allows us to understand in detail the contributions of a single cell type to the overall immune response. In our study, we observed that expression of LAG-3 is expressed by a greater proportion of CD8+ T cells than CD4+ T cells and CD69 follows an opposite pattern. Based on these observation,s we hypothesized that CD8+ T cells would have a more suppressed phenotype than CD4+ T cells. Although we did not observe this to be the case, our experiments were limited by the purity of the population. The best studied ligand for LAG-3 is MHC class II which would not have been present within our cell cultures due to the removal of APCs through sorting. The lack of the MHC class II as a ligand may have prevented the suppressive phenotype from being induced by the LAG-3 found on the surface of these CD8+ T cells. However, this provides the opportunity to follow this protocol but add another

experimental condition where exogenous MHC class II is added in order to induce LAG-3 function and observe the reduction in cytokine production.

Another limitation of our experiment was the inability to examine co-expression of surface markers (LAG-3 and CD69) with cytokine production. Unfortunately, because of the required use of Golgi stop and Golgi plug in order to preform intracellular staining for cytokine production, we would be unable to observe CD69 surface expression. Additionally, we have previously observed that Golgi stop and Golgi plug can alter LAG-3 expression either through interactions with matrix metalloproteases or with newly synthesized protein.

4.6 General Discussion – Major Findings

This thesis focused on understanding early regulations mechanisms and kinetics of LAG-3 expression. The majority of current knowledge on LAG-3 expression has been generated in murine studies which do not accurately reflect the human immune system. The results of this thesis depict early expression of LAG-3 following stimulation under the control of various mechanisms. Consistent with murine studies [87], we found that pre-formed stored of LAG-3 are present within the cell facilitating rapid expression and that surface LAG-3 is cleaved by matrix metalloproteases. We also observed that LAG-3 was expressed in an anergic environment created by ionomycin stimulation. We found that early LAG-3 expression coincides with expression of activation marker CD69 but is not stably expressed until later, 16 hours post stimulation. Finally, we showed that a greater proportion of CD8+ T cells express LAG-3 than CD4+ T cells.

The function of LAG-3 is complex and contradictory. Membrane bound LAG-3 is associated with inhibition and exhaustion through the disruption of calcium flux mediated activation [86],

conversely, sLAG-3 has been shown to act as a ligand for MHC class II to induce cytokine production and cellular maturation [104-107] and has also been used in cancer therapy [110, 123, 124] and as an adjuvant [111]. Additionally, LAG-3 expression is not increased during several chronic viral infections [120, 121], including HIV [51, 112, 113], even though exhaustion markers are typically expressed in this setting [56, 63]. By expanding our understanding of LAG-3 regulation we are able to better understand the balance of immune activation and inhibition. The majority of exhaustion markers, including PD-1, have situationally been described as markers of cellular activation [155-159]. Although we know that LAG-3 has functions that contribute to the inhibition of T cell activity, it is possible that the impact of early LAG-3 alone on inhibition is negligible compared to the many activating signals generated by the immune response. Early LAG-3 expression may play a more significant role in maintaining cell populations [132] and APC activation [104-106].

4.6.1 Future Directions

This thesis has examined and defined early expression kinetics on LAG-3 on bulk T cells as well as the large CD4+ and CD8+ T cell subsets. Further investigation of LAG-3 expression on other cell types could be warranted given that other immune inhibitory markers, such as TIM-3, have been shown to elicit different functions of different cell types. Additionally, LAG-3 is known to play a large role on Tregs in murine studies but the impact and importance of LAG-3 on human Tregs is not as well studied.

An additional avenue that requires further studies is the LAG-3 signal transduction pathway. LAG-3 associated proteins are not well studied and any insight into their targets or mechanism of action would greatly benefit our understanding of LAG-3. The contribution of LAG-3 to

inhibition or eventually exhaustion is not well characterized either. In order to assess the magnitude of LAG-3 mediated cellular inhibition, it would be feasible to study changes in cytokine production, proliferation, calcium flux, and TCR signalling on LAG-3+ cells in the presence of MHC class II to act as a ligand.

Since LAG-3 has been observed to be more highly expressed by CD8+ T cells than CD4+ T cells, it would be interesting to examine the primary LAG-3 ligand on these cells. As recent studies have found that LSECtin [79] and galectin-3 [80] , in addition to MHC class II, act as ligands for LAG-3, it is possible that a non-MHC class II protein is the primary ligand of CD8+LAG-3+ T cells as CD8+ T cells do not classically interact with MHC class II APCs. It would be interesting to observe if the presence of LAG-3 on the surface of CD8+ T cells was sufficient to facilitate and interaction between CD8+ T cells and MHC class II APCs.

sLAG-3 as a ligand has been studied for potential use as an adjuvant based on the immune activating signal through MHC class II, however, there is no current knowledge of the balance of signals generated when surface LAG-3 is engaged by MHC class II. The balance of the diverging signals resulting from the LAG-3-MHC class II interaction may ascribe to the importance of rapid LAG-3 expression following stimulation. It is also possible that the balance of these signals depends on the cellular or immune environment, or other undescribed co-stimulatory signals.

4.7 Hypothesis and Conclusion

The hypothesis of this thesis was that soluble and membrane LAG-3 will be rapidly expressed from an intracellular store following stimulation. Membrane LAG-3 will decline over time due to metalloprotease cleavage, which will concurrently increase soluble LAG-3.

Consistent with our hypothesis, early LAG-3 was found to be normally expressed despite inhibiting *de novo* protein synthesis. Additionally, surface expression of LAG-3 was observed to be higher when matrix metalloprotease cleavage was inhibited. Unfortunately, we were unable to measure the contribution of matrix metalloprotease cleavage to sLAG-3 due to the limit of detection of our ELISA.

We further observed the rapid expression of LAG-3 in a time course which followed our hypothesized expression pattern of rapid expression followed by a decline in surface expression. From this time course we also observed that a greater proportion of CD8+ T cells express LAG-3 than CD4+ T cells and that sLAG-3 may act as an activation marker of CD8+ T cells.

4.7.1 Significance

This thesis sought to expand the understanding of surface and soluble LAG-3 protein expression. Through understanding of LAG-3 expression kinetics we are able to better understand how LAG-3 interacts with the immune response. Human LAG-3 was found to use similar regulatory methods as mice, including presence of a pre-formed store to facilitate rapid expression and the use of matrix metalloproteases to cleave surface LAG-3 into sLAG-3. Based on the observations outlined in this thesis, the immune system places an importance on expressing surface LAG-3 rapidly following activation. Early expression of an immune inhibitory marker is unexpected and raises questions about when the inhibitory capacity of LAG-3 is most important; early expression may help to regulate strength of response and prevent robust activation and apoptosis. This work will hopefully inspire future research into the early applications of immune inhibitory markers in assisting with proper immune control.

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