

2020

Immune checkpoint expression in SIV-infected rhesus macaques treated with TLR7 agonists

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BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

**IMMUNE CHECKPOINT EXPRESSION IN SIV-INFECTED RHESUS
MACAQUES TREATED WITH TLR7 AGONISTS**

by

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B.S., Loyola University Chicago, 2017

Submitted in partial fulfillment of the
requirements for the degree of
Master of Science

2020

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ABSTRACT

While the human immunodeficiency virus (HIV) can be managed with antiretroviral therapy (ART), there is no cure for the disorder. If ART is discontinued, viral RNA levels rapidly increase in most individuals due to the presence of a cell-mediated hidden replication competent viral burden known as the viral reservoir. In order to successfully cure this disease, a mechanism to eliminate the viral reservoir must be developed. Preliminary research completed using a toll-like-receptor agonist 7 (TLR7) has shown favorable results supporting this goal. In a simian immunodeficiency virus (SIV) model, dosing rhesus macaques (RMs) with TLR7 agonists resulted in the development of controlled viremia. A controlled RM is a SIV positive animal that is able to maintain an undetectable viral load without continued therapeutic intervention. In cases of controlled SIV/HIV, viral RNA no longer replicates despite the discontinuation of all treatment. This implies that the viral reservoir is either completely eliminated or severely reduced.

In this study, we quantified expression levels of several immune checkpoint and activation markers including CD69, CD39, CXCR5, TCF7, PD-1, PD-L1, TIGIT, CTLA-4, Tim-3, and Lag-3 on isolated peripheral blood mononuclear immune cells (PBMCs) [including CD4+ T cells, CD8+ T cells, natural killer (NK) cells, and B cells] in both controlled and non-controlled RMs. Our goal was to identify possible mechanisms by which controlled RMs are able to successfully modulate the host immune response after

discontinuing TLR7 agonist treatment. The subjects each received one of two different TLR7 agonists (GS-9620 and GS-986). Isolated peripheral blood mononuclear cells (PBMCs) were obtained from two controlled RMs and two non-controlled RMs. Samples were analyzed using flow cytometry to identify and quantify levels of markers above.

Expression levels of PD-1 and PD-L1 were elevated in PBMCs obtained from non-controlled RMs when compared to levels seen in controlled RMs. In contrast, levels of TIGIT and CTLA4 were downregulated in samples obtained from the controlled RMs. This suggests that immune checkpoint markers responsible for viral control and SIV/HIV pathogenesis have different functional roles. Additionally, the controlled RMs showed high expression of CD69 and CD39 on B cells and increased levels of CXCR5 on CD4+ T cells. This suggests that newly activated B cells likely contribute to the observed improvements in immune function.

The results obtained provide favorable support for the potential role of immune checkpoint blockade as an HIV-specific immunotherapy that may contribute to the development of a controlled population. However, it is worthwhile to note that this study was completed using a relatively small sample size (n=4). Thus, interpretations of the findings herein must be replicated with a larger sample prior to forming any definitive conclusions.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen Presenting Cell
ART	Antiretroviral Therapy
CD28	Cluster of Differentiation 28
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte Associated Protein 4
CXCR5	C-X-C Motif Chemokine Receptor 5
gp120	Glycoprotein 120
HIV	Human Immunodeficiency Virus
Lag-3	Lymphocyte Activation Gene-3
MHC	Major Histocompatibility Complex
NFAT	Nuclear Factor of Activated T Cells
NK	Natural Killer
NRTI	Nucleoside Reverse Transcriptase Inhibitors
PBMC	Peripheral Blood Mononuclear Cells
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Death Ligand 1
PHA	Phytohaemagglutinin
PRR	Pathogen Recognition Receptors
RM	Rhesus Macaques
RT	Room Temperature

SIV	Simian Immunodeficiency Virus
TCF7	T Cell Factor 7
T _{CM}	T Cell Central Memory
T _{EM}	T Cell Effector Memory
TCR.....	T Cell Receptors
Tfh.....	T Follicular Helper Cells
TIGIT	T Cell Immunoreceptor with Immunoglobulin and ITIM domains
TIM-3.....	T Cell Immunoglobulin and Mucin-domain Containing-3
TLR.....	Toll-like Receptor
TLR7	Toll-like Receptor Agonist 7
Treg.....	T Regulatory Cells

INTRODUCTION

Human immunodeficiency virus (HIV)

Using electron microscopy, scientists were first able to identify the human immunodeficiency virus (HIV) in 1983, though it was not officially named until 1986 (Greene et al., 1986). This 9-kb RNA virus would go on to impact international conversation in an immeasurable way as scientists and members of the public studied this new disease (Greene et al., 1996). The 25 million deaths worldwide attributed to HIV are secondary to reduced immune function and through associated comorbid conditions including HIV-associated neurocognitive disorders and cancers (Sharp et al., 2011; Beck et al., 2018; Dandapani et al., 2010). While therapeutic research has dramatically increased and improved the lifespan of HIV+ individuals, a curative treatment does not yet exist (Evans et al., 2013). Much of the research conducted today is done on a simian immunodeficiency virus (SIV) nonhuman primate model (Evans et al., 2013).

SIV Model

Preliminary research completed using an SIV model for HIV allowed scientists to better understand events that contributed to viral transmission and disease pathogenesis (Evans et al., 2014). Years of research resulted in the development of a well-controlled, highly characterized, and easily reproducible non-human primate model for HIV (Beck et al., 2018). Interestingly, though African monkeys are the natural host of SIV, they are typically non-pathogenic (reviewed in Evans et al., 2014). As such, researchers today frequently utilize SIV-infected Asian macaques in their work. Macaques share many

physiologic and immunologic characteristics with humans. Both SIV and HIV are chronic progressive infections. Kinetically, both disorders can be characterized by an acute peak of viremia followed by decline. Furthermore, both non-human primates and humans develop a viral reservoir shortly after acute infection. The viral reservoir is a pool of latent infected cells and is frequently studied. Using a non-human primate model allows researchers to study the molecular characteristics of the reservoir and to test the efficacy of aggressive reservoir-targeted therapeutic treatments while tightly regulating a number of experimental parameters (dose frequency, duration, route).

HIV Viral Life Cycle

The HIV virus will first enter a cell when a cell-surface envelope glycoprotein binds to a receptor (CCR5 or CXCR4) (reviewed in Goodsell et al., 2015). The resulting conformational change allows the virus to fuse through the targeted cell's membrane. Inside the cell, the viral RNA will be transcribed into DNA upon activation of a reverse transcription complex. Through integration, the newly replicated viral DNA inserts itself into the host cell's DNA. The viral particle will assemble and bud from the cell and enter the bloodstream.

Stages of Infection

It has been well established that there are three stages of HIV infection. After initial infection, the virus will predominantly target and infect CD4⁺ T cells. A patient will likely be symptomatic within one to six weeks, presenting with signs of fever, fatigue, and weight

loss as their viral load rapidly increases (reviewed in Aavani et al., 2019). The concentration of CD4⁺ T cells will decline while CD8⁺ T cells and antibody levels increase and attempt to fight the viral infection. This acute stage of infection will last for approximately 14 days. For the next 6 to 15 years, a patient will remain in an “asymptomatic stage.” During this stage, there is a slower decline in CD4⁺ T cells. However, the HIV particle is still actively targeting lymphoid tissue which results in rapid lymphocytic degradation and regeneration. A patient’s viral load, or measured number of viral particles found in plasma, is approximately 10⁴ copies per milliliter of plasma during the asymptomatic stage. In the third and final stage of infection, a patient has progressed to acquired immunodeficiency disorder syndrome (AIDS). All immune responses rapidly decline, including antibody production, CD8⁺ T cell function, and CD4⁺ T cell concentrations. At this stage, additional comorbid conditions will likely develop and consequently, death is imminent as the immune system struggles to successfully target invading pathogens.

Current Treatment Options

Preventing Viral Replication (ART)

Current treatment options include antiretroviral therapy (ART), a combination therapy that can result in the reduction of viral RNA to an undetectable level while also improving immune system function by increasing CD4⁺ T cell count (reviewed in Pau et al., 2014). ART functions by stopping viral replication during one of the six stages of the HIV life cycle. The effects of different agents that are commonly found in a therapeutic

cocktail are detailed in **Table 1**. Though ART has successfully prolonged survival, it is not a curative treatment.

Therapeutically Targeting the Viral Reservoir

A viral reservoir consists of a pool of HIV-1 viral particles that reside in CD4⁺ T-cells and likely forms during peak viremia (Liu et al., 2020; Thompson et al., 2017). Currently, there is a limited understanding of the expression level of surface proteins on reservoir cells, though this is an important area of study that can potentially lead to a curative treatment for HIV (Liu et al., 2020). This reservoir of cells often forms in well-protected lymphoid tissues which create an additional physical and molecular barrier against therapeutic agents (Cory et al., 2019). In addition, the reservoir has a half-life of approximately 44 months, requiring over 70 years total to decay to zero without therapeutic intervention (Liu et al., 2020).

Since these latent cells are not effectively treated by ART, the viral reservoir maintains a persistent repository of viral particles (Aawani et al., 2019; Pau et al., 2014). These latent cells can reactivate at any time. If ART is discontinued or temporarily paused, an otherwise undetectable patient will likely start presenting with detectable plasma levels of HIV due to clonal expansion of HIV-1 infected cells in the viral reservoir (Pau et al., 2014). Many factors can contribute to the discontinuation of ART therapy, including forgetting to take medications, travel, psychological disturbances, as well as limited access to healthcare (Shubber et al., 2016). Thus, studying reservoir targeted eradication therapies

is critical in developing a curative treatment for HIV. Identifying markers that are present on cells in the viral reservoir is an important step in this process.

Currently, the viral reservoir is not being treated by any publicly available therapeutic treatments. In addition to the therapies preventing viral replication (top of **Table 1**), there are three primary experimental categories of therapeutic treatments to target the viral reservoir (bottom of **Table 1**). The first method is informally referred to as “kick and kill” (reviewed in Cory et al., 2019). Cells in the reservoir are re-activated (“kicked”) and the newly activated virus is subsequently destroyed by the immune system (“killed”). Histone deacetylase inhibitors (HDACs) are frequently studied as a potential therapeutic agent that can support the “kick and kill” model. This model can lead to a sterilizing cure for HIV, which implies that all detectable viral particles are eliminated. A second strategy that can lead to a functional cure for HIV is to prevent reservoir cells from reactivating, which effectively eliminates any potential functionality of the hidden HIV particles without the use of ART. At present, Tat inhibitors are being evaluated to validate this model. Finally, developing mechanisms to increase the concentration of ART in well protected lymphoid tissue can result in an overall decrease in latent HIV concentrations. Presently, P-gp inhibitors are being studied to support this theory. Though the discussed options are limited, they provide a promising start that can lead to a more definitive cure for this disease.

Table 1: Molecular agents commonly used or studied in HIV therapeutic development

HIV Life Cycle	Drug Class	Function
Preventing Viral Replication		
Replication	Nucleoside Reverse Transcriptase Inhibitors (NRTI)	Phosphorylated NRTIs block the conversion of viral RNA into double stranded DNA by incorporating into the nucleotide analog and blocking the function of reverse transcriptase (Pau, 2014)
	Non-nucleoside Reverse Transcriptase Inhibitors (NNRTI)	Inhibits the function of reverse transcriptase by changing the structural conformation of the enzyme (Pau, 2014)
	Protease Inhibitors (PI)	Halts the function of HIV proteases which prevents the formation of mature virions (Pau, 2014)
Integration	Integrase Strand Transfer Inhibitors (INSTI)	Prevents the formation of covalent bonds between host and viral DNA by blocking the integrase enzyme (Pau, 2014)
Cell entry	CCR5 Antagonist	Binds to CCR5, a cell membrane receptor, which effectively prevents HIV from invading a CD4 T cell (Pau, 2014)
	Fusion Inhibitor	Binds to gp41 (glycoprotein) which prevents the fusion of the viral and cellular membrane (Pau, 2014)
Targeting the Viral Reservoir		
Resting state	Histone Deacetylase Inhibitors (HDAC)	Reactivate latent CD4+ T cells and increase HIV RNA concentrations allowing the virus to be targeted by the immune system (Archin, 2017 and Cory, 2019)
	TAT Inhibitors	Inhibits the function of HIV transcription factors in latent CD4+ T cells (Cory, 2019)
	P-gp Inhibitors	Inhibits the function of P-glycoprotein, a drug efflux transporter, effectively allowing for ART agents to enter lymphoid tissues (Robillard, 2013 and Thompson, 2017)

Immune Response

Innate Immunity

Host-genome encoded innate immune mechanisms are non-specific and include physical barriers, proteins, regulatory cytokines, chemokines, and other enzymes (Chaplin et al., 2006). The innate immune response to viral infection is triggered by binding between pathogen recognition receptors (PRRs) and pathogen associated molecular patterns (PAMPs) (Chaplin et al., 2006; Altfeld et al., 2015; Mogensen et al., 2010). Toll-like receptors (TLRs) are a family of PRRs that are essential in the human innate immune response (as reviewed in Morgenson et al., 2010). The ten identified TLRs that are membrane-bound receptors expressed on innate immune cells and function in recognizing broad categories of pathogenic material, including glycoproteins, lipoproteins, DNA, and RNA.

This paper studied molecular changes in rhesus macaques (RM) after administration of a TLR7 agonists. TLR7 can be found in endosomal compartments of innate immune cells and recognize nucleic acids. Specifically, single-stranded viral RNA serves as a ligand for TLR7 and binding results in activation of the innate immune response (Diebold et al., 2004). HIV-1 encodes for several TLR7 activating ligands (Chang et al., 2009). Once a ligand binds to the TLR-7 receptor, two transcription factors (nuclear factor kappa light chain enhancer of activated B cells and interferon regulatory factor 7) translocate to the nucleus and stimulate the production of cytokines, including interferon alpha (Bam et al., 2016). After the cytokines bind to cytokine-specific receptors, the

production of a number of different antiviral proteins begin. Bam et al. (2016) found that GS-9620, an oral TLR-7 analog, induces the production of antiviral proteins, specifically, interferon alpha, that effectively stops HIV replication in PBMC at or prior to the reverse transcription step.

A study conducted by Lim et al. (2018) on a non-human primate model demonstrated that administration of a TLR7 agonist can potentially serve as a “kick and kill” therapeutic agent. Treated subjects developed transient viral blips and had measurable increases in interferon-activated gene production. The increased immune response combined with induced transient viremia contributed to noticeable reductions in the viral reservoir of treated RMs. Thus, it is hypothesized that the TLR7 agonists used in this study may have a functional role in eliminating the viral reservoir. Additionally, the HIV set point, or the measured viral load after infection, decreased in RMs that were treated with TLR7 agonists. Two subjects were aviremic for >24 months after treatment discontinuation.

Adaptive Immunity

In contrast to the innate immune system, the adaptive immune system specifically targets invading antigens (reviewed in Chaplin et al., 2006; Molnar et al., 2012). Millions of different antigen receptors, known as T-cell receptors (TCRs), can be created using germline-encoded genes and are found on T-cells, a broad class of immune cells. The TCRs will recognize a class I or class II major histocompatibility complex (MHC) molecule. Class I MHC molecules are found on cell surfaces and express fragments of proteins that

are internalized in the cell. Class I molecules will bind to CD8⁺ T cells. Class II molecules are found on antigen-presenting cells (including but not limited to dendritic cells and macrophages) and express fragments of extracellular proteins largely obtained from a pathogenic source. Class II molecules will bind to CD4⁺ T cells.

When activated by MHC II binding, CD4⁺ T cells will become T helper cells which then stimulate B cells and cytotoxic T cells (CTLs) (reviewed in Molnar et al., 2012; Martin et al., 2018). B cells are white blood cells that will produce antibodies when activated. CTLs release cytokines that eventually lead to cellular apoptosis. When a CD8⁺ T cell binds to MHC I, it becomes a CTL and will induce cellular apoptosis. Naïve CD4⁺ and CD8⁺ T cells can further differentiate into memory T cells. Memory T cells have previously encountered an antigen and are able to mount an immediate immune response through rapid cell proliferation. There are two classes of memory cells: central memory (T_{CM}) and effector memory (T_{EM}). The classes differ in the cell surface markers they express. Central memory cells tend to possess surface markers that localize to secondary lymphoid organs (i.e. lymph nodes) and consequently, are thought to primarily function in fighting systemic infections. On the other hand, effector memory cells possess a large number of chemokine receptors that resultantly lead them to sites of inflammation. These cells are generally found in peripheral tissues and are more cytotoxic in nature than central memory cells.

A number of viruses have developed mechanisms to downregulate MHC I expression and thus, hamper the adaptive immune response (reviewed in Kerkau et al., 1997). Though HIV-1 infection induces rapid proliferation of CD8⁺ T cells, infected cells are able to evade CD8⁺ T cell mediated destruction. HIV-1 encodes for a viral protein U gene which normally functions in facilitating the release of a HIV particle from cells. Kerkau et al. (1997) found that this gene additionally interferes in an early step of class I MHC molecular formation which results in a reduction of MHC I molecules on infected cells. Without MHC I, CD8⁺ T cells are unable to recognize HIV-infected cells and can no longer maintain normal destructive function.

Interestingly, the innate immune system utilizes the same degradative effects to recognize and destroy infected cells (as reviewed in Molnar et al., 2012). Natural killer (NK) cells innate immune cells that induce apoptosis by releasing cytolytic granules. If an NK cell comes across a “healthy” cell with an MHC I molecule, it will ignore it and move on. However, if it comes across a cell that does not possess an MHC I molecule, it will operate under the assumption that the cell is infected and self-degraded its MHC I molecule. The NK cell will subsequently release function in destroying this cell.

HIV actively targets and infects any cells that contain a CD4 receptor, including CD4⁺ T cells, macrophages, and monocytes (as reviewed in Aavani et al., 2019). However, the viral particle will predominantly target CD4⁺ T cells. As described above, CD4⁺ T cells have a critical role in the adaptive immune response as they are able to stimulate B

cells and CTLs. When CD4⁺ T cells are infected by the viral particle, they are no longer able to function as immune cells and instead primarily function in replicating the viral particle. However, if a CD4⁺ T cell is not infected by the virus, it attempts to maintain normal function and fight off the pathogenic infection. Aavani et al. (2019) developed a mathematical model of this conflicting dynamic and identified a gradually diminishing cyclical pattern. Because CTLs are stimulated by CD4⁺ T cells and viral load, they respond by destroying CD4⁺ T cells. Without CD4⁺ T cells to induce the production of CTLs, the overall concentration of these molecules decrease. In summary, the adaptive immune response is significantly limited in cases of HIV infection as both CD8⁺ T cell and CD4⁺ T cells are not able to maintain normal functioning.

CD69

Effector cells are plasma cells (which are derived from fully differentiated B cells) and CTLs (reviewed in Molnar et al., 2012; Janeway et al., 2005). Memory cells are able to rapidly differentiate into effector cells upon re-exposure to a pathogen. Additionally, upon activation, some naïve T cells will form into effector cells. The expression levels of many cell surface molecules will change after naïve or memory cells transition into effector cells. CD69 serves as an early activation marker and is one of the first cell surface markers displayed by CTLs and plasma cells. In the innate immune system, CD69 also serves as an activation marker and is present on activated NK cells. This marker is exclusively seen on effector cells and will not be expressed on naïve or memory cells. The CD69 marker consists of a 24-kDa polypeptide with N-linked oligosaccharides and is a phosphorylated disulfide linked homodimer (Krowka et al, 1995). Using flow cytometry and CD69

antibodies, researchers are able to easily identify populations of activated CTLs, B cells, and NK cells.

T cell Exhaustion

Chronic viral infection results in the development of an exhausted lymphocytic state (Gupta et al., 2015). Typically, some effector T cells will form into memory T cells. However, in cases of T cell exhaustion, effector cells are unable to form into memory cells and instead lose functionality and proliferative capabilities (reviewed in Khaitan et al., 2011). Additionally, the effector cells that are present are no longer fully responsive to stimuli. Exhausted CD8⁺ T cells can be further divided into two classes which are categorized by the surface transcription factors they express (reviewed in Gupta et al., 2015). CD8⁺ T cells that express the transcription factor T-bet are progenitor cells. Progenitor cells have not reached a state of terminal exhaustion and that can still proliferate into functioning CTLs, though the proliferative potential and effector functions may be markedly reduced than what can be observed in healthy individuals. On the other hand, cells that express eomesodermin (eomes) in high concentrations have likely reached a stage of terminal exhaustion and cannot regain normal functionality. Both T-bet⁺ CD8⁺ T cells and Eomes⁺ CD8⁺ T cells express unique concentrations and compositions of inhibitory receptors, known as immune checkpoint molecules, on their surface.

Immune Checkpoint Molecules

Immune checkpoint molecules are inhibitory receptors that are located on T cells. Immune checkpoint blockade therapies were first studied while evaluating mechanisms that induce T cell activation and have been found to have an important role in regulating the immune response (Wei et al., 2018). Exhausted T cells can be characterized by the expression of a number of inhibitory immune checkpoint molecules including PD-1, Lag3, Tim3, and CTLA4 on T cells (Khaitan et al., 2011). In cases of infection, blocking inhibitory immune checkpoint markers can potentially improve immune response as immune checkpoint mediated inhibitory signals directed at T-cells are no longer functional (Wei et al., 2018). Thus these markers can be used as a targeted immunotherapy in cases of chronic viral infection.

Many forms of cancerous cells utilize immune checkpoint molecules to weaken the immune response (reviewed in Wykes et al., 2018). Currently, antibodies that directly target immune checkpoint markers expressed on exhausted cells are being used as a therapeutic treatment for cancer, with the hope of improving the immune response. In theory, blocking these markers can result in reversal of T cell exhaustion in chronic HIV infection. It is important to note that experimental use of immune checkpoint inhibitors have occasionally been linked to retinal toxicity (in macaques), and autoimmune toxicities, including colitis, myocarditis, and pneumonitis. In addition, the use of immune checkpoint inhibitors is a costly endeavor. As such, before implementing the use of immune checkpoint inhibitors in HIV, additional studies need to be completed to identify specific

marker expression at various stages in the disease process. The next sections herein evaluate the structure and function of immune checkpoint molecules that are theorized to contribute to the HIV/SIV immune response, with special emphasis placed on T cell exhaustion. Immune checkpoint molecule expression levels in an SIV model are thought to mimic the expression levels seen in HIV+ samples. Thus, we will evaluate expression levels of each of the immune checkpoint markers discussed in SIV-infected rhesus macaques.

PD-1

PD-1 (programmed cell death protein 1) is a member of the cluster of differentiation 28 (CD28) family (Akinleye et al., 2019). It is present on activated T cells and B cells and is encoded by the PDCD1 gene (Zheltkova et al., 2019; Akinleye et al., 2019). (reviewed in Akinleye et al., 2019). Tasuku Honjo discovered this immune checkpoint molecule in 1992 while studying apoptotic immune cells (reviewed in Akinleye et al., 2019). He was able to successfully clone this 288-amino acid receptor from a murine model. In 1998, further studies were completed in a lupus-like murine model and it was found that PD-1 negatively regulates immune responses (Okazaki et al., 2007). When a ligand (PD-L1 or PD-L2) binds to this receptor, the proliferative and effector functions of T cells decrease (Bekerman et al., 2019). Most PD-1 research has focused on its role in cancer immunology, as it was found that tumors frequently evade T-cell mediated responses through PD-1 signaling (Akinleye et al., 2019).

In models of untreated HIV, PD-1 was found to be upregulated in on central memory and regulatory CD4⁺ and CD8⁺ T cells (reviewed in Wykes et al., 2018). Though levels of PD-1 decrease after ART initiation, the overall expression level of this immune checkpoint remains elevated when compared to a HIV- individual. Time of initiation of ART does not seem to affect expression levels in anyway. Clinically, the elevated expression of PD-1 after initiation of ART has been linked to oxidized low-density lipoproteins which may increase a patients' risk of stroke or vascular damage. At a molecular level, elevated levels of PD-1 are associated with shorter time to viral rebound after ART discontinuation.

Previous research completed using an SIV model has shown that blocking PD-1 resulted in an increased number of CD8⁺ T cells and improved functioning of these cells (Zheltkova et al., 2019; Wykes et al., 2018 Gupta et al., 2015). An expansion in CD8⁺ T cells is directly linked to reduction in the SIV RNA and prolonged survival (Zheltkova et al., 2019; Bekerman et al., 2019; Wykes et al., 2018). There were no adverse effects observed when administering anti-PD-1 therapy in an SIV model if the subject was on ART (Wykes et al., 2018). However, it is important to note that using PD-1 as a diagnostic or therapeutic marker is challenging as it has been found to be normally expressed on a number of a number of different cells, including up to 60% of a healthy individual's memory CD8⁺ T cell population (Gupta et al., 2015). In this thesis, we will quantify expression levels of PD-1 in TLR-7 treated SIV⁺ rhesus macaques. Based on the concepts

described above, we know that overexpression of PD-1 implies decreased immune function, specifically in CD8⁺ T cells.

PD-L1

Freeman et al. (2000) reported that programmed death ligand 1 (PD-L1) is expressed by antigen presenting cells and is a ligand for PD-1. Expression levels were predominantly seen on interferon γ stimulated human peripheral blood monocytes, human dendritic cells, murine dendritic cells, heart, and lung tissues. Freeman found that exposure to cytokines induced the upregulation of PD-L1 and B7-1 protein. B7-1 is a stimulatory protein that activates T cell function. Its counterpart, B7-2 is, is constitutively on monocytes but can be induced in other APCs (including B cells, dendritic cells, and macrophages). The balance between PD-L1 and B7-1/2 directly control the level of T cell activation. Like PD-1, research with PD-L1 has primarily focused on cancer immunotherapy (Akinleye et al., 2019).

In an ex-vivo model developed using HIV⁺ blood donors, using anti-PD-L1 resulted in increased proliferation of CD4⁺ and CD8⁺ T lymphocytes (Zheltkova et al., 2019). Since CD4⁺ T lymphocytes are targeted and killed by the HIV virus, increasing the functional concentration of CD4⁺ cells can result in viral expansion. Therefore, it is assumed that the viral load is reduced only if the number of activated CD4⁺ T cells remain below a specific threshold. In addition, while B-cells uncommonly express the PD-1 receptor, they too benefit from anti-PD-L1 (Akinleye et al., 2019). Reduced PD-L1/PD-1 binding effectively reduces PD-1 mediated exhaustion in this cell population

(Zheltkova et al., 2019). B cell exhaustion presents in a very similar manner as T cell exhaustion, with exhausted B cells exhibiting decreased ability to proliferate in response to stimulation (Fauci et al., 2014). The antibodies produced by the activated B cells can help neutralize the increased viral spread caused by the greater concentration of functional CD4⁺ T cells. We will quantify PD-L1 expression in a TLR7 treated rhesus macaque model. After reviewing the factors addressed above, our assumption is that monkeys that exhibit better viral control will express lower levels of PD-L1.

CXCR-5

Chemokines are a family of cytokines that primarily function in recruiting leukocytes and effector cells to inflammatory or infectious sites (reviewed in Janeway et al., 2005). They have a chemoattractive function in both the innate and adaptive immune system. Chemokine receptors are integral membrane proteins with seven integrated helices that signal through a G-protein coupled mechanism. Structurally, chemokines that bind to a CXCR receptor contain two cysteine residues separated by amino acid and are expressed on a range of different cell types.

The chemokine receptor, CXCR5, has been found to be expressed in elevated concentrations on T-bet⁺ CD8⁺ T cells (He et al., 2016). Thus, presence of this marker may be indicative of the presence of T-bet⁺ T cells. He et al. (2016) demonstrated that T-bet⁺ CD8⁺ cells that express CXCR5 showed greater therapeutic potential than CXCR5-negative cells. A number of factors may contribute to this observation. First, He et al. demonstrated that this subset of cells was able to migrate into B cell follicles. While CD8⁺

T cells are not frequently observed in lymphoid follicles, Valentine et al. (2019) observed that CXCR5⁺ CD8⁺ T cells maintain function similarly to CD4⁺ T cells in antibody-mediated response to infection. Second, when He et al. administered anti-PDL1, a noticeable reduction in viral load was observed. Preliminary research completed using T-bet⁺ CD8⁺ T cells has shown that this subpopulation is responsive to anti PD-L1 therapy (Gupta et al., 2015). Finally, assuming the marker is indicative of T-bet⁺ CD8⁺ T cells, the enhanced immune function may be secondary to a decrease in the number of T cells that have reached a stage of terminal exhaustion. In summary, quantifying expression of CXCR5 in cases of chronic infection can provide useful insight in observed immune function as well information on the amount of T-bet⁺ CD8⁺ T cells present. We expect CXCR5⁺ CD8⁺ cells to be increased in animals that demonstrate improved viral control.

CD39

Gupta et al. (2015) found CD39 is a marker of terminal, irreversible CD8⁺ T cell exhaustion in HIV. CD39 is an acidic glycoprotein which contains 24kDa of N-linked oligosaccharides (Kansas et al., 1991). Co-expression with PD-1 on CD8⁺ T cells, is a sign of T cell exhaustion and expression levels of the two markers have been found to be proportional to the viral load (reviewed in Gupta et al., 2015). I We will quantify the expression of both PD-1 and CD39 in order to determine the amount of exhausted CD8⁺ T cells in SIV⁺ rhesus macaques. Our assumption is that macaques that demonstrate better viral control will have lower expression of CD39, which would suggest fewer exhausted CD8⁺ T cells.

Tim-3

T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) is a glycoprotein that is frequently co-expressed with PD-1 on CD8⁺ T cells (Larsson et al., 2013; Jones et al., 2008). In an HIV model, Tim-3 levels were found to be up-regulated on CD8⁺ T cells which corresponded with increased viral loads and decreased concentrations of CD4⁺ T cells (Jones et al., 2008). In addition, the CD8⁺ Tim-3 T cells demonstrated decreased functionality with reduced cytokine production and proliferation. When anti-Tim-3 was administered, the reversal of these effects was seen. This suggests that Tim-3 serves as an immune checkpoint molecule that is present on exhausted CD8⁺ T cells. We will quantify Tim-3 expression in SIV⁺ rhesus macaques, with the expectation that macaques that demonstrate better viral control will have lower levels of Tim-3.

CTLA-4

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is an immunoglobulin protein that is expressed on activated T cells along with CD28, a costimulatory protein (reviewed in Larsson et al., 2013). CTLA-4 is overexpressed on CD4⁺ T cells in cases of HIV infection (reviewed in Kaufmann et al., 2007). Levels of CTLA-4 expression indirectly correlated with CD4⁺ T cell function and directly correlates with disease progression. An expected overall reduction in cytokine production was observed in subjects that overexpressed CTLA-4 on CD4⁺ T cells. This observation is expected because CD4⁺ T cells function in stimulating cytokine production by activating B cells and CTLs. Mechanistically, CTLA-4 functions by competing with CD28 to bind to B7-1 and B7-2 (Kaufmann et al., 2007; Graydon et al., 2019). Kaufmann et al., (2007) identified

increased CD4⁺ T cell proliferation and cytokine production when CTLA-4 was blocked. Currently, ipilimumab is an FDA-approved drug for metastatic melanoma that blocks the CTLA-4 receptor (Wei et al., 2018). In this study, we will quantify levels of CTLA-4 in SIV⁺ rhesus macaques. Macaques demonstrating better viral control are expected to express lower levels of CTLA-4.

TIGIT

T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) is a marker that is found on natural killer cells, CD8⁺ T cells, and CD4⁺ T cells (Chew et al., 2016). TIGIT was found to be overexpressed in pre- and post-ART treated individuals. Elevated levels of TIGIT on CD8⁺ T cells are believed to be indicative of T cell exhaustion. There was a observed decrease in CD4⁺ T cell counts and increased plasma viral loads when TIGIT was overexpressed. Additionally, TIGIT is frequently co-expressed with PD-1 on chronically exhausted CD8⁺ T cells. Administration of anti-TIGIT and anti-PD-1 resulted in an improvement of CD8⁺ T cell function. In this thesis, we quantify levels of TIGIT in a SIV⁺ rhesus macaque model. We hypothesize that TIGIT will be downregulated in macaques that demonstrate enhanced viral control.

TCF7

Transcription factor 7 (TCF7) is theorized to have a critical role in maintaining T-cell function in cases of immune cell exhaustion (reviewed in Utzschneider et al., 2016). Though there are a limited number of studies evaluating the function of TCF7, preliminary work found that T cells expressing TCF7 maintained significant proliferative capabilities.

As such, TCF7 may be indicative of exhausted T cells that still maintain progenitor capabilities (cells have not reached full state of exhaustion). Since these cells lack effector functionality, they are unable to directly reduce the viral load. However, the cells were able to produce TCF7- progenitor cells that possess effector capabilities. TCF7- progenitor cells are limited in their ability to expand independently. This led to the conclusion that TCF7⁺ cells have a stem cell-like function and are able to preserve proliferative potential in cases of chronic infection. In addition, blocking PD-1 expression in TCF7- cells can result in improved effector-function capabilities in these cells. These findings positively support the potential for TCF/PD-1 mediated therapeutic intervention in cases of chronic infection. In this thesis, we quantify TCF7 expression levels in SIV⁺ rhesus macaques. Our hypothesis is that macaques that are able to better control viral infection will have greater levels of TCF7 present.

Lag-3

Lymphocyte activation gene 3 (Lag-3) is an immune checkpoint molecule that can be found on T cells, NK cells, B cells, and plasmacytoid dendritic cells (Graydon et al., 2019). In 1990, a preliminary study was published that addressed the functional and structural organization of Lag-3, a member of the immunoglobulin superfamily (Triebel et al., 1990). This human gene is 2-kb in size. Triebel and coworkers compared the gene sequences that encode for Lag-3 to CD4 and found they shared similar peptidic sequences and intron/exon organization. In addition, both the gene encoding for Lag-3 and CD4 are found on the distal short-arm of chromosome 12.

In cases of HIV-1, Lag-3 is associated with rapid disease progression and a high viral load (reviewed in Graydon et al., 2019). Since Lag-3 is an immune checkpoint molecule, it does not directly contribute to disease progression but instead is associated with immune activation. However, unlike other immune checkpoint molecules that largely rely on an inhibitory motif to function, Lag-3 utilizes an intracellular mechanism. Specifically Lag-3 likely inhibits the intracellular mechanisms utilized by CD4+ T cells and CD8+ T cells.

Additionally, elevated levels of Lag-3 were found in areas where the HIV viral reservoir is prominent, including lymph nodes. Blocking Lag-3 is hypothesized to reverse latency. Scientists have demonstrated that blocking Lag-3 for a short period of time resulted increased formation of memory T cells in cases of chronic viral infection. Studies completed using animal models have found that combined PD-1/Lag-3 blockade is more effective than blocking just PD-1 or just Lag-3. In this thesis, we will quantify expression of Lag-3 in a SIV+ rhesus macaque model. Our assumption is that lower levels of Lag-3 will be observed in macaques that are able to better control a viral infection.

Aims of Present Study

Aim 1

In the first part of our study, we exclusively focused on TCF-7. Our aim was to complete an antibody titration assay in order to identify the ideal concentrations of TCF7 antibody that can be used to elicit a signal in a non-human primate sample. Once an ideal

concentration can be identified, we will quantify the amount of TCF7 present in an SIV-infected rhesus macaque model in order to determine if TCF7 has a significant control in maintaining viral control. We compared TCF7 expression levels on rhesus macaques (RM) and human immune cells using an antibody mixture containing various dilutions of anti-TCF7 antibody.

Aim 2

In the second part of our study, we evaluated the expression levels of the immune checkpoint and activation markers described at the end of the previous section in SIV-infected RMs. The study population had previously been treated with two different TLR7 agonists (GS-986 and GS-9620) and we selected two controlled and two non-controlled RMs from this population (**Table 2**). A controlled RM is a SIV positive animal that is able to maintain an undetectable viral load without continued therapeutic intervention (Graydon et al., 2019). We hypothesized that immune checkpoint markers, including PD-1, PD-L1, CD39, Tim-3, CTLA-4, TIGIT, and Lag-3 would be downregulated in some immune cells in the controlled RM population. We hypothesized that CD69, an activation marker, would be expressed in equal amounts on both controlled RMs and non-controlled RMs since all cells used in this thesis were stimulated by phytohaemagglutinin (PHA). Finally, we hypothesized that CXCR-5 and TCF-7, which are markers that are present on “revivable” exhausted T cells, would be upregulated in controlled RMs.

METHODS

Animals, SIV Challenge and Vaccination Protocol

All (n=4) SIV-infected monkey samples used in this study were obtained from prior study published by Lim et al. (2018). Briefly, Indian-origin, outbred, young adult, male, specific pathogen-free RMs that did not express the class I alleles Mamu-A*01, Mamu-B*08, and Mamu-B*17 (which are associated with enhanced virus control) were infected using a repeated low-dose intrarectal challenge with the SIVmac251 strain. The preformulated ART cocktail contained two reverse transcriptase inhibitors, TFV (20 mg/ml) and FTC (50mg/ml), plus the integrase inhibitor DTG (2.5 mg/ml). This ART cocktail was administered once daily at 1 ml/kg body weight via the subcutaneous route. CD4⁺ and CD8⁺ T cell counts, CD4⁺/CD8⁺ T cell ratio, and animal weights were monitored regularly once or twice per week. The endotoxin-free TLR7 agonists GS-986 and GS-9620 were provided by Gilead Science. The formulated concentrations of GS-986 and GS-9620 administered to macaques every other week were: GS-9620 at 0.15 mg/kg and GS-986 at 0.10 mg/kg for a total of either 7 doses (GS-9620) or 19 doses (GS-986) (**Table 2**). Two weeks after the last TLR7 dose, ART was discontinued in all groups to monitor viral rebound.

For the TCF7 antibody titration assay, Stemcell Technologies (Vancouver, Canada) provided human leukopak obtained from healthy donors. Naïve rhesus blood samples were provided by Wisconsin National Primates Research Center (Madison, WI).

Immune cell staining and analysis

Peripheral blood mononuclear cells (PBMCs) were isolated by ficoll density gradient centrifugation from two naïve humans, two naïve RMs, two controlled RMs and two non-controlled RMs (**Table 2**) following manufacturer's instruction (GE Healthcare Life Sciences, Marlborough, MA). Two controlled RMs and two non-controlled RMs were further stimulated with 2 µg/mL of phytohemagglutinin-M (PHA-M) in RPMI-1640 media supplemented with 10% heat-inactivated fetal bovine serum and 2% antibiotic cocktails which included penicillin and streptomycin at 37°C in a humidified 5% CO₂ atmosphere for four days. Cells were resuspended in 1X phosphate-buffered saline (PBS) containing LIVE/DEAD Fixable Aqua or Blue Dead Cell Stain (Biolegend, San Diego, CA) for exclusion of dead cells. Human Fc-block (BD Bioscience, Franklin Lakes, NJ) was added to avoid non-specific Fc binding and incubated for 10 minutes at room temperature (RT) in the dark. Cells were first stained with fluorescent-conjugated antibody cocktails (addressed in **Table 3**) with the exception of CTLA-4 and TCF7 and then stained with biotin-conjugated PD-L1 (house-made, Bristol-Myers Squibb, New York, NY) followed by streptavidin-conjugated PE/Cy7 (BD Bioscience) at RT in the dark. Cells were washed once and permeabilized with TF Fix/Perm Buffer (BD Bioscience) and incubated for 50 minutes at 4°C in the dark. The cells were washed twice with TF Perm/Wash Buffer (BD Bioscience) then resuspended in TF Perm/Wash Buffer containing CTLA-4 and TCF7 antibodies for 30 minutes at RT in the dark. Cells were washed twice with TF Perm/Wash Buffer (BD Bioscience) prior to being fixed with 1% paraformaldehyde (ThermoFisher, Waltham, MA) then transferred to 5mL round-bottom tubes. Samples were run on the

Symphony™ flow cytometer (BD Bioscience) or LSRII™ flow cytometer. All flow cytometric analysis was conducted by Flowjo v. 10 (FlowJo, LLC, Ashland, OR) and graphical figures were generated by Prism v. 8 (GraphPad, San Diego, CA).

Table 2: Experimental Sample Parameters

Treatment	Identifier	Controlled/Non-controlled
GS-986 (0.10 mg/kg)	280-10	Non-controlled
	344-10	Controlled
GS-9620 (0.15 mg/kg)	341-10	Non-controlled
	177-10	Controlled

Table 3: Antibody Panel

Fluorescent Marker	Antibody	Clone	Vendor	Application
FITC	Lag-3	Goat polyclonal	R&D Systems	
BB700	PD-1	E12.1	BD Bioscience	
PerCP/Cy5.5	CD38	CD28.2	BD Bioscience	TCF7 validation
BB790	CD45RA	549	BD Bioscience	
PE	NKG2a	Z199	Beckman Coulter	
PE	TCF7	S33-966	BD Bioscience	TCF7 validation
PE-eF610	TIGIT	MBSA43	ThermoFisher	
PE-Cy5	CXCR5	MU5UBEE	ThermoFisher	
PE-Cy5.5	CD20-2H7	2H7	ThermoFisher	
BV421	CTLA-4	BNI3	BD Bioscience	
V450	CD3-SP34.2	SP34.2	BD Bioscience	TCF7 validation
BV510	CD4	L200	BD Bioscience	
BV570	CD56	HCD56	BioLegend	
BV605	CD39	TU66	BD Bioscience	
BV650	CD197	G043H7	BioLegend	
BV650	CD8	RPA-T8	BD Bioscience	TCF7 validation
BV711	CD95	DX2	BD Bioscience	
BV711	CD4	L200	BD Bioscience	TCF7 validation
BV750	VISTA	MIH65.rMAb	BD Bioscience	
BV786	CD28	CD28.2	BD Bioscience	
BUV396	CD45	D058-1283	BD Bioscience	

BUV496	HLA-DR	G46-6	BD Bioscience	
BUV563	CD8	RPA-T8	BD Bioscience	
BUV615	TCF7	S33-966	BD Bioscience	
BUV737	CD69	Fn50	BD Bioscience	
BUV737	CD14	M5E2	BD Bioscience	TCF7 validation
BUV805	CD14	M5E2	BD Bioscience	
APC	TIM-3	344823	R&D Systems	
APC	CD95	DX2	BD Bioscience	TCF7 validation
A700	CD3-SP34.2	SP34.2	BD Bioscience	
APC-Cy7	CD16	3G8	BD Bioscience	

RESULTS

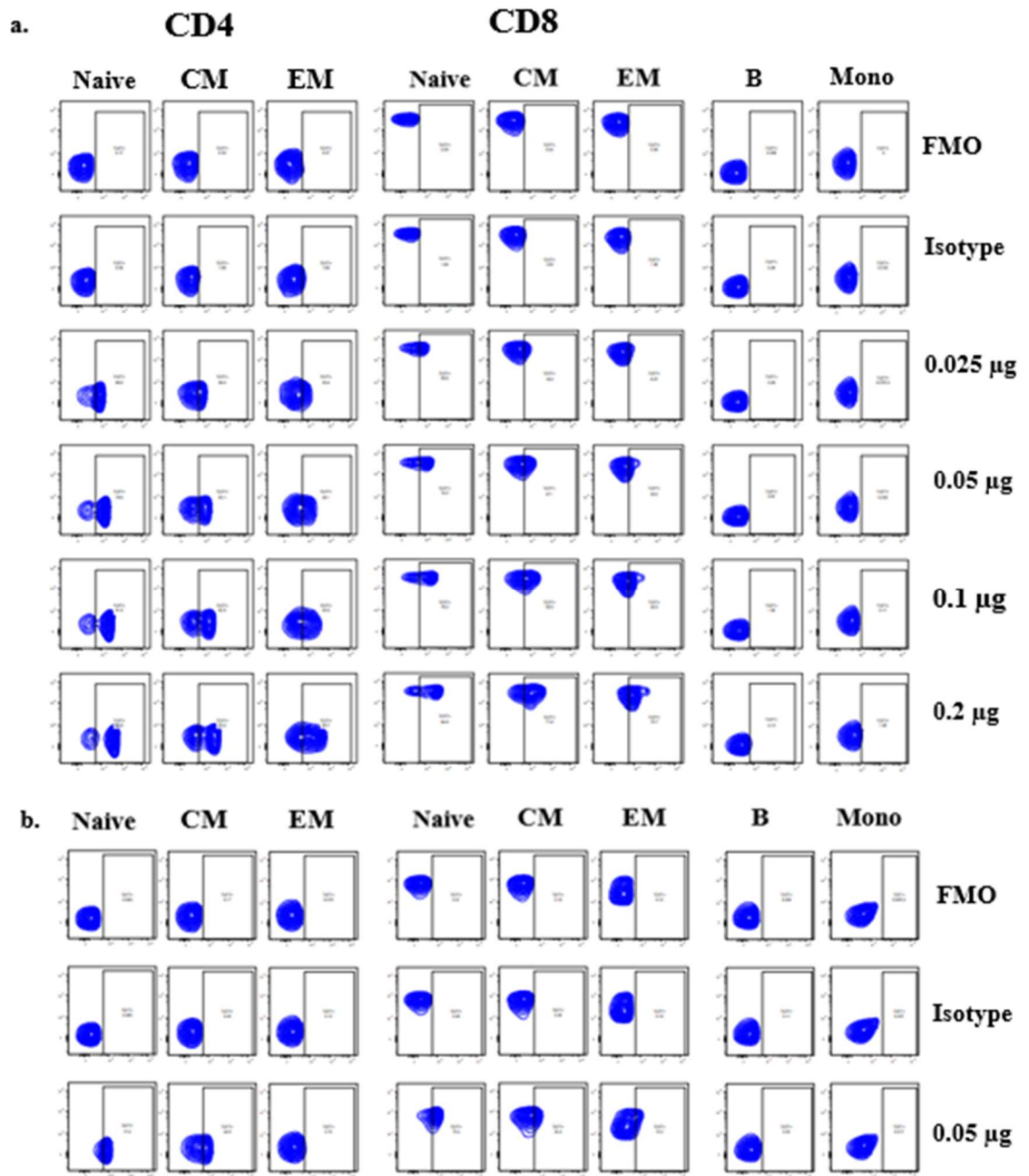
Aim 1: Antibody Validation Assay – TCF7

Currently, there are a limited number of antibody clones commercially available for TCF7 for human subjects. The available antibodies are not fully validated for either human or RM subjects. Thus, we selected a TCF7 clone (S33-966) from BD Bioscience, which was originally developed as an anti-mouse antibody and has not been tested using a RM model. We tested this antibody clone using PBMCs from two naïve humans and two RMs to in order to answer a number of questions. First, we wanted to identify whether this clone can react with endogenous TCF7 in humans. Second, we wanted to quantify the optimal antibody concentration of this clone in human subjects and apply this concentration to a RM model. And finally, we wanted to validate the reactivity of TCF7 antibody in RM subjects and compare its expression level between naïve human subject and naïve rhesus subjects.

TCF7 was originally reported to be highly expressed in naïve T memory cells (approximately 60-80%), medium to low expression on central memory (CM)/effector memory (EM) T cells (approximately 10-40%), and at undetectable levels in B cells and monocytes under normal physiological condition (Utzschneider et al., 2016). We first tested human PBMCs with several different concentrations of TCF7 (0.02 µg/mL, 0.01 µg/mL, 0.005 µg/mL, and 0.0025 µg/mL) and included an isotype control and negative control (referred as **Fluorescence Minus One**, FMO). Using the isotype control, we were able to set an inclusion gate for TCF7 that did not include any background fluorescence.

We detected positive populations of TCF7 with all tested concentrations of TCF7. The isotype control and FMO did not induce significant fluorescence on either CD4⁺ or CD8⁺ naïve, CM, or EM cells. (**Figure 1a**).

When testing the two highest concentrations (0.01 µg/mL and 0.02 µg/mL), a strong signal and clear separation of the TCF7 population on naïve and central memory CD4⁺ and CD8⁺ T cells was observed. Unexpectedly, when testing the two concentrations, we found that TCF7 was also expressed on B cells and monocytes. Utzschneider et al. determined these cells were not supposed to express TCF7. The positive TCF7 signals observed on B cells and monocytes may be attributed to non-specific binding of antibodies on these cells. As such, non-specific binding may also result in undesirable false positive signals on true target cells as well. Therefore, we determined the minimal antibody concentration that elicits a strong fluorescent signal and clear separation is 0.005 µg/mL for human subjects. Next, we tested 0.005 µg/mL of TCF7 antibody with naïve rhesus samples and found clear separation on CD4⁺ and CD8⁺ T memory (naïve, CM and EM) cells and a limited amount of background fluorescence in B cells and monocytes (**Fig. 1b**). Naïve human and naïve RM also showed similar expression levels of TCF7 in all cells evaluated (CD4⁺ naïve, CD4⁺ CM, CD4⁺ EM, CD8⁺ naïve, CD8⁺ CM and CD8⁺ EM) (**Fig. 1c, Table 4 and 5**).



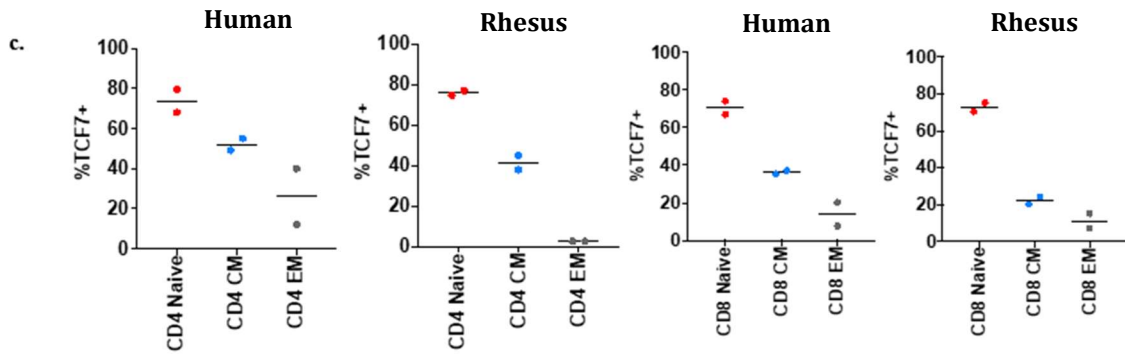


Figure 1. TCF7 Antibody Panel Optimization: **A.** Expression of TCF7 in a human PBMC sample was measured using varying antibody concentrations, an isotype control, and a negative control (FMO). Specifically, expression was measured in B cells (CD3-CD20+), monocytes (CD3-CD20-NKG2a+), central memory, and effector cells. **B.** Expression of TCF7 was measured in a naïve rhesus macaque sample using 0.05 µg of the antibody, an isotype control, and a negative control (FMO). Specifically, expression was measured in B cells (CD3-CD20+), monocytes (CD3-CD20-NKG2a+), central memory, and effector cells. **C.** Comparison of TCF7 expression in humans versus rhesus macaque samples, with alternating presentations of human and rhesus macaque graphs. Closed circles are indicative of each individual subject and a horizontal line marks the average of the two subjects.

Table 4: Average TCF7 Expression Levels in a Human Sample

(% of total cell population)

	CD4 Naïve	CD4 CM	CD4 EM	CD8 Naïve	CD8 CM	CD8 EM	B	Mono
FMO	0.085	0.53	0.41	0.145	0.425	0.73	0.094	0
Isotype	0.73	1.7	1.95	1.06	1.425	1.815	0.38	0.009
0.025 µg	65.4	37.15	15.22	57.55	15.95	6.12	0.545	0.008185
0.05 µg	73.85	52.2	26.15	70.6	36.25	13.95	1.695	0.04
0.1 µg	75.5	59.6	38.1	75.5	53.05	27.65	3.75	0.105
0.2 µg	79.2	68.05	56.7	81.35	68.5	54.2	8.065	1.55

Table 5: Average TCF7 Expression Levels in a Rhesus Macaque Sample (% - Percent)

	CD4 Naïve	CD4 CM	CD4 EM	CD8 Naïve	CD8 CM	CD8 EM	B	Mono
FMO	0.084	0.2	0.116	0.16	0.23	0.135	0.019	0.011
Isotype	0.093	0.275	0.145	0.2	0.33	0.17	0.0985	0.0225
0.05 µg	75.9	41.45	2.725	72.95	22.4	11.285	0.31	0.0295

Aim 2: Controlled versus non-controlled immune checkpoint expression

An optimized antibody panel (**Table 3**) was applied to isolated PBMC samples obtained from two non-controlled and two controlled TLR7 treated RMs. The lymphocyte population was first gated based on its size and granularity (**Figure 2**), then immune cells were defined as follows; CD4⁺ T cells (CD3⁺CD4⁺CD14⁻CD20⁻CD45⁺NKG2a⁻), CD8⁺ T cells (CD3⁺CD8⁺CD14⁻CD20⁻CD45⁺NKG2a⁻), B cells (CD45⁺CD3⁻CD14⁻CD20⁺NKG2a⁻), NK cells (CD45⁺CD3⁻CD14⁻CD20⁻CD8⁺NKG2a⁺).

CD69

The average percent difference in expression level of CD69 on B cells is 22.95% greater in controlled RMs (**Fig. 3a, Table 6 and 7**) compared to non-controlled RMs. Additionally, there is an average increase of 3.05% of CD69 on CD4⁺ T cells as well on CD8⁺ T cells (>1.4%) in controlled groups. This marker was found on over 90% of NK cells in both controlled and non-controlled RMs at 94.7% and 96.9% of the total cell population respectively.

CD39

The greatest expression of CD39 was found on CD8⁺ T cells with nearly identical expression found on controlled (65%) and non-controlled RMs (65.2%) (**Fig. 3b, Table 6 and 7**). Expression patterns in controlled and non-controlled samples were similar in NK cells with an expression level of approximately 25% in each sample. CD39 was expressed in greater frequency on CD4⁺ T cells in non-controlled RMs, with an average difference of 6.35%. However, when comparing the two non-controlled RMs, we found that subject 280-

10 expressed approximately 32.1% more of this marker which may have upwardly skewed the non-controlled data average. B cells obtained from controlled RMs expressed 27.43% more CD39 than non-controlled RMs (**Fig. 3b**).

CXCR5

90.15% of B cells express CXCR5 in controlled RMs with an average difference of 10.2% in expression levels between the controlled and non-controlled samples (**Fig. 3c, Table 6 and 7**). In addition, CD4⁺ T cells in controlled RMs expressed an average of 19.35% more CXCR5 than non-controlled RMs. Expression levels of CXCR5 on CD8⁺ T cells was 1.565% greater in the controlled population. There was a minimal difference in CXCR5 expression in NK cells, with the non-controlled RMs expressing an average of 0.985% CXCR5 more than the controlled RMs.

TCF7

TCF7 levels are expressed in greater frequency on CD4⁺ T cells obtained from our controlled sample compared to that seen in non-controlled RMs, with an average difference of 20.5% (**Fig. 3d, Table 6 and 7**). Additionally, expression levels of TCF7 are elevated in CD8⁺ T cells in controlled RMs with an average difference of 14.35% between the controlled and non-controlled samples. NK cells from controlled RMs presented with an average of 11.3% more TCF7 than non-controlled RMs. Finally, the presentation on B cells was marginally greater on the controlled sample, with an average difference of 3.305%.

PD-1

CD4⁺ T cells, CD8⁺ T cells, and B cells from controlled RMs all expressed lower levels of PD-1 than that of the non-controlled population (**Fig. 3e, Table 6 and 7**). Non-controlled RMs had a higher expression overall of PD-1 on CD4⁺ T cells (16.45%), CD8⁺ T cells (0.5%), and B cells (8.05%). While NK cells expressed 1.05% more PD-1 in controlled RMs, the overall concentration of PD-1 on both controlled and non-controlled RMs was low with an average expression of 6.43% in the non-controlled population and an average of 7.48% in the controlled population.

PD-L1

Non-controlled RMs generally expressed a greater frequency of PD-L1 than that of controlled RMs which shared similar expression tendencies as PD-1 (**Fig. 3f, Table 6 and 7**). Non-controlled RMs showed higher expression of PD-1 on NK cells and CD8⁺ T cells with an average of 32.15% and 14.95% more than what was seen in the controlled population. The difference in expression levels on CD4⁺ T cells was relatively small in comparison, with non-controlled RMs expressing an average of 3.25% more PD-L1 than controlled RMs. Interestingly, the controlled B cell sample did express a greater overall concentration of PD-L1 than that of non-controlled sample, with an increase of 3.6% in expression level.

TIGIT

TIGIT was expressed in greater frequency on NK cells, CD4⁺ T cells, CD8⁺ T cells (16.295%, 7.45%, and 4.6%) in controlled RMs (**Fig. 3g, Table 7**). Non-controlled RMs expressed an average of 1.03% more TIGIT on B cells than cells obtained from the controlled sample.

CTLA-4

CTLA-4 was present in elevated frequencies in all controlled RM immune cells when compared to cells from non-controlled samples (**Fig. 3h**). CD4⁺ T cells expressed an average of 15.3% more CTLA-4 in controlled RMs than that of non-controlled RMs. Similarly, CD8⁺ T cells from controlled RMs expressed an average of 13.65% more CTLA-4 than non-controlled RMs. Controlled RM B cells expressed an average of 10.3% more CTLA-4 than non-controlled B cells. Finally, NK cells expressed 1.86% more CTLA-4 in controlled RMs than non-controlled RMs.

Tim-3

Overall, the difference in expression levels of Tim-3 between controlled and non-controlled RMs in the immune cells studied were minimal. CD4⁺ T cells and B cells expressed greater concentrations of the marker in controlled RMs than non-controlled RMs (0.1575% and 1.085%) (**Fig. 3i, Table 6 and 7**). CD8⁺ T cells obtained from non-controlled RMs expressed marginally more Tim-3 with an average increased expression of 0.045% when compared to expression levels in controlled RMs. NK cells did not express Tim-3.

Lag-3

CD4⁺ T cells, CD8⁺ T cells, and NK cells expressed slightly more Lag-3 in non-controlled RMs than controlled RMs (0.055%, 0.19%, and 0.355% respectively) (**Fig. 3j, Table 6 and 7**). B cells expressed more Lag-3 in the controlled RM population than the non-controlled RMs, with the difference in the average expression level being 3.755%.

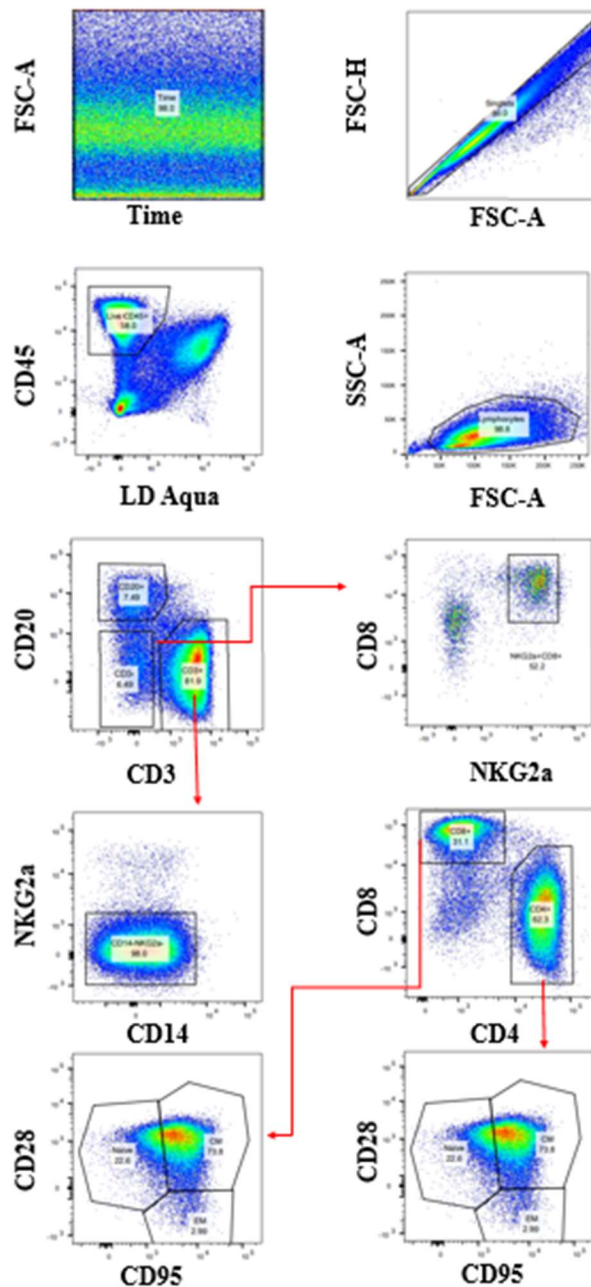
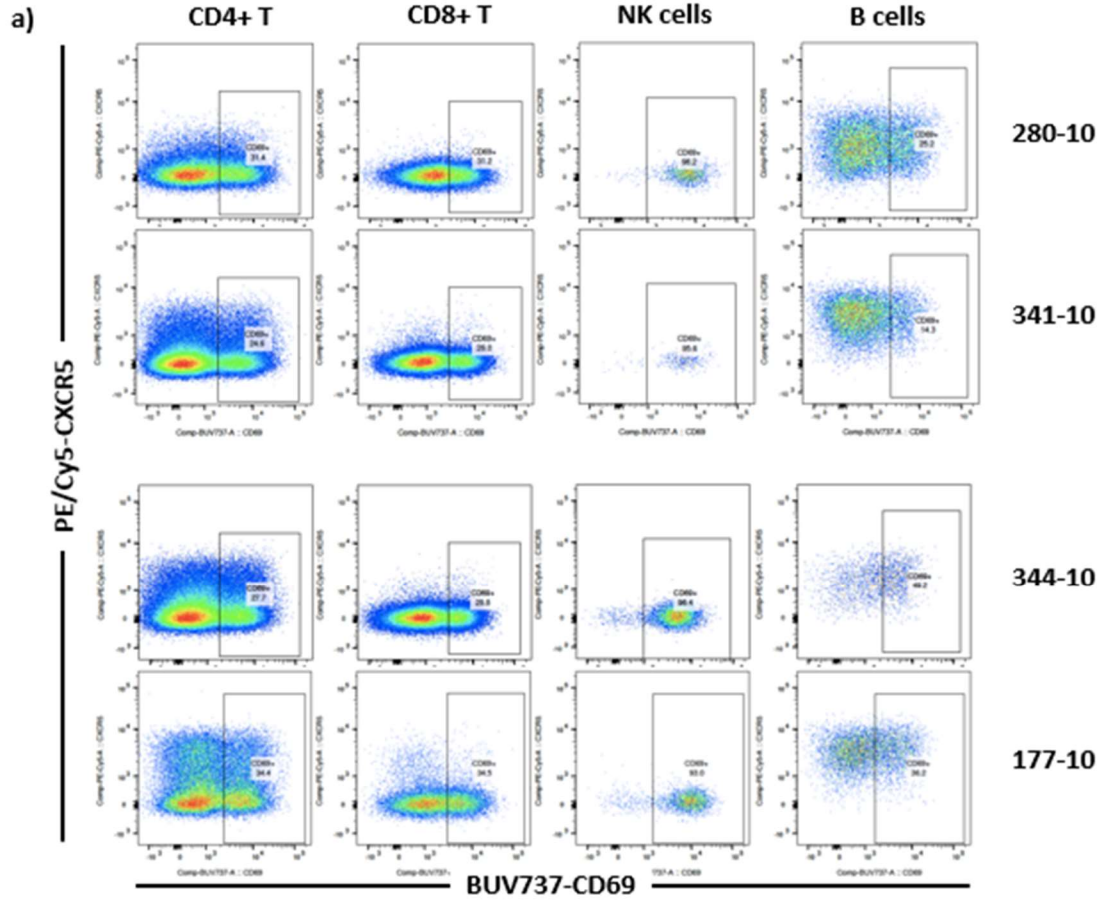


Figure 2. Gating Strategy:

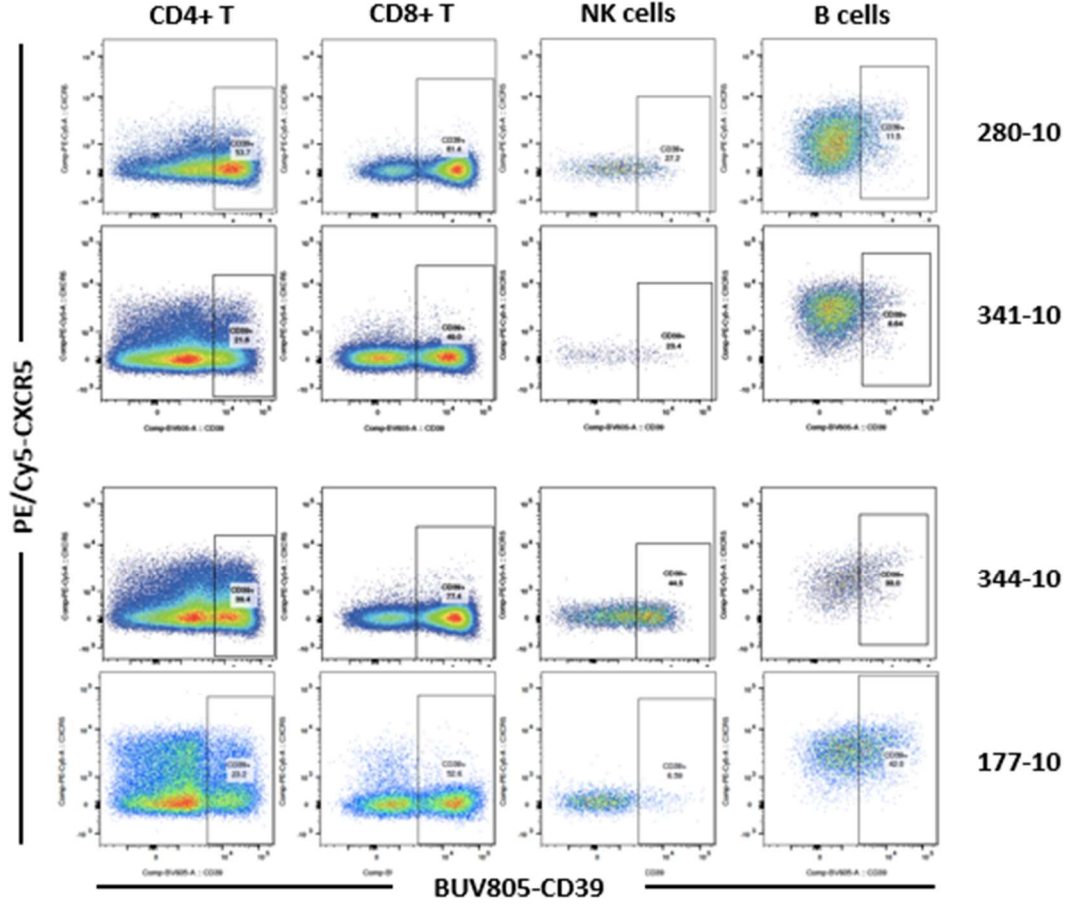
Isolated PBMC samples were gated using the pictured protocol. Doublets were excluded from our study. Live cells were identified using live-dead aqua. Lymphocytes were isolated from the live cell population and were further subdivided into CD3⁻CD20^{dim}, CD3⁻CD20⁺, and CD3⁺ cells. NK cells were separated from the CD3⁻ population, co-expressing NKG2a and CD8. CD8⁺ and CD4⁺ cells were identified from the CD3⁺CD14⁻NKG2a⁻ cells. Finally, both the CD8⁺ and CD4⁺ cells were divided into naïve cells, central memory cells, and effector memory cells.

CD69



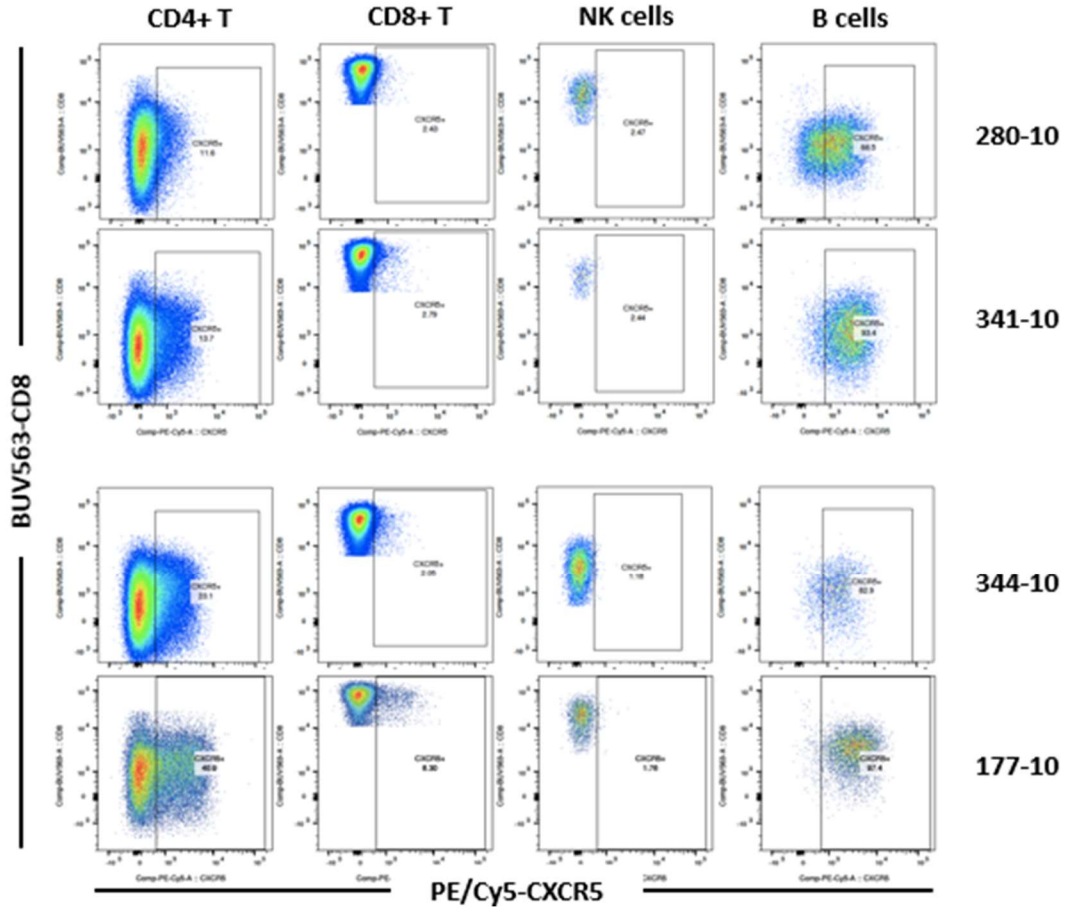
CD39

b)



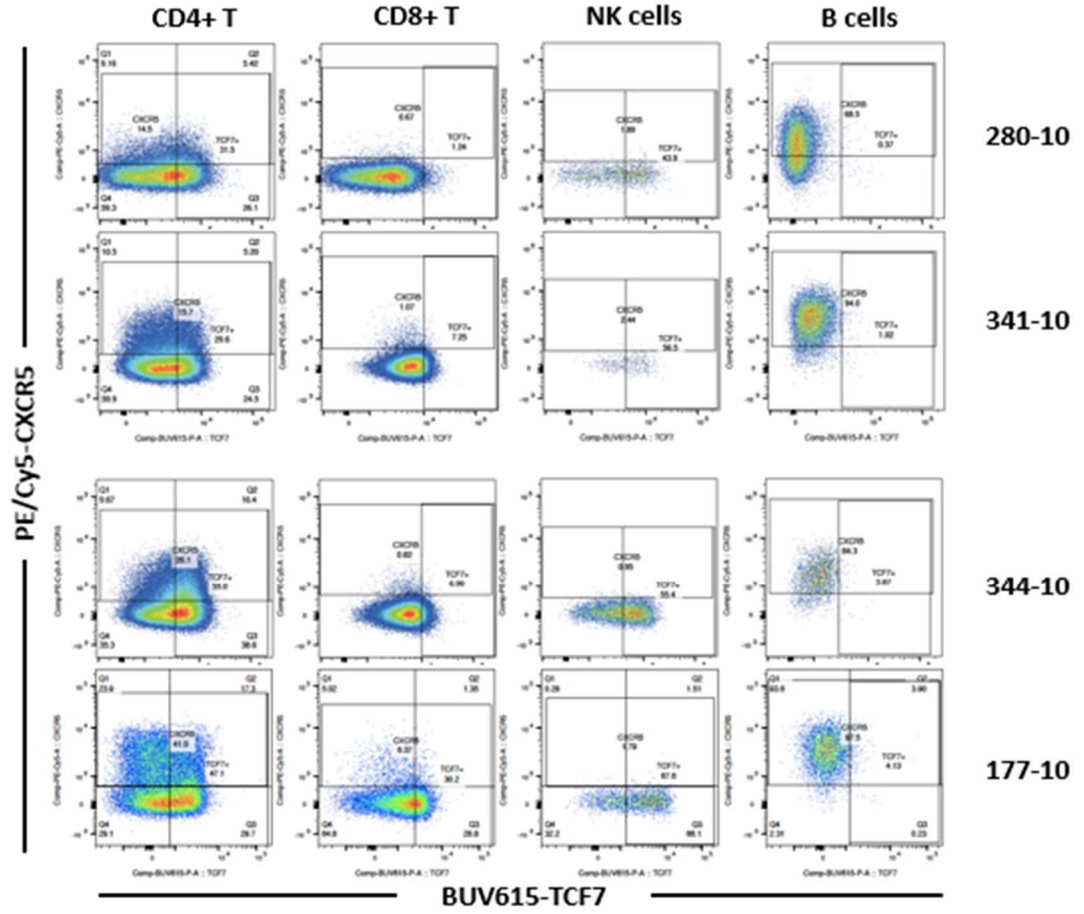
CXCR5

c)



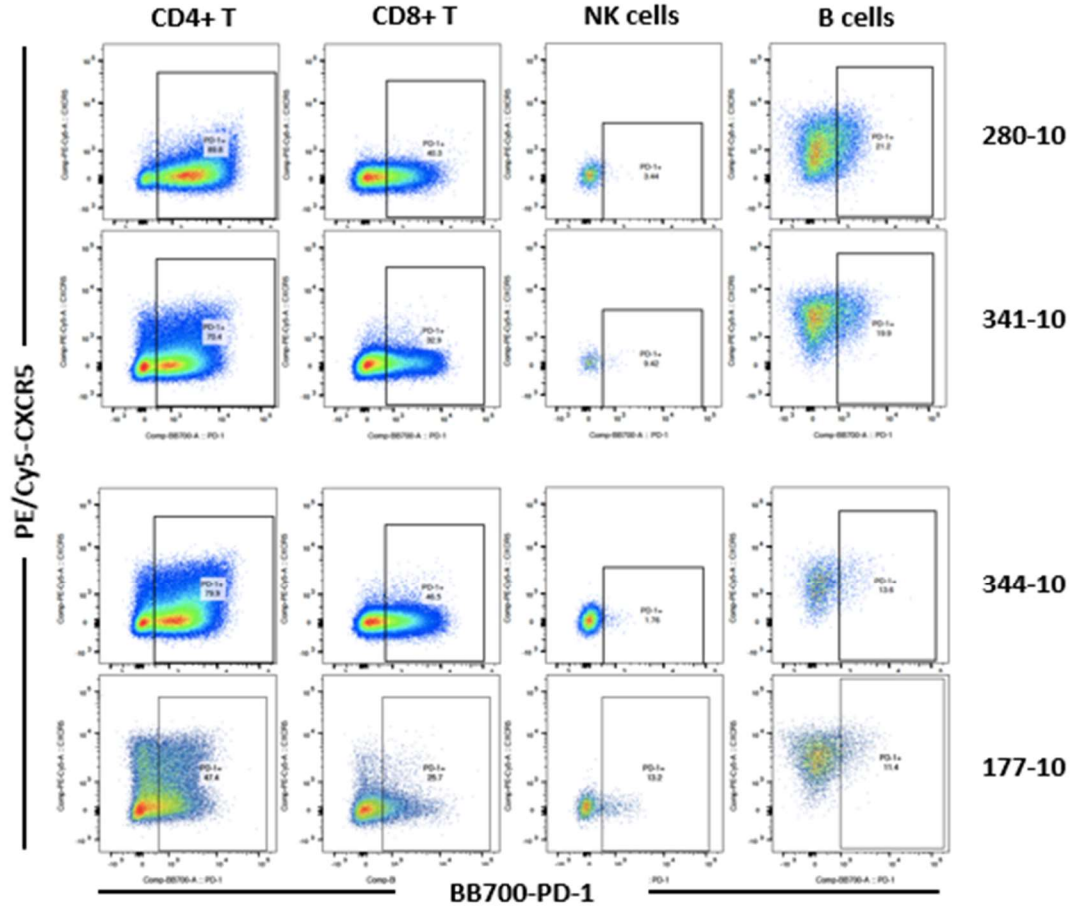
TCF7

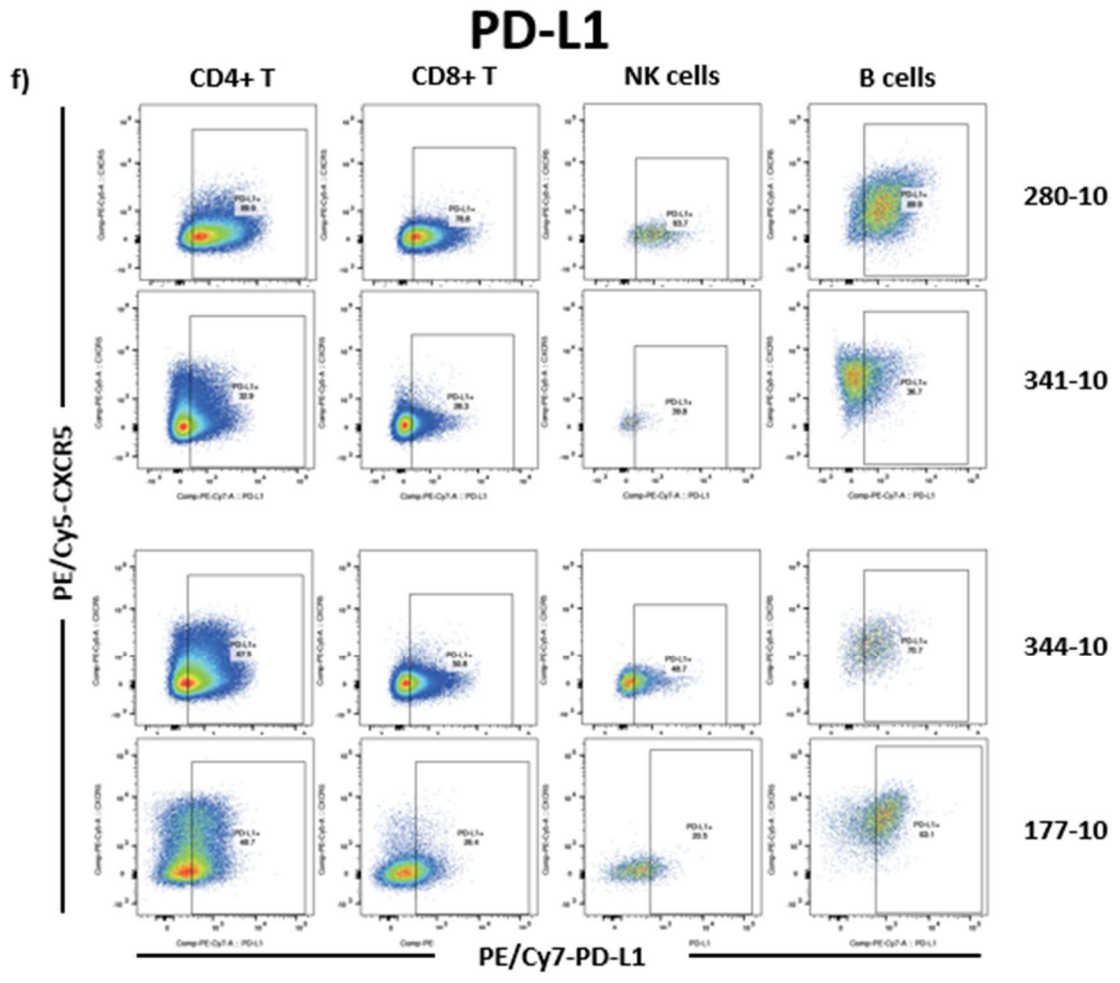
d)



PD-1

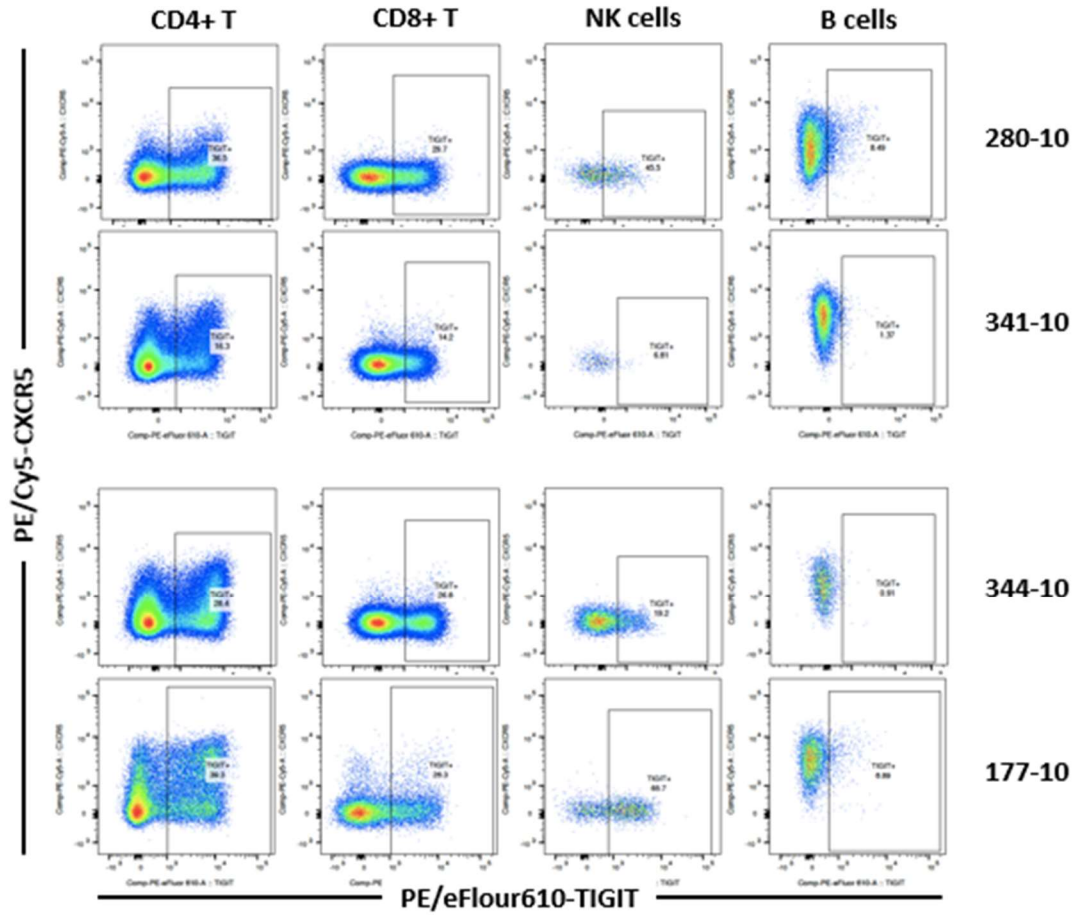
e)





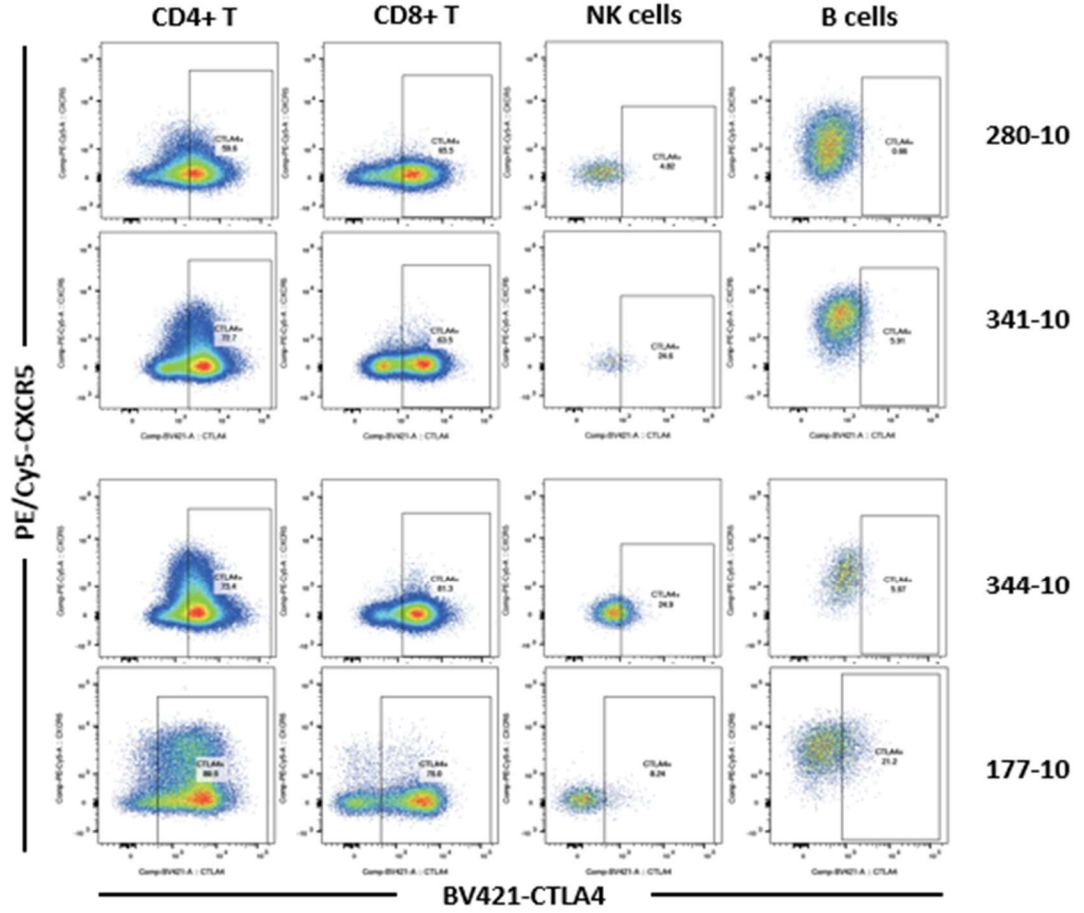
TIGIT

g)



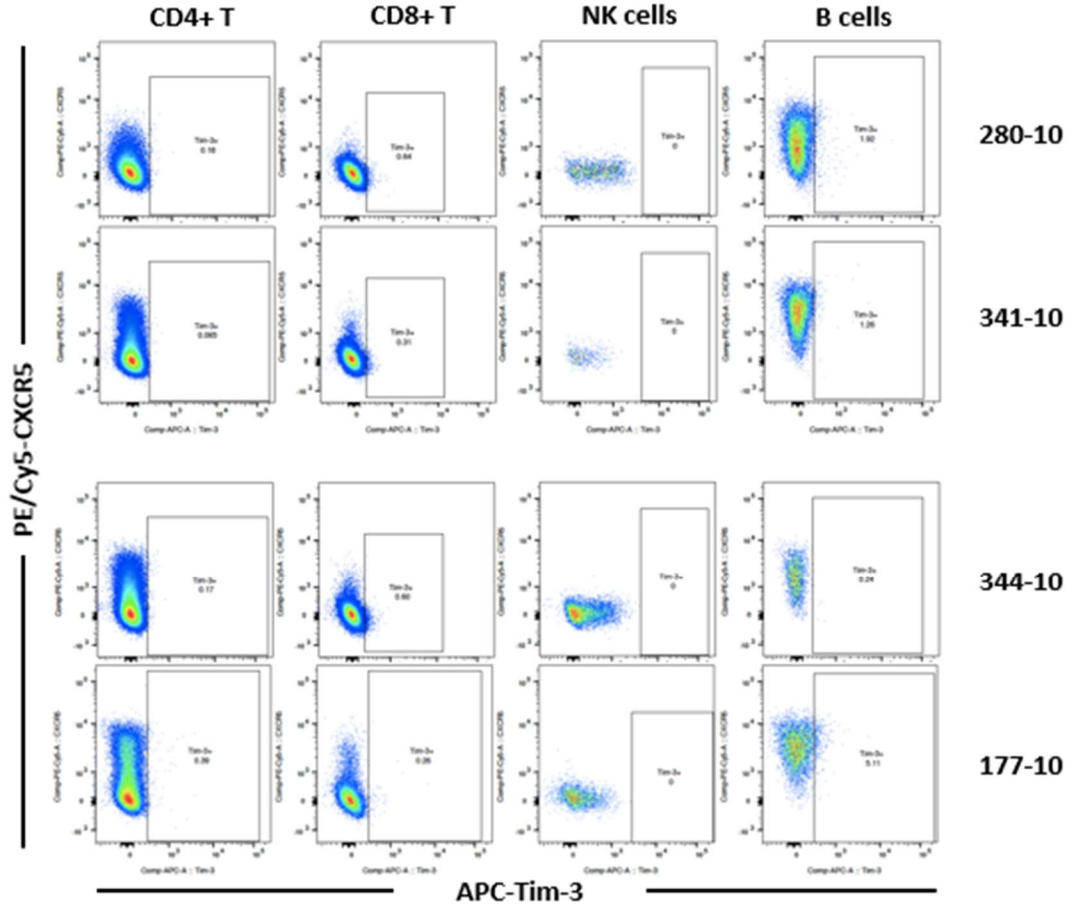
CTLA4

f)



Tim-3

g)



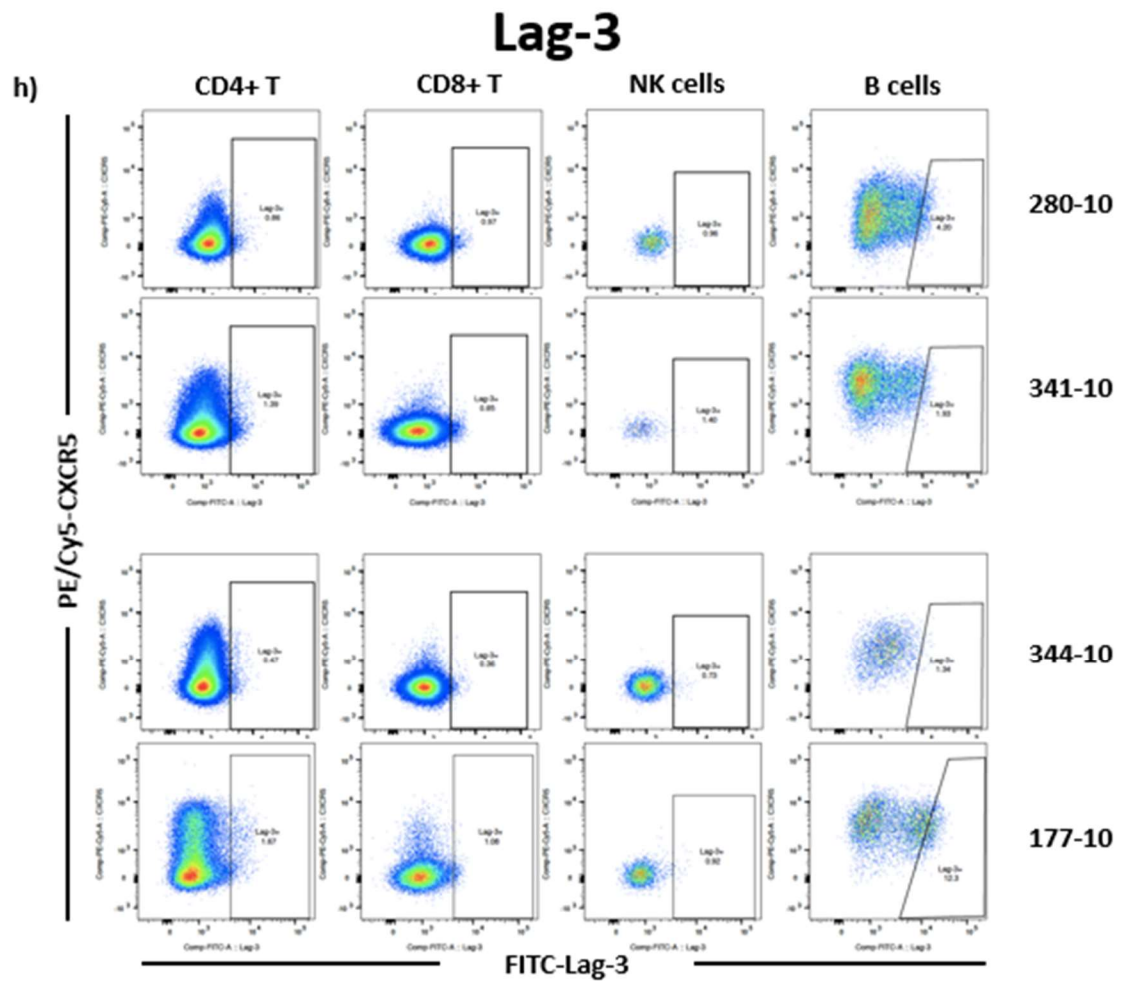


Figure 3. Immune Checkpoint Expression Levels in TLR7 treated ART-suppressed Rhesus Macaques: Immune checkpoint expression levels obtained via flow cytometric analysis of the following activation and immune checkpoint markers: **a.** CD69 **b.** CD39 **c.** CXCR5 **d.** TCF7 **e.** PD-1 **f.** PD-L1 **g.** TIGIT **h.** CTLA-4 **i.** Tim3 **j.** Lag3

Table 6: Average expression levels of immune checkpoint markers in a controlled and non-controlled RM samples. (% of total cells)

		CD4+ T cells	CD8+ T cells	NK cells	B cells
CD69	Non-controller	28	28.6	96.9	19.75
	Controller	31.05	30	94.7	42.7
CD39	Non-controller	37.65	65.2	25.3	10.07
	Controller	31.3	65	25.545	37.5
CXCR5	Non-controller	12.65	2.61	2.455	79.95
	Controller	32	4.175	1.47	90.15
TCF7	Non-controller	30.55	4.245	50.2	0.695
	Controller	51.05	18.595	61.5	4
PD-1	Non-controller	80.1	36.6	6.43	20.55
	Controller	63.65	36.1	7.48	12.5
PD-L1	Non-controller	61.35	53.55	66.75	63.3
	Controller	58.1	38.6	34.6	66.9
TIGIT	Non-controller	26.4	21.95	26.155	4.93
	Controller	33.85	26.55	42.45	3.9
CTLA4	Non-controller	66.15	64.5	14.71	3.285
	Controller	81.45	78.15	16.57	13.585
Tim-3	Non-controller	0.1225	0.475	0	1.59
	Controller	0.28	0.43	0	2.675
Lag-3	Non-controller	1.125	0.91	1.18	3.065
	Controller	1.07	0.72	0.825	6.82

Table 7: Differences between the average expression levels of immune checkpoint markers in non-controlled RM and controlled RM samples.

	CD4+ T cells	CD8+ T cells	NK cells	B cells
CD69	3.05	1.4	-2.2	22.95
CD39	-6.35	-0.2	0.245	27.43
CXCR5	19.35	1.565	-0.985	10.2
TCF7	20.5	14.35	11.3	3.305
PD-1	-16.45	-0.5	1.05	-8.05
PD-L1	-3.25	-14.95	-32.15	3.6
TIGIT	7.45	4.6	16.295	-1.03
CTLA4	15.3	13.65	1.86	10.3
Tim-3	0.1575	-0.045	0	1.085
Lag-3	-0.055	-0.19	-0.355	3.755

DISCUSSION

Due to the limited number of TCF-7 antibody clones available for human and RM models, we first attempted to determine an optimal TCF7 antibody concentration that will elicit a signal in a non-human primate sample. Once the optimal concentration was identified, we quantified the amount of TCF7 present in controlled and non-controlled RMs. Additionally, we identified the expression levels of a number of immune checkpoint and activation molecules and compared expression levels between controlled and non-controlled RMs to have better understanding of the role of immune checkpoints and HIV/SIV pathogenesis.

An antibody titration assay was performed to determine the optimal concentration of TCF-7 antibody for use in flow cytometry analysis to quantify TCF7 expression in controlled and non-controlled RMs. We found the optimal concentration in humans was 0.05 μg and continued to use this concentration throughout our study. Analysis of obtained data indicated that at 0.05 μg concentration, TCF7 was predominantly expressed on CD4⁺ and CD8⁺ naïve cells. Both human and rhesus macaques showed similar expression levels of TCF7 on each memory subtype of CD4⁺ and CD8⁺ T cells. This supported data obtained by Utzschneider et al, who identified TCF7 expression on CD4⁺ and CD8⁺ naïve T cells to be approximately 90% (Utzschneider et al., 2016). We were then able to use our validated TCF-7 antibody to identify expression in controlled and non-controlled RMs. It is hypothesized that TCF-7 has an important role in maintaining T cell function in cases of immune exhaustion. Specifically, it is believed that cells expressing this marker possess

“progenitor-like” properties and are able to function in proliferating the immune response in cases of chronic infection. This theory was supported by the data obtained in our study, with controlled RMs expressing more TCF-7 on CD4⁺ and CD8⁺ T cells than non-controlled RMs.

We evaluated the expression levels of two activation markers, CD69 and CD39, in CD4⁺ T cells, CD8⁺ T cells, NK cells, and B cells. Our objective was to identify differences in activation between controlled RMs and non-controlled RMs after antigenic re-stimulation. CD69 has been identified as a general immune activation marker for T, B, and NK cells (Janeaway et al., 2005). Additionally, CD69 acts as a co-stimulatory molecule on T cells resulting in enhanced proliferation and function (Borrego et al., 1999). CD39 is also an activation marker in lymphocytes, predominantly on B cells, and it has a key role in immunoglobulin class switch recombination (Schena et al, 2013). On average, the controlled RMs expressed more CD69 on CD4⁺ T cells (3.05%), CD8⁺ T cells (1.4%), and NK cells (2.2%). Since the difference in expression levels of the CD69 marker is marginal between the two study subsets, there is likely a similar level of activation in controlled and non-controlled RMs. Strikingly, CD69 and CD39 were found to be expressed in higher concentrations on B cells in the controlled group when compared to the non-controlled RMs which may be indicative of a greater number of active B cells producing antibodies resulting in a proliferative immune response.

We then evaluated the expression levels of several immune checkpoint molecules including PD1, PDL1, CTLA4, and TIGIT. Previous research has shown that these four markers are indicative of HIV-mediated T cell exhaustion and rapid viral progression (Boyer et al, 2018; Wykes et al, 2018). Our data showed an overall decrease of PD-1 and PD-L1 in CD4⁺ T cells, CD8⁺ T cells, and NK cells in controlled RMs. In contrast, the expression levels of CTLA-4 and TIGIT increased in controlled RMs. As such, it is likely that TIGIT and CTLA-4 do not contribute to overall viral control while PD-1 and PD-L1 have a more substantial role in viral control mediated by TLR7 treatment. Of interest, subjects treated with GS-9620 analog showed a greater decrease in expression of PD-1 and PD-L1 in the controlled population. This is likely secondary to structural differences between the GS-986 analog and GS-9620 agonist. Additionally, one RM from the GS-9620 treatment group presented with the lowest level of expressed PD-1. A previous study conducted using this subject showed lower levels of viral SIV RNA overall post-ART treatment (Lim et al., 2018) which may indicate that this subject is intrinsically better at controlling a viral infection (**Appendix I, Fig. 4**).

CXCR5 is expressed in high concentrations on cells that typically reside in the center of lymphoid aggregates near inflamed tissue (Rao et al, 2018). These cells are known as T follicular helper (Tfh) cells and are characterized by the surface expression of several markers, including CXCR5, PD1, and ICOS (Rao et al, 2018). The CXCR5 present on Tfh cells will ligate with CXCL13 ultimately resulting in B cell differentiation and antigen production in lymphoid organs (Locci et al., 2013). Cagigi et al. (2008) reported that HIV+

individuals express a lower amount of CXCR5 on B cells due to a decreased concentration of CD4⁺ T cells. Our data shows increased expression of CXCR5 on CD4⁺ T cells in controlled RMs when compared to non-controlled RMs, with an average increase of 10.35%. This may be indicative of an increase in functional CD4⁺ Tfh-like cell and improved viral control. One RM from the controlled group presented with the greatest concentration of CD4⁺ CXCR5 (40.9%).

In conclusion, we found controlled RMs showed lower expression of PD-1 and PD-L1 on several immune cells, particularly NK and T cells, which are responsible for controlling viremia and inducing an optimal immune response. Additionally, controlled RMs showed higher expression of activation markers on B cells, as well as an increase of CXCR5 on CD4⁺ T cells. Thus, there may be a connection between functional B cell activation and improved viral control. An important factor to consider while reviewing the results from this study is the number of study subjects that were analyzed (n=4). We recommend repeating this study with a larger number of subjects to obtain more precise insights and statistically significant results. Additionally, we did notice lower overall expression levels of Tim-3 and Lag-3 in our panel. While the exact reason for this is unknown, we believe that it may be secondary to the quality of the antibody that was used. Replicating this experiment with Tim-3 and Lag-3 antibodies obtained from a different manufacturer or with different reagents will likely be beneficial.

APPENDIX I

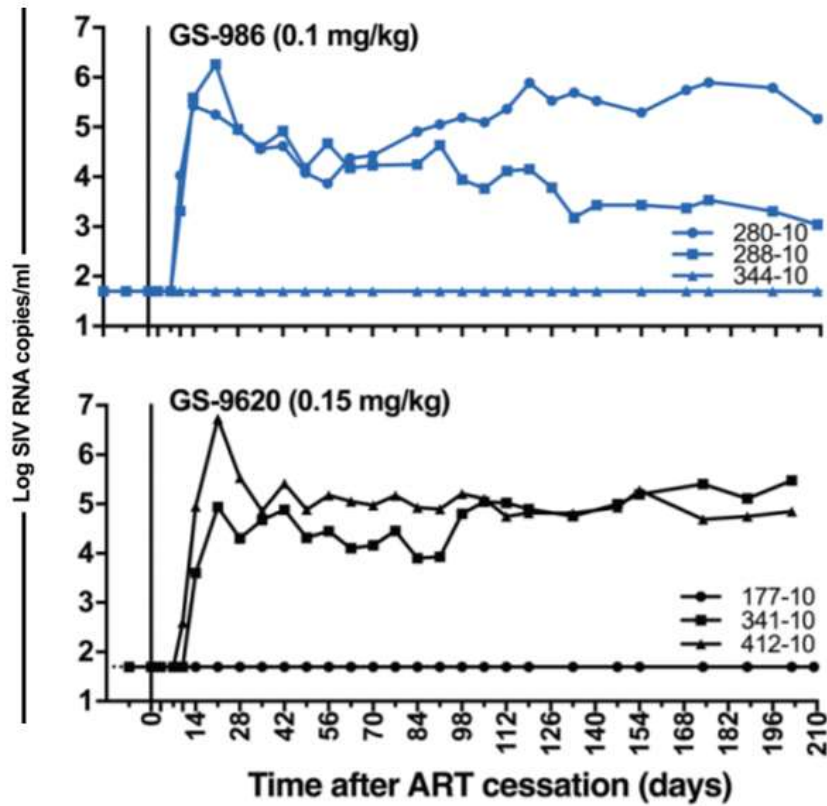


Figure 4. SIV RNA Rebound Kinetics in SIV-Infected RMs After ART Cessation: Viral rebound kinetics after stopping ART in groups of RMs treated with either GS-986 (0.1 mg/kg; n=3; blue) or GS-9620 (0.15 mg/kg; n=3; black). Log plasma virus RNA was assessed between days 1 and 210 after ART cessation. Figure adapted from Lim et al., 2018.

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