Impact of CCR5Δ32/Δ32 Allogenic Hematopoietic Stem Cell Transplant on Adaptive Immune Parameters in HIV Infection

by

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Abstract

Introduction: Human Immunodeficiency Virus (HIV) is a chronic viral infection without a cure. While antiretroviral medications allow people with the infection live long and healthy lives, treated HIV remains associated with a degree of immune exhaustion that is linked to poor clinical outcomes. One individual, the so called "Berlin Patient", was cured of HIV with a CCR5Δ32/Δ32 stem cell transplant. It remains unclear how such a transplant impacts the long standing immune exhaustion of HIV. Purpose: To examine changes in immune phenotype and function after a CCR5Δ32/Δ32 stem cell transplant in the context of HIV infection. **Methods:** A person living with HIV received a CCR5Δ32/Δ32 allogenic hematopoietic stem cell transplant for chronic myeloid leukemia. The inducible viral reservoir in peripheral blood was quantified both pre and post-transplant. Immune exhaustion marker expression on T cells and B cell subsets were determined by polychromatic flow cytometry. T and B cell responses to HIV, cytomegalovirus (CMV), and influenza antigens were determined by ELISPOT and ELISA. We compared immune phenotype, immune function, and proviral reservoir to a matched local cohort. **Results:** Full donor engraftment was achieved and the HIV proviral reservoir was reduced, but not eliminated. At 9 months post-transplant, the recipient had an increase in phenotypically exhausted $CD8^+$ T cells and immature B cells, but also an increase in activated $CD8^+$ T cells and a decrease in exhausted memory B cells. $CD4^+$ T cells showed variable changes in immune exhaustion While anti-HIV T cell responses were greatly diminished, anti-HIV B cell responses were not after transplant. Finally, B cell responses against CMV and influenza remained low, while anti-CMV T cell responses remained high. **Conclusion:** Taken together, these data suggest that a stem cell transplant with HIV-resistant stem cells may not reduce all immune exhaustion, but HIV reservoir is more limited. The transplanted immune cell phenotype is influenced by factors beyond HIV, including other viral infections post-transplant. Further examination of people living with HIV receiving CCR5Δ32/Δ32 stem cell transplants will further delineate the role of reversible immune exhaustion in HIV and other clinical outcomes.

List of Abbreviations and Symbols Used

α – Alpha

Ab – Antibody

ACD – Acid Citrate Dextrose

AEC - 3-Amino-9-ethylcarbazole

AIDS – Acquired Immunodeficiency Syndrome

alloHSCT – Allogenic Hematopoietic Stem Cell Transplant

AM – Activated Memory

AML – Acute Myeloid Leukemia

APC – Antigen Presenting Cell

ART – Antiretroviral Treatment

ARV – Antiretroviral

ATG – Anti-Thymocyte Globulin

β – Beta

BCR-ABL1 – Breakpoint Cluster Region–Abelson Murine Leukemia Viral Oncogene Homolog 1

bp – Base Pair

BSA – Bovine Serum Albumin

ºC – Degree Celsius

CCR – C-C Chemokine Receptor

CD – Cluster of Differentiation

CDC – Centers for Disease Control

CEF – Cytomegalovirus, Epstein-Barr Virus, Influenza Virus

CML – Chronic Myeloid Leukemia

CMV - Cytomegalovirus

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats

CO_2 – Carbon Dioxide

CXCR – C-X-C Chemokine Receptor

Δ - Delta

DC – Dendritic Cell

D/C - Discontinued

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic Acid

ELISA – Enzyme Linked Immunosorbent Assay

ELISPOT – Enzyme Linked Immunospot

Env – Envelope

FBS – Fetal Bovine Serum

FMO – Fluorescence Minus One

γ – Gamma

g – G-Force

Gag – Group-Specific Antigen

GALT – Gut Associated Lymphoid Tissue

gp – Glycoprotein

HA – Hemagglutinin

HBV – Hepatitis B Virus

HCV – Hepatitis C Virus

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV – Human Immunodeficiency Virus

HLA – Human Leukocyte Antigen

hr -Hour

HRP – Horse Radish Peroxidase

I/T – Immature/Transitional

ICISTEM - International Collaboration to guide and Investigate the potential for HIV cure by Stem Cell Transplantation

IFN – Interferon

IgG – Immunoglobulin G

IL – Interleukin

kB - Kilobase

KIR - Killer-cell immunoglobulin-like receptor

KLH – Keyhole Limpet Hemocyanin

M – Molar

mg – Milligram

min - Minute

mL – Milliliter

MRC-5 – Medical Research Council cell strain 5

NK – Natural Killer

NP - Nucleoprotein

OD – Optical Density

PBMC – Peripheral Blood Mononuclear Cell

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PD-1 – Programed Cell Death Protein 1

PHA – Phytohaemagglutinin

PMA – Phorbol 12 myristate 13 acetate

pmol – Picomole

R848 – Resiquimod

Rev – Regulator of Expression of Virion Proteins

RM – Resting Memory

RNA – Ribonucleic Acid

RPMI – Roswell Park Memorial Institute Medium

RT – Reverse Transcription

SCT – Stem Cell Transplant

sec - Second

Tat – HIV Trans-Activator

TBE – Tris-Borate EDTA

TBI – Total Body Irradiation

TCR – T Cell Receptor

TE – Tris-EDTA

Tfh – T Follicular Helper

Th – T Helper

TILDA – Tat/Rev Limiting Dilution Assay

Tim-3 - T-cell immunoglobulin and mucin-domain containing-3

TKI – Tyrosine kinase inhibitor

TLM – Tissue-like Memory

TMB - 3,3',5,5'-Tetramethylbenzidine

TNF – Tumor Necrosis Factor

Treg – T Regulatory Cell

Tris – Tris-aminomethane

Tween-20 – Polysorbate 20

V - Volts

VL – Viral Load

Vpu – Viral Protein U

µg – Microgram

µL – Microliter

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Chapter 1: Introduction

1.1 Human Immunodeficiency Virus in Human Health

Human Immunodeficiency Virus (HIV) is a pathogen that causes a chronic, incurable infection. As a member of *Retroviridae*, HIV establishes chronic infection by integration of its genome into that of the host. This stabilizes the latent form of the genome and allows for latency to be maintained for extended periods of time, up to years (A. J. Murray, Kwon, Farber, & Siliciano, 2016). HIV infects the key regulator of adaptive immunity, the $CD4^+$ T Cell, leading to dysregulation of both cell-mediated and humoral immunity over the course of the infection. As such, chronic HIV infection leads to an increased risk of other infections, especially by opportunistic pathogens, and an increased risk of cancer (Calcagno et al., 2015).

HIV is a significant public health threat. Since the first recognized human cases of severe immunodeficiency arose in 1981, HIV is estimated to have infected 76.1 million people and lead to 35 million HIV-related deaths by the end of 2016 (UNAIDS, 2017). As of 2016, about 37 million people worldwide are infected and 1.1 million people die every year due to HIV-related complications (UNAIDS, 2017). This global epidemic has also disproportionately affected particular populations. Men who have sex with men, people who inject or use drugs, transgender individuals, and people who work in the sex industry, and sexual contacts of these groups are at risk for HIV acquisition, especially if they live in endemic areas such as Africa (Ortblad, Lozano, & Murray, 2013). Up until 1987, HIV infection was effectively a death sentence due to a lack of effective treatments. With the introduction of highly potent antiretrovirals (ARVs) and increased efforts to treat and prevent HIV globally, it is now a chronic, manageable illness. People with HIV are living longer than ever due to ARVs and these medications are thought to have prevented 1.2 million deaths in 2016 alone (UNAIDS, 2017). While ARVs have revolutionized care, unequal access to delivery of care makes HIV treatment a challenge even in the current era (Ortblad et al., 2013).

1.2 The HIV Virus: Structure and Replication

As a retrovirus, HIV is an enveloped RNA virus. It features two major glycoproteins on its envelope, gp41 and gp120, and has its genome surrounded by a

protein capsid. It also carries a reverse transcriptase and an integrase, two proteins that are essential for initial infection of target cells. Figure 1.1 illustrates the key events of HIV infection of a new cell and reactivation.

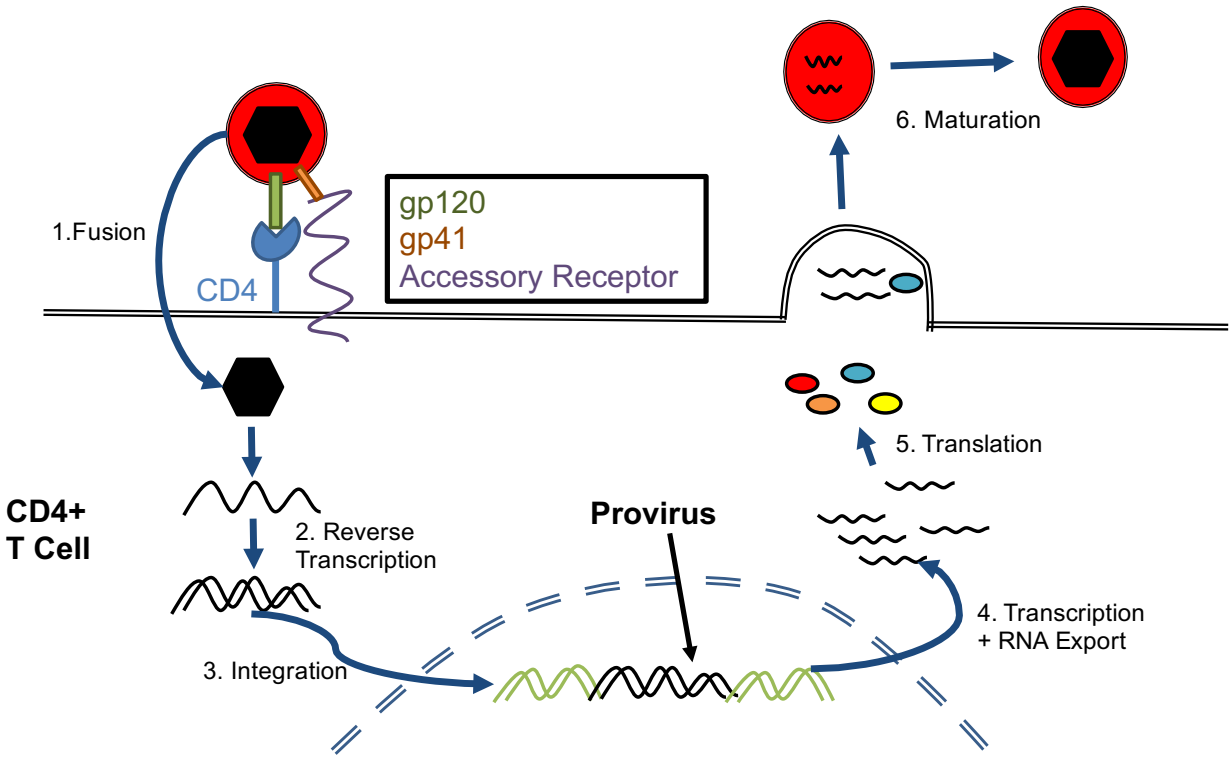


Figure 1.1: Key events in the Infection of a New Cell by HIV.

Upon nearing a target cell, most often a $CD4^+$ T cell, gp120 will bind to CD4 and an accessory cell surface protein, either CCR5 or CXCR4. This allows for viral entry via endocytosis, where conformational changes in the viral envelope proteins allow for fusion with the host membrane and release of the capsid into the cytoplasm (Root & Hamer, 2003). Viral tropism is defined by which of these coreceptors these virus uses for entry, with R5 tropic requiring CCR5 and X4 tropic requiring CXCR4. After the virus uncoats, the reverse transcriptase converts the RNA genome into a DNA genome. This is an error prone process, which allows for the introduction of mutations, such as drug resistance mutation, into the genome that can be passed to progeny virions (Abram et al., 2014). Once inside the nucleus, the integrase protein interacts with the host genome and integrates the HIV genome into the host (Hazuda et al., 2000). At this point, the virus may remain latent as a provirus for an extended period of time (Rothenberger et al., 2015).

This provirus waits until the cell reactivates through T cell receptor signaling so that it can begin its own replication (Kim, Mbonye, Hokello, & Karn, 2011). From this point, replication continues much like other viruses. HIV first produces early proteins that enhance replication, such as Tat and Rev (Feinberg, Baltimore, & Frankel, 1991; Zapp & Green, 1989). The virus then transitions to producing later stage proteins, such as Env to make envelope proteins and Gag to make the capsid. The virus then assembles at the plasma membrane and buds out to form a progeny virion (Ivanchenko et al., 2009). Finally, a protease performs the final maturation step inside the virion to form a fully infectious virion (Göttlinger, Sodroski, & Haseltine, 1989).

1.3 Early Events in HIV Infection

HIV is a blood-borne virus transmitted from person to person when HIV-infected fluids (including blood, semen, breast milk, and vaginal secretions) contact breaks in the epithelium (Maartens, Celum, & Lewin, 2014). Upon contact, such as through sex or use of contaminated needles, HIV enters the stroma of the epithelium. Here, virions may infect $CD4^+$ T cells in the mucosal surface lamina propria or bind to dendritic cells (DC) to bring them to other sites of $CD4^+$ T cell residency (Geijtenbeek et al., 2000). To enter these cells, HIV binds CD4 and either CCR5 or CXCR4 as coreceptors to enter the cytoplasm of the T cell where it can begin active replication or establish active infection by use of viral reverse transcriptase and integrase proteins (Farnet & Haseltine, 1990). In latency, these proteins convert and insert the HIV genome into the host genome. The infection begins to spread locally as $CD4^+$ T cells in the lamina propria begin to activate as HIV can reactivate in these cells and produce new virion (Meng et al., 2000). Over the course of days, DCs attach to HIV virions and latently infected $CD4^+$ T cells make their way to lymph nodes (Geijtenbeek et al., 2000). These $CD4^+$ T cell rich sites allow for amplification of HIV virions, dissemination of latently infected cells systemically, and establishment of the proviral reservoir (Pasternak et al., 2008). Overtime, latently infected T cells can escape these lymph nodes to seed other sites with an HIV reservoir, such as the gut or testes (Jenabian et al., 2016; Q. Li et al., 2005).

After several weeks of viral amplification in secondary lymphoid organs, HIV RNA is detectable in plasma, followed shortly after by detection of HIV structural and

non-structural proteins (Feinberg et al., 1991). This is associated with acute HIV syndrome in many, though not all, HIV infected persons. Acute HIV syndrome is an initial period of illness associated with initial infection with HIV and can feature myalgia, fever, and pain (Daar, Pilcher, & Hecht, 2008). While up to 75% of people may present with symptoms consistent with acute HIV syndrome, they are typically nonspecific and often not diagnosed as acute HIV syndrome (Daar et al., 2008). More specific findings, such as cervical lymphadenopathy, may occur in only 39% of people, with variance based on sex, likely route of transmission, and use of intravenous drugs (Daar et al., 2008). During this period, $CD4^+$ T cell counts drop dramatically and HIV viral load increases. While this is not clearly understood, it is thought that both viral proteins, host self defense mechanisms, and pro-inflammatory cytokines play a role rather than a cytopathic effect of the virus (Westendorp et al., 1995) (Herbeuval et al., 2005; Monroe et al., 2014). Within 12 weeks of initial infection, peak viremia passes and $CD4^+$ T cell numbers recover partially, though virus remains detectable in blood and other fluids (Goonetilleke et al., 2009). This is due to both $CD8^+$ T cell control of virally infected cells and regeneration of lost $CD4^+$ T cells (McCune et al., 2000) (J. Cao, McNevin, Malhotra, & McElrath, 2003). $CD4^+$ T cell numbers never recover to pre-infection levels as the central memory T cell population that is responsible for regeneration becomes exhausted and shrinks in size (Day et al., 2006; Okoye et al., 2007). Without treatment, $CD4^+$ T cells continue to die over the course of 2 to 3 years while HIV viremia rebounds. The relative deficit of $CD4^+$ T cell numbers and function leads to both an inability to control HIV and also an increased susceptibility to opportunistic infections from pathogens such as *Pneumocystis jirovecii* and *Cryptococcus neoformans* (Maartens et al., 2014).What immune cells are still present have reduced response to antigenic challenge (Sokoya, Steel, Nieuwoudt, & Rossouw, 2017)**.** This immunosuppression eventually leads to death, usually linked to infection or cancer if treatment is not started. Persistent immune activation and inflammatory cytokines however can lead to increased mortality to non-infectious or cancer causes, such as cardiovascular disease (Sinha et al., 2016).

The advent of ARV therapy has drastically changed the outlook of chronic HIV infection. ARVs pharmacologically target different parts of the HIV viral lifecycle by inhibiting HIV entry, integration replication or viral procession. The main commercially

available steric or non-steric targets of therapy (from entry through to integration) are CCR5, reverse transcriptase, protease, and integrase. New targets are being researched as well, such as the recently discovered capsid inhibitor (Thenin-Houssier et al., 2016). The development of antiretroviral resistance has led to the standard of practice of treating HIV using 3 drugs with at least 2 different mechanisms of blocking HIV replication. While there is variation in first line therapy due to differences in HIV resistance, comorbidities, side effects, and personal preference, a regimen of 2 nucleoside inhibitors with either an integrase inhibitor or a protease inhibitor with a pharmacokinetic enhancer is typical (Department of Human Health & Services, 2018).

Unlike other chronic viral infections, like HCV, ARVs cannot cure HIV. This is because the ARVs cannot stop the initial formation of proviral reservoir. As well, ARVs are unable to remove integrated HIV DNA already in immune cells, leading to persistence of the reservoir in the face of highly effective therapy (Maartens et al., 2014). This is an issue because chronic HIV infection, even one that is well treated, is associated with increased risk of a number of diseases, such as cardiovascular disease, due to an increase in inflammation and immune exhaustion (Paisible et al., 2015; Tenorio et al., 2014). These contribute to a reduction in quality of life for the individual and increased burdens on the health care systems that treat them (Crothers et al., 2005; Quiros-Roldan et al., 2016). While ARV therapy has revolutionized how we treat HIV, the lack of HIV cure with these medications and the persistence of the health issues noted above highlight the need to understand the HIV-induced immune changes that persist during highly active therapy. Only with better understanding of these changes can we move forward on improving strategies for living well with HIV as we age, and also offer new avenues of exploration for cure.

1.4 Immune Changes in HIV Infection

HIV directly infects the immune system. It is not surprising therefore that HIV infection is associated with perturbations in immune system function. Broadly, HIV induces “immune exhaustion”. This is a low function state characterized by upregulation of surface proteins associated with suppression of immune function, downregulation of surface proteins associated with increases in immune function, and an inability for these

cells to properly perform their functions when challenged, whether that be secreting cytokines or producing antibodies. (Khaitan & Unutmaz, 2010). While many of the mechanisms behind immune exhaustion remain elusive, chronic antigenic stimulation of immune cells is thought to induce exhaustion (Kahan, Wherry, & Zajac, 2015). This exhaustion is observed in both a phenotypic and functional manner. Phenotypically, immune exhaustion is associated with differential expression in activating and inhibitory makers on $CD4^+$ T Cells, $CD8^+$ T cells, and B cells (Kaufmann et al., 2007; Moir et al., 2008; Tavenier et al., 2015). While these markers are cell type dependent, the overall impact is that the increased presence of inhibitory markers, combined with fewer activating makers, shifts the signaling balance such that cells are less likely to react to antigen and perform their prescribed functions. Functionally, T cells produce less IFN-γ and B cells produce less antibody, amongst other changes (Lane et al., 1983). Clinically, immune exhaustion is associated with a decreased ability to clear cancer cells, decreased responsiveness to vaccines, and increased susceptibility to infections (El-Far et al., 2008; Malaspina et al., 2005; Moir & Fauci, 2014; Zarour, 2016). Given the broad immune effects that immune exhaustion has on the body, better understanding immune exhaustion within HIV infection can allow us to better combat the immune changes that help lead to poor immune health in those living with HIV.

1.4.1 Acute HIV Infection

Immune exhaustion starts during the acute phase of HIV infection. The rapid changes induced by the acute phase lay the groundwork for broader immune dysfunction in the chronic phase. Due to the fact that most HIV cases are not diagnosed until after this phase, much of our work has come either from animal models or from limited studies of acutely infected individuals. As such, many of the mechanisms behind these changes are not well understood. Interestingly, this phase is defined by immune activation even as cells are actively dying. HIV predominately infects $CCR5^+$ $CD4^+$ T cells, which either are activated or have newly transitioned into the memory phenotype, and takes advantage of active replication happening in these cells (Douek et al., 2002). While many of these cells die, either due to their short half-life or cell mediated lysis, a number of them survive to form the reservoir or produce more HIV in this acute burst of reproduction. This is especially pernicious in the gut associated lymphoid tissue (GALT), where the large

number of memory $CD4^+$ T cells provide ample target and lead to subsequent depletion of the $CD4^+$ T cell compartment (Q. Li et al., 2005), (Wang et al., 2007). The presence of HIV induces $CD8^+$ T cell responses, especially with regards to IFN-γ production. Activated $CD8^+$ T cells, alongside plasmacytoid dendritic cells, produce type I IFNs in mass due to high levels of HIV RNA (Sokoya et al., 2017). While this does help bring HIV under control, this pro-inflammatory state induces cell death amongst both HIV-infected and bystander cells, depleting the memory T cell compartment even further. It also suppresses T cell function and induces formation of immunosuppressive T regulatory cells (Sokoya et al., 2017). Finally, B cells become markedly deformed in acute infection. They exhibit polyclonal activation, leading to greatly elevated levels of IgG and formation of immune complexes that further induce immune activation. As well, the pro-inflammatory state induced in HIV infection also induces B cell apoptosis, leading to depletion of the memory B cell compartment (Amu et al., 2014). Overall, acute HIV infection is associated with dysregulated immune activation and depletion of crucial cell populations that set the stage for immune dysfunction later on.

1.4.2 Chronic HIV Infection

In the absence of treatment, the immune changes of acute infection progress as immune cells die off or become exhausted. The advent of ART has changed this picture dramatically. More recent work has gone into understanding the changes HIV infection induces in the presence of ART. While research has shown that some of the immune exhaustion induced by HIV is reversible, there are numerous cell-specific differences that influence long term immune exhaustion in the era of ART therapy (Moir, Chun, & Fauci, 2011).

1.4.2.1 $CD4^+$ T Cells

$CD4^+$ T cell exhaustion is frequently a focus of HIV research, as these cells are the primary target of HIV infection and they are critical in regulating the adaptive immune response. While ART initiation helps halt the massive cell death seen in acute infection and slow future $CD4^+$ T cell death, $CD4^+$ T cell levels never rebound to the levels seen pre-infection (Maartens et al., 2014). This is particularly true in the GALT, where the $CD4^+$ T cell population is heavily depleted and never is fully reconstituted

(Chun et al., 2008). As well, $CD4^+$ T cells have higher turnover rates during treated and untreated HIV infection when compared to uninfected individuals (Lempicki et al., 2000). This turnover is associated with a shift in the memory $CD4^+$ T cell population toward more terminally differentiated T cells that lack expression of CD28 and gain expression of the exhaustion marker CD57 (Vivar et al., 2011). These terminally differentiated cells lack proliferative capacity and have reduced functionality in response to antigenic challenge (Vivar et al., 2011). Increases in these populations would naturally reduce the ability for these memory T cells to help initiate future immune responses to pathogens, thereby contributing to the immunosuppression seen in chronic HIV.

In addition to reduced numbers of $CD4^+$ T cells, the remaining $CD4^+$ T cells present an exhausted phenotype. In addition to CD57 mentioned above, a number of other markers have been associated with $CD4^+$ T cell exhaustion. These include Programmed Cell Death Protein 1 (PD-1), T-cell immunoglobulin and mucin-domain containing-3 (Tim-3), and CD152 (CTLA-4) (Antonelli et al., 2010; Day et al., 2006; Jones et al., 2008). These markers all have emerging roles in downregulating T cell responses through various pathways. PD-1 and CTLA-4 both affect CD28 signaling, while CTLA-4 also acts by disrupting TCR signaling (Day et al., 2006; Kaufmann et al., 2007). Tim-3 negatively regulates the expression of T cell cytokines such as IFN-γ and IL-2 (Hastings et al., 2009). Upregulation of these markers leads to greater $CD4^+$ T cell suppression by preventing activation and function in response to antigenic challenge. The regulatory mechanisms are not well understood, but it is known that chronic antigenic stimulation and T cell activation in chronic HIV infection is associated with higher expression of these molecules (Antonelli et al., 2010). In treated HIV infection, this chronic activation comes in part from background HIV replication but also through other mechanisms such as microbial translocation due to the destruction of the GALT (Brenchley et al., 2006; Cohen Stuart et al., 2000). Increased exposure to microbial products is thought to further activate the immune system in chronic HIV, leading to greater expression of immune exhaustion markers (Brenchley et al., 2006). While the molecular mechanisms of immune exhaustion are still unknown, it is clear that certain T cell immune exhaustion markers are upregulated in HIV and work to suppress $CD4^+$ T cell function after partial reconstitution by ART.

The effects of immune exhaustion in $CD4^+$ T cells directly impacts both $CD8^+$ T cells and B cells, as both populations require $CD4^+$ T cell help at some point in the development of their respective immune responses. While decreased numbers of $CD4^+$ T cells is partly to blame, functional differences in $CD4^+$ T cells have been observed as well. Exhausted $CD4^+$ T cells express fewer functional cytokines, such as IL-17, IL-2, IL-6, and IFN-γ (Hastings et al., 2009). These are important as they can trigger immune responses and help other arms of the adaptive immune system respond to pathogens. Furthermore, T cell exhaustion is also associated with increased expression of other pro-inflammatory cytokines, such as TNF, IL-1β, and IL-6 (Davalos, Coppe, Campisi, & Desprez, 2010). These help to further maintain a state of immune activation that drives immune exhaustion. Overall, $CD4^+$ T cells in chronic HIV are marked by increased levels of immune activation that help drive the formation of exhausted, less functional populations that are hyporesponsive to antigenic challenge. Such exhaustion has downstream effects on cell types that rely on $CD4^+$ T cell help for function.

1.4.2.2 $CD8^+$ T Cells

While not directly infected by HIV, $CD8^+$ T cells are impacted by HIV infection and remain persistently elevated in chronic HIV infection even with successful ART. While these levels are known to be influenced by many host and environmental factors, such as age and CMV serostatus, none of these fully explain the persistent elevation of $CD8^+$ T cells seen in HIV infection (Wei Cao, Mehraj, Kaufmann, Li, & Routy, 2016). These expanded cells tend to be memory or effector cells rather than naïve cells and have markers of persistent activation similar to $CD4^+$ T cells (Weiwei Cao et al., 2009). Furthermore, these expanded cells are not just from the HIV-specific compartment. Rather, it is a broad, non-specific activation of $CD8^+$ T cells (Doisne et al., 2004). The mechanisms behind this in ART are still unclear, but the persistent exposure to a pro-inflammatory environment rather than exposure to particular antigens may drive this (Bastidas et al., 2014). Activation is a normal response to antigen presentation, but the persistent activation of these cells can be detrimental by furthering a persistent pro-inflammatory state that helps induce exhaustion amongst many immune cell types (Youngblood et al., 2013). This is important, as persistent $CD8^+$ T cell elevation is

associated with poor clinical outcomes beyond HIV related disease, including cardiovascular disease, renal disease, and respiratory disease (Serrano-Villar et al., 2014).

Even though the $CD8^+$ T cells are expanded and activated in chronic HIV infection, this persistent activation also leads to an exhausted phenotype. Phenotypic immune exhaustion of $CD8^+$ T cells features many of the same markers as $CD4^+$ T cells, including PD-1 and Tim-3, though ARV therapy does help reduce the expression of these markers (Jones et al., 2008; Youngblood et al., 2013). This translates functionally to decreased IFN- γ production in response to antigenic challenge as well as decreased perforin levels, which work together to create $CD8^+$ T cells that are suboptimal at killing virally infected and tumor cells (Day et al., 2006). This contributes to the increase risk of infections and cancers seen in HIV^+ people on ART (Day et al., 2006). As well, coexpression of these markers further impacts immune exhaustion, forming populations of highly exhausted immune cells that become less functional with the expression of each additional marker (Blackburn et al., 2009). Therefore, like $CD4^+$ T cells, expression of inhibitory markers remains in chronic HIV infection in $CD8^+$ T cells and is associated with poor functionality within this group.

1.4.2.3 B Cells

Chronic HIV infection is associated with a number of changes in B cells even with ARV induced control. While the acute phase is marked by hypergammaglobulimia, ART helps induce normalization of this aberrant B cell response (Moir et al., 2010). Like T cells however, chronic antigenic stimulation leads to a shift in memory populations (Amu et al., 2014). Table 3.1 describes the characterization of these populations by surface marker expression.

Table 1.1: Select Peripheral B Cell Populations by Immune Marker Expression. All populations also express CD19.

Population	CD10	CD20	CD21	CD27
Immature/Transitional	+	+	+/-	-
Naïve	-	+	+	-
Resting Memory	-	+	+	+
Activated Memory	-	+	Low	+
Tissue-like Memory	-	+	Low	-

Typically, the largest populations of peripheral B cells include naïve and resting memory B cells, both of which are ready to respond to antigenic challenge. In chronic HIV, there is a shift away from these cell types. While this is established in acute infection, with destruction of memory B cell populations, these are not corrected with ART and the mechanisms for this perturbation and its maintenance are not well understood (Amu, Ruffin, Rethi, & Chiodi, 2013). What is known is that there is an expansion of rarer and less functional memory populations, in particular activated memory and tissue-like memory (TLM) B cells (Moir et al., 2008). Activated memory B cells have a low proliferative capacity, but secrete large amounts of IgG and may play a role in the hypergammaglobulimia seen in acute HIV (Notermans et al., 2001). On the other hand, TLM B cells show a reduced potential to proliferate and produce antibodies in response to challenge (Malaspina et al., 2005)**.** This is of particular note because HIV-specific B cells tend to be enriched in the TLM subset and this may partly explain why there are variable levels of anti-HIV antibody expression amongst people living with HIV (Moir et al., 2008). Expansion of these two populations is associated with poor responsiveness to potent vaccines (Malaspina et al., 2005). There is also a normalization of immature/transitional B cells, which are short lived and immature, after an increase in acute HIV (Malaspina et al., 2006). While B cells are not targets for HIV infection, there

are broad changes to subset balance that are associated with poor clinical outcomes in the age of ART.

Even though there is noticeable B cell dysfunction, antibodies against HIV are produced by many people in response to infection. These antibodies can be seen as early as a week after infection as the infection transitions from the acute to chronic phases. Despite this, these non-sterilizing antibodies are particularly poor at neutralizing HIV (Tomaras et al., 2008). During the first months of infection, neutralizing antibodies are produced but have limited epitope specificity, and the pressure they elicit on HIV drives divergent evolution of gp41 and gp120, the two envelope proteins predominately targeted by anti-HIV antibodies (Bunnik, Pisas, van Nuenen, & Schuitemaker, 2008). Overall, B cell dysfunction is largely reversed upon initiation of ART, yet longstanding memory perturbations and the unclear generation of an effective anti-HIV antibody response allow for continued dysfunction against HIV and bystander antigens.

1.4.3 Immune Exhaustion in Other Conditions

Immune exhaustion is not solely a problem associated with HIV. It can be related to other chronic conditions, such as cancer and chronologic aging, but chronic viral infections are set apart by the speed in which this exhaustion happens. Chronologic aging is also associated with PD-1, Tim-3, and CTLA-4 expression and is thought to lead to decreased vaccine responsiveness and increased risk of infection in older people (Canaday et al., 2013; Lee et al., 2016). Immune exhaustion is also associated with other chronic viral infections, such as HCV and CMV (McMahan et al., 2010; Simanek et al., 2011). CMV is of particular importance, as up to 60% of the adult population is latently infected with this herpes virus (Staras et al., 2006). CMV infects epithelial cells and establishes latency in other cell types, especially myeloid progenitor cells, though rarely causes clinical disease unless immunocompromised (O'Connor & Murphy, 2012). CMV-associated immune exhaustion is similar to that of HIV, where T cells express more exhaustion markers and clonal expansion of this population may prevent new memory cells directed against other antigens from maturing (Margolick et al., 2018). This has led to some debate as to how much of the $CD8^+$ T cell exhaustion is due to HIV versus CMV in coinfected individuals (El-Far et al., 2008). Finally, many cancers are associated with

immune exhaustion, as the exhausted phenotype is associated with decreased immune surveillance and less functional activity towards tumor cells (Zarour, 2016). Immune exhaustion in cancer and the use of checkpoint inhibitors, including a PD-1 antagonist and a CTLA-4 antagonist, have been developed and are used in clinics currently (Hamid et al., 2013) (Hodi et al., 2010). While these are very recent developments and not much is known about the long-term effects of these drugs, the lessons learned from the use of these drugs may help inform future treatments of HIV. The field of immune exhaustion in HIV is constantly evolving as insights from other diseases inform us on the role of shared immune proteins and the impact of therapies on other immunosuppressive conditions.

1.4.3.1 Immune Exhaustion and Chronic Myeloid Leukemia

Of particular importance for this thesis is the hematologic cancer Chronic Myeloid Leukemia (CML). This is a myeloid lineage cancer associated with translocation of the *BCR-ABL1* oncogene, forming the "Philadelphia Chromosome", that leads to constitutive expression, resistance to apoptosis, and a blockade to differentiation, amongst other effects. While pharmaceuticals, particularly tyrosine-kinase inhibitors (TKIs), slow disease progression and extend the life expectancy of those living with this disease, they are not able to induce remission in all cases (Ross et al., 2013). While immune exhaustion in CML is due to a number of different cell types, there are shared features with immune exhaustion seen in HIV. Studies into the effects of tyrosine kinase inhibitors have shown elevated PD-1 expression on T cells in CML patients and a subsequent decrease in expression of PD-1 after TKI induced CML (Hughes et al., 2017). This also was associated with an increase in both T cell and NK cell function after discontinuing TKI therapy (Hughes et al., 2017). At the same time, people living with CML have decreased memory B cell populations and reduced responsiveness to vaccination, which is a similar phenotype to that of chronic HIV (de Lavallade et al., 2013). The B cell defects are thought to be more due to the immunosuppressive effects of TKI rather than the CML itself (de Lavallade et al., 2013). As such, the effects of CML may have varying effects of immune exhaustion. Despite this, the literature surrounding CML is lacking with regards to the expression of other immune exhaustion markers, such as CTLA-4, Tim-3, and less functional B cell subsets such as TLM B cells. While much still remains unknown about immune exhaustion in CML, there are clear similarities within B cell function and PD-1

expression that mimic those seen in HIV infection. It is not known whether HIV and CML act cumulatively to exacerbate this immune dysregulation.

1.5 HIV Provirus and Reservoir Analysis

The immune exhaustive effect of HIV is directly linked to its ability to persist long after acute infection has resolved. Such latency allows the virus to reactivate if not suppressed pharmacologically. As $CD4^+$ T cells and macrophages are the primary targets for HIV infection, HIV reservoirs can be located wherever these cells are located. These include lymph nodes, mucosal associated lymphoid tissues, central nervous system and spleen (Chun et al., 2008). Proviral burden has been associated with accelerated clinical progression in chronic HIV and therefore, estimating proviral load may be prognostic immunologically and clinically.

One of the current challenges to proviral load is getting tissue to test from non-blood borne sites such as spleen, GALT and the central nervous system. Knowledge of the HIV reservoir in humans largely comes from peripheral blood and may be an underestimate, as they exclude cells that are resident in other tissues. Second, the tests we currently have for identifying proviral reservoir have issues providing a true answer for the size of the reservoir. Culture-based assays, such as the quantified viral outgrowth assay, assess infectious virus production, yet fail to identify cells with non-inducible HIV DNA. Research has shown that this could be up to 99% of cells, with only 12% of those harboring replication competent virus (Ho et al., 2013). On the other hand, PCR based assays are able to detect provirally-infected cells regardless of their ability to make infectious virion, which may overestimate the reservoir when asking about the likelihood of virus infecting nearby uninfected cells (Hodel, Patxot, Snäkä, & Ciuffi, 2016). The lack of consistent tests, combined with the inability to feasibly test all reservoir locations, makes reservoir quantification difficult. Development of more consistent tests that are able to examine all competent provirus would allow us to better understand the true burden of provirus in chronic HIV and help monitor treatments that look to reduce this factor associated with negative clinical outcomes. For now, the most reliable use of the various tests is longitudinally within the same individual or the compare groups, as opposed to an individually meaningful test.

1.6 Attempts at HIV Cure

Given the global burden of HIV and the long-term health impacts of chronic HIV infection even with ART, research has been keenly focused on finding a cure for the infection. As mentioned previously, one of the major hurdles to do this is identifying all infected cells and developing techniques to remove these cells. As well, the integrated genome of HIV makes it a difficult target for removal. This makes it different than HCV, where an unintegrated genome and better understood localization of infected cells have allowed for the development of curative antivirals. One proposed theory was to eliminate all immune cells in the body and then reconstitute the immune system. This would eliminate the reservoir and allow for an uninfected immune system to be formed. The discovery that a 32 base pair deletion in the CCR5 gene, which is a obligate co-receptor for HIV viral entry into cells, could provide resistance to HIV further fueled interest in this mechanism. On immune cells, CCR5 is involved with migration of leukocytes release of inflammatory cytokines (Allers & Schneider, 2015). The Δ32 mutation is a naturally occurring mutation and while people living with this allele are generally healthy and not considered immune compromised, there have been studies showing the mutation is associated with a higher chance of disease with West Nile Virus but also protection from chronic Hepatitis B infection (Glass et al., 2006; Thio et al., 2006). Given the lack of overall disease associated with this mutation and the variable effects on immunity, this mutation was thought to be a candidate for HIV cure.

In 2009, a team from Germany described a case in which a person living with HIV was given a stem cell transplant for acute myeloid leukemia. The "Berlin Patient" received CCR5Δ32/Δ32 stem cells that in theory should be resistant to infection by his R5-tropic virus. After myeloablative conditioning and ART stoppage at the beginning of the transplant, the patient had no viral rebound and has remained so for over a decade (Hütter et al., 2009). Further analysis of this case revealed that not only did the donor derived Δ32 cells effective repopulate the GALT, an important HIV reservoir that is known for resistance to chemotherapy and radiation, there were no host derived $CD4^+$ T cells remaining in the GALT (Allers et al., 2011). This is despite persistence of CCR5 wild type macrophage persisting in tissue, which represent a potential reservoir (Allers et al., 2011). As well, viral nucleic acid could not be detected in tissue at 45 months after

transplant and anti-HIV antibody levels declined, in keeping with viral cure (Allers et al., 2011). While this case is important as a proof of concept that eradicating the viral reservoir *in vivo* would lead to functional cure, there has been a lack of analysis into persistent immune exhaustion seen in chronic HIV and whether or not this stem cell transplant has changed them. As well, despite the promise that this case provided, researchers have not been able to replicate the same outcome in any other individual. This is due to a number of factors, such as the low prevalence of the CCR5Δ32/Δ32 mutation as well as the risks associated with stem cell transplant. As such, the Berlin Patient provides a good starting point for understanding how to cure HIV but highlights the need for more analysis into the impacts of cure attempts on other known HIV pathologies.

Other groups have tried to cure HIV in other ways. One way was to treat acute HIV very early in the hopes of preventing the formation of long lasting viral reservoir. The Mississippi Baby was treated aggressively with ART just after birth and retained undetectable viral load while off ART for 18 months before having a viral load rebound (Persaud et al., 2013). A more recent study examined treatment interruption in a group of people living with HIV who started ART during their acute HIV infection, which is much earlier than most people (Sáez-Cirión et al., 2013). After treatment interruption, there was long term control of HIV viremia, though no eradication of reservoir, and stable $CD4^+$ T cell levels. There was also a reduction of T cell activation, which may contribute to less immune activation. They also exhibit lower proviral reservoirs and less of their proviral reservoir in long-lived T cell compartments (Sáez-Cirión et al., 2013). These studies further show that disrupting the reservoir compartment is critical for better survival without ART. Newer technologies, such as CRISPR and zinc finger endonucleases, aim to reduce reservoir sizes in people living with HIV in a less drastic way than stem cell transplant, but the technology is very new (Ophinni, Inoue, Kotaki, & Kameoka, 2018). Furthermore, HIV seems to be able to mutate out of at least the zinc finger method suggesting that these therapies either will have to target multiple parts of the genome at once or find some way to avoid the ability for HIV to mutate its genome to avoid therapies (De Silva Feelixge et al., 2016). While these recent advancements have been promising, there remains a lack of information surrounding the long term immune impacts of attempting to cure HIV.

1.7 An Individual with HIV and CML

Given the lack of replication of the Berlin Patient situation, and outstanding questions surrounding the impacts of such a transplant on other pathologies within HIV infection, it important that similar cases be repeated so that we can better understand these transplants and their effects on HIV and immune exhaustion. We identified a 58 year old male living with HIV who also had chronic myeloid leukemia and was a candidate for a CCR5Δ32/Δ32 stem cell transplant. Their HIV was also serotyped as R5 tropic, meaning the stem-derived target cells would likely be resistant to the circulating dominant virus. These all align very well with the Berlin Patient case and provide a similar case to the one performed in 2009. An observation of the immune impacts of this transplant could provide insights into the changes in immune exhaustion after such a transplant, which is important and stem cell transplant is the only feasible option for proviral reservoir reduction in individuals who did not start ART in acute infection.

1.8 Study Rationale

While we have learned much about immune exhaustion in the context of HIV infection, not much is known about how this immune exhaustion impacts attempts at clearing HIV infection with the current models of HIV cures by stem cell transplant. Furthermore, there is very little knowledge about how levels of immune exhaustion change after stem cell transplant in HIV^+ people. This stem cell transplant case provides us with a unique opportunity to further our knowledge into the association between immune exhaustion and HIV viral reservoirs after stem cell transplant.

1.9 Research Questions, Study Hypotheses, and Objectives

Question 1: Does a CCR5Δ32/Δ32 stem cell transplant result in a depletion of HIV^+ viral reservoir in peripheral blood $CD4^+$ T cells?

Hypothesis: CCR5Δ32/Δ32 stem cell transplant will result in a depletion of HIV^+ viral reservoir in peripheral blood $CD4^+$ T cells.

Objectives: Evaluate stem cell transplant efficacy and immune cell levels by clinical tests. Quantify the peripheral blood reservoir by Tat/Rev Limiting Dilution Assay.

Question 2: Do T and B cells become less phenotypically exhausted after CCR5Δ32/Δ32 stem cell transplant?

Hypothesis: T and B cells will become less phenotypically exhausted after stem cell transplant.

Objectives: Quantify the size of phenotypically exhausted T and B cell subsets in peripheral blood via flow cytometry.

Question 3: Do HIV-specific T and B Cell responses decrease post-transplant?

Hypothesis: HIV-specific T and B Cell responses will decrease post-transplant, in keeping with reduction in proviral reservoir.

Objective: Quantify the number of HIV-specific IFN-γ and antibody secreting cells by ELISPOT.

Question 4: Do bystander T and B Cell responses increase post-transplant?

Hypothesis: Bystander T and B Cell responses will increase post-transplant.

Objectives: Quantify the number of Influenza-specific IFN-γ and antibody secreting cells by ELISPOT. Quantify the number of CMV-specific IFN-γ secreting cells by ELISPOT. Quantify the relative levels of anti-CMV and anti-influenza antibody by ELISA.

Chapter 2: Methods and Materials

2.1 Participant Recruitment

ICISTEM-0021 was recruited from the outpatient HIV Clinic at the Victoria General Hospital in Halifax, Nova Scotia, Canada. Individuals were included in this study if they were adults living with HIV and had a clinical indication for stem cell transplant while they were excluded if they were unable to give informed consent. Only 1 person was included in this arm of the study. ICISTEM-0021 was included because they had CML that had progressed to the accelerated phase but was pharmacologically induced to the chronic phase with nilotinib. This, combined with an increasing *BCR-ABL1* transcript level, made them a candidate for a stem cell transplant under the standard of care protocol. Ethics approval for the alloHSCT study was obtained through the Nova Scotia Health Authority. ICISTEM-0021 gave informed consent for both the stem cell transplant and for research-only activities both pre-transplant and post-transplant.

11 participants for the comparator group were recruited from an ongoing natural history study at the same site from 2015-2018. Cohort characteristics are described in Table 4.3. Participants were included if they were male, were HBV- and HCV-, were between the ages of 40 and 76, had been living with HIV for 12 to 28 years, and had a HIV viral load of under 400 copies/mL. Ethics approval for this study was obtained through the Nova Scotia Health Authority. All participants gave informed consent for research and all consented for their blood products to be used in immune studies.

2.2 ICISTEM-0021 Allogenic Hematopoietic Stem Cell Transplant (alloHSCT)

Stem cell transplant was performed as per a modified standard clinical protocol for the Nova Scotia Health Authority reflecting ICISTEM-0021's HIV status and renal function. Briefly, nilotinib therapy was discontinued on day -4, the participant received total marrow irradiation on day -6, -5, and -4 followed by 60 mg/kg effective body weight cyclophosphamide on day -3 and -2. Rabbit anti-thymocyte globulin was infused on day -1 at 0.5 mg/kg and on day 0 and +1 at 2 mg/kg. On day 0, 6.6×10^8 $CD34^+$ cells were transfused from an allogenic donor via peripheral transfusion. Recipient characteristics and HLA typing are presented in Tables 4.1 and 4.2. 3.75 mg/m^2 methotrexate was given on day +1 for graft versus host disease prophylaxis. Cyclosporine was initiated on day +14 due to acute kidney injury that was thought to be partially immune mediated. Further

immunologically relevant details of the peri-transplant clinical course are described in section 3.1.

2.3 Collection of Peripheral Blood Mononuclear Cells

85 mL of whole blood was collected in ACD tubes (BD Biosciences, Franklin Lakes, USA) as per standard clinical protocol for venous collection. Plasma and packed blood cells were aliquoted for future use. The remaining blood was diluted 1:1 in PBS (Thermo-Fisher, Waltham, USA) and separated using Leucosep Tubes (VWR, Randor, USA) and Lympholyte-H (Cedarlane, Burlington, Canada). Cells were washed once with PBS (Gibco, Waltham, USA) and then twice with PBS+2% FBS (Thermo-Fisher, Waltham, USA) before being counted in methylene blue (STEMCELL Technologies, Vancouver, BC, Canada). PBMCs were isolated and frozen down in Cell Recovery Media (Gibco) for future use. All samples were biobanked in -20ºC or liquid nitrogen as appropriate.

Leukapheresis samples were collected from peripheral blood following the standard clinical leukapherisis protocol. Blood was removed from the bag after collection and aliquoted into 50 mL tubes. No plasma or packed blood cell aliquots were taken. PBMCs were isolated using Leucosep tubes as above.

2.4 Western Blot

The Genetic Systems HIV-1 Western Blot Kit (Biorad, Hercules, USA) was used to detect anti-HIV antibodies peri-transplant. Briefly, specimen diluent from the kit was prepared with triple distilled H_2O. Strips were removed using clean forceps and loaded into an Medtec Autoblot (Biorad) along with samples, controls, wash buffer, and substrate. 15 µL of samples was used in each run. The samples were run as per the recommended protocol. At the end of the run, excess liquid was drained off the strips and the samples were air dried on a clean towel. Strips were transferred to a prepared worksheet and interpreted by a trained technician in relation to the low and high positive controls.

2.5 Tat/Rev Limiting Dilution Assay (TILDA)

The TILDA assay was used to determine HIV proviral reservoir size in peripheral $CD4^+$ T Cells as previously described in (Procopio et al., 2015). Briefly, $CD4^+$ T cells were isolated from previously isolated PBMCs using the EasySep Human CD4 Negative

Selection Kit and the BigEasy magnet (STEMCELL Technologies) using the recommended protocol. 100,000 cells were removed prior to and after separation to verify separation success. Cells were stained using the protocol in Section 2.6 with the antibody mix listed in Appendix 2, Table 1.4 and read on a BD FACSCanto II (BD Biosciences). Cells were rested for 12 hrs in RPMI 1640 +2 mM L-glutamine (Invitrogen, Waltham, MA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO), 20 μM beta - mercaptoethanol (Sigma-Aldrich), 10mM HEPES (Invitrogen), 100U/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen), 100 ng/mL PMA (Sigma-Aldrich), and 1 μg/mL ionomycin (Sigma-Aldrich) [from here on referred to as lymphocyte media]. Cells were counted, serially diluted in PBS (Sigma-Aldrich) + 10% FBS, and plated in a 96-well PCR plate (Bio-Rad, Hercules, CA) at the following cell concentrations: 18000 cells/well, 9000 cells/well, 3000 cells/well, and 1000 cells/well. RNA from ACH-2 cells (NIH AIDS Reagent Program, Maryland, USA) was isolated using the RNEasy Mini Kit (Qiagen, Hilden, Germany) and was treated with DNAse using the Ambion RNAse-free DNAse Kit (Invitrogen). ACH-2 RNA was diluted to 1000 cells/uL and plated in duplicate at 1:10, 1:100, and 1:1000 dilutions. A duplicate of PBS + 10% FBS was used as a no-template control. ROX Buffer and master mix containing the tat1.4 and rev primers (Integrated DNA Technologies, San Jose, USA) (Described in Appendix 2, Table 1.5) and Superscript III Platinum Taq (Invitrogen) was added. Reverse transcription PCR was performed on a Bio Rad C1000 Touch Thermocycler with the following steps: 50˚C for 15 min, 95˚C for 2 min, and 24 cycles of 95˚C for 15 sec, 60˚C for 4 min. Post-amplification, the samples were diluted 1:4 with RNAse free, DNAse free water (Life Technologies, Carlsbad, USA) and 4 uL of samples was plated into a new 96-well plate. Master mix containing ssoAdvanced Probes Super Mix (Bio Rad) and the tat2 and rev primers (Integrated DNA Technologies) (Described in Appendix 2, Table 1.5) was added to each well. Real-time PCR was run on a Bio Rad C1000 Touch Thermocycler outfitted with the CFX96 Real Time system under the following conditions: 95˚C for 2 min followed by 45 cycles of 95˚C for 10 sec and 60˚C for 20 sec. PCR data was analyzed using CFX Manager and the maximum likelihood method was used to estimate the number of positive cells/million $CD4^+$ T Cells (Hu & Smyth, 2009).

2.6 Flow Cytometry

Immune phenotyping was assessed by flow cytometry. Briefly, cells were thawed in lymphocyte media and counted in trypan blue (Sigma-Aldrich). Cells were rested in 12 well plates (Corning, Corning, USA) overnight at 37ºC and 5% CO_2. Antibody mixes (see Appendix 2, Tables 1.1, 1.2, and 1.3 for antibody clones and suppliers) and appropriate controls were prepared on ice in 4 mL polystyrene tubes (Corning) the day prior to staining and stored at 4ºC away from light. The next day, cells were removed and washed with lymphocyte media. Cells were counted in trypan blue and washed with cold flow buffer (BD Biosciences). Cells were resuspended at appropriate concentrations for the panels and 200 µL was aliquoted into appropriate tubes with antibodies in the dark. Compensation controls using appropriate beads (BD Biosciences) and antibodies were also prepared. The tubes were covered in foil and incubated at 4ºC for 30 minutes. The cells were washed twice with cold flow buffer and resuspended in 100 µL of Cytofix/Cytoperm (BD Biosciences). The cells were incubated in the dark for 30 minutes at 4ºC and then washed twice with Perm/Wash Buffer (BD Biosciences). 70 µL of intracellular antibody mix diluted in Perm/Wash Buffer or 70 µL Perm/Wash Buffer was added to the cells and incubated for 30 min at 4ºC in the dark. The cells were washed twice more with Perm/Wash Buffer before being resuspended in 300 µL cold flow buffer. Tubes were kept at 4ºC and covered with foil until reading on the BD LSRFortessa (BD Biosciences). Isotype controls were run using the appropriate anti-mouse Ig kappa beads (BD Biosciences). Gating was done on FlowJo version 10.5 (BD Biosciences), using FMOs run during optimization of the panel to determine gates. A Tim-3 FMO was run for each individual to determine the gate for this marker.

2.7 Enzyme Linked Immunospot Assay (ELISPOT)

2.7.1 IFN-γ T Cell ELISPOT

To determine the number of antigen-specific IFN-γ secreting T cells, the BD IFN-γ ELISPOT Set was used (BD Biosciences). Briefly, included plates were prewet with 50 µl of 70% ethanol before being washed with 200 µL PBS (Gibco) 3 times. NA/LE Purified Anti-Human IFN-γ Antibody was diluted as per the kit in PBS and 100 µL was added to each well and allowed to incubate at 4ºC overnight. Cells were thawed the next morning in lymphocyte media and rested for 6 hours at 37ºC and 5% CO_2 in a 15

mL tube. The plate was washed once with 200 µL PBS and then twice with 200 µL lymphocyte media before being blocked with 200 µL lymphocyte media for 2 hrs. Cells were counted, suspended at $2x10^6$ cells/mL, and 100 µL of cells were added to each well. Activators were suspended in lymphocyte media at the following concentrations: 3 µg/mL for HIV-1 Consensus B Gag and HIV-1 Consensus B Env (NIH AIDS Reagent Program), 4 µg/mL CMV pp65 and IE-1 (Miltenyi Biotec), 4 µg/mL for CEF Control Peptide Pool (NIH AIDS Reagent Program, Bethesda, USA), 5 µg/mL for PHA-L (Sigma-Aldrich), and 0.12% DMSO (Sigma-Aldrich) in lymphocyte media as a negative control. 100 µL of activator media was added to wells containing cells in triplicate and the plate was incubated at 37ºC and 5% CO_2 overnight. The plate was decanted and washed twice with 200 µL sterile H_2O (Sigma-Aldrich) and three times with 200 µL PBS+0.05% Tween-20 (Sigma-Aldrich) wash buffer and allowed to incubate at room temperature in the dark for 2 hours with 100 µL of biotinylated Anti-Human IFN-γ antibody diluted in PBS + 10% FBS (Sigma-Aldrich). Plate was then washed three times with wash buffer and 100 µL of Streptavidin-HRP diluted in PBS+10% FBS was added. Plate was incubated at room temperature in the dark for one hour before being washed four times with wash buffer and twice with PBS. AEC substrate (BD Biosciences) was diluted as manufacturer's instructions and 100 µL was added to each well. Plate was incubated in the dark at room temperature for 45 minutes before being thoroughly washed under tap water. Plates were dried overnight in the dark and counted on CTL Imunospot Core Reader (CTL, Cleveland, USA).

2.7.2 B Cell ELISPOT

Antigen-specific antibody secreting cells were determined using the B cell ELISPOT as described by (Moir et al., 2008). Briefly, cells were thawed in lymphocyte media and rested for 6 hours at 37ºC and 5% CO_2 . Cells were counted in trypan blue, resupended at $2x10^6$ cells/mL, and added to a 75 cm^2 flask (Corning). Activator media was made from lymphocyte media containing 2 µg/mL R848 (Sigma Aldrich) and 0.02 µg/mL IL-2 (Cedarlane). Activator media was added in equal volume to the volume of cells and cells were activated at 37ºC and 5% CO_2 for 72 hrs. Immunolon-HA filter plates (Millipore, Burlington, USA) were prewet with 50 µl of 70% ethanol before being washed with 100 µL PBS (Gibco) three times. 100 µL of antigen was added to wells in

triplicate at the following concentrations: 10 µg/mL for HIV-1 gp41 and HIV-1 gp120 (NIH AIDS Reagents), 5 µg/mL for 2015-2016 Flulaval (GlaxoSmithKline, Brentford, UK), 5 µg/mL for Goat Anti-Human IgG H+L (Jackson Immunoresearch), and 5 µg/mL for KLH (Millipore). Plates were sealed with parafilm and allowed to incubate at 4ºC overnight. The following day, plates were decanted and washed twice with lymphocyte media before being blocked for 2 hours in 200 µL lymphocyte media. Cells were removed from flasks and counted in trypan blue before being resuspended at $2x10^6$ cells/mL. After decanting the plate 100 µL of cells were added to each well and incubated at 37ºC and 5% CO_2 overnight. The next morning, the plate was washed twice with sterile H_2O before 100 µL of 1µg/mL Biotin-SP-conjugated AffiniPure Goat Anti-Human IgG (Jackson Immunoresearch) was added; Plate was incubated in the dark for 2 hours at room temperature before being washed three times with PBS+0.05% FBS wash buffer. ELISPOT Blue Colour module (R&D Biosystems, Minneapolis, USA) was used to develop the plate as per the manufacturer's instructions. Plate was dried overnight in the dark and counted on the CTL Immunospot Core reader (CTL, USA).

2.8 Enzyme Linked Immnosorbent Assay

Antibody titers in plasma were determined using the ELISA. MRC-5 cell lysate and CMV lysate (described in Appendix 4) were diluted 1:500 in pH 9.2 carbonate-bicarbonate buffer (Sigma-Aldrich, St. Louis, USA). Clear Immulo-2 HB 96-well round bottom plates (VWR) were coated with 50L of either MRC-5 cell lysate, CMV lysate, 2015-2016 season Flulaval (GlaxoSmithKline), or PBS (Gibco). Plates were covered in parafilm and incubated overnight at 4ºC. Plates were washed three times with PBS+0.1% Tween (Sigma-Aldrich) wash buffer and then blocked with PBS+1% BSA (Sigma-Aldrich) for 60 minutes. Plasma was thawed on ice and spun at 10,000 g for 2 minutes at 4ºC to pellet debris. Plasma was diluted 1:500 and vortexed gently. Plate was washed 6 times with wash buffer and 100 µL of plasma was added to appropriate wells. After 90 minutes of incubation at room temperature, the plate was decanted and washed 6 times with wash buffer. Peroxidase AffiniPure Goat Anti-Human H+L IgG (Jackson Immunoresearch, West Grove, USA) was diluted to 16 ng/mL in wash buffer and 100 µL was added to each well. The plate was incubated for 60 minutes at room temperature. After incubation, plate was washed 6 times with wash buffer and 100 µL room

temperature TMB (Sigma-Aldrich) was added. The plate was incubated for 40 minutes before being stopped with 50 µL 1M H_2SO_4 (Sigma-Aldrich). The plate was read at 450 nm and 570 nm on an Epoch Microplate Spectrophotometer (Biotek, Winooski, USA).

Chapter 3: Results

3.1 Clinical and Immunologic Events during Full Engraftment of Donor Stem Cells after matched CCR5 Allogeneic Stem Cell Transplant

ICISTEM-0021 was identified as a stem-cell transplant candidate after progressing to accelerated phase of CML. After comparing ICISTEM-0021 to the international stem cell registry, a CCR5Δ32/Δ32 donor was identified. Table 3.1 describes match criteria for ICISTEM-0021, including CCR5 status. The donor was a 10/10 HLA class I and II match, had the CCR5Δ32 mutation at both loci, and was CMV negative. With patient consent, the peripheral-derived $CD34^{+}$, CCR5Δ32/Δ32 stem cells were used for a curative intent stem cell transplant, with additional consent for research procedures to investigate the effects of immune reconstitution with CCR5Δ32/Δ32 stem cells.

Table 3.1: Pre-transplant characterization of recipient and donor immunologic factors.

Factor	ICISTEM-0021	Donor
Sex	Male	Female
HLA Match	N/A	10/10
CMV serostatus	Positive	Negative
Blood Group (red blood cell antigen; ABO system)	O+	AB+
CCR5	Wild Type/Wild Type	Δ32/Δ32

Table 3.2: Recipient class I and II HLA. The donor was a 10/10 match across these loci.

HLA Class I	
Locus	**Allelic variants**
A	01:01, 02:01
B	08:01, 15:01
C	03:04P, 07:01P
HLA Class II	
Locus	**Allelic variants**
DRB1	03:01, 04:01
DQB1	02:01, 03:02

Figure 3.1 shows the important clinical and immunologic events pre-transplant and for the first 9 months post-transplant in the context of $CD4^+$ T cell count, $CD8^+$ T cell count, and HIV viral load. Pre-transplant and 9 months post-transplant counts along with reference ranges are found in Table 3.3.

Prior to transplant, ICISTEM-0021 maintained an undetectable HIV viral load using abacavir, lamivudine, and dolutegravir (Figure 3.1A). CML was controlled using nilotinib for approximately 1.25 years prior to transplant. $CD4^+$ T cells were within the normal range at 836 cells/μL while $CD8^+$ T cells were slightly above the reference range at 890 cells/μL. CD4:CD8 ratio also remained normal at 0.94.

In the peri-transplant period, viral load was monitored daily for the first 30 days. During this time, the antiretrovirals were separated into individual components to allow for adjustments based on renal function (Figure 3.1B). HIV viral load remained undetectable during this time period. Due to renal insufficiency, only one dose of methotrexate was given on day 1 post-transplant, with a 75% dose reduction. Cyclosporine was not initiated until day 14 post-transplant for the same reason.

The initial transplant period was marked with acute kidney injury, *Clostridium difficile* infection, and multi-factorial acute respiratory failure. This was treated with methylprednisone followed by tapered prednisone. There were two episodes of skin-predominant graft versus host disease treated with oral prednisone. Oral herpes simplex virus was treated with acyclovir at day 37 after transplant. Clinical CMV reactivation

occurred on day 35 post-transplant, with detectable CMV viral load in blood, and valgancyclovir was started on day 50 post-transplant. Cyclosporine was discontinued on day 70 post-transplant. No other major complications arose after this point through to 18 months post-transplant. The CML associated *BCR-ABL1* translocated oncogene was <0.001% on post-transplant day 100, consistent with CML cure (Jabbour et al., 2011).

Immune cell counts initially decreased after transplant in response to total marrow irradiation (Figure 3.1A). At the time of discharge, day 30 post-transplant, $CD4^+$ and $CD8^+$ T cell counts were low at 24 cells/μL and 36 cells/μL respectively. There was a notable increase in $CD8^+$ T cells detected on day 160 post-transplant to 1941 cells/μL while $CD4^+$ T cells remained low at 356 cells/μL. At 9 months post-transplant, $CD4^+$ T cell levels remained low at 176 cell/μL while $CD8^+$ T cells had further risen to 2120 cells/μL and CD4:CD8 T cell ratio decreased out of the reference range to 0.08.

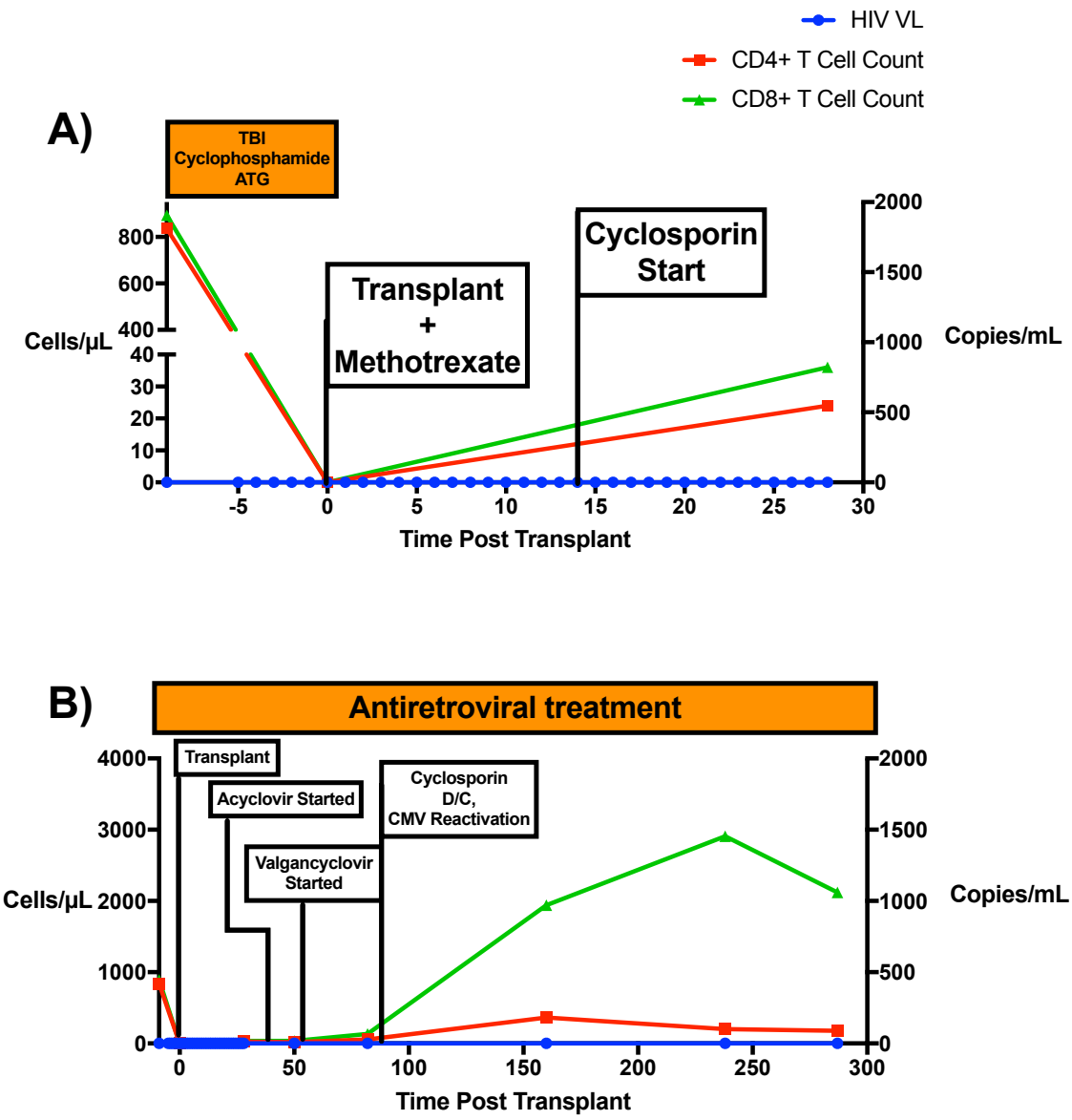


Figure 3.1: Key immunologic and clinical changes peri-transplant. (A) $CD4^+$ T cell counts, $CD8^+$ T cell counts, and HIV viral load during the first 30 days after transplant with the initiation of immunosuppressive regimens and conditioning regimen. (B) $CD4^+$ T cell counts, $CD8^+$ T cell counts, and HIV viral load during the first 9 months after transplant with immunosuppressive treatments and antiretroviral treatments annotated. Legend in (A) the same for all panels. *VL = Viral Load*, *D/C = Discontinued; TBI = Total body irradiation*

3.1.1 Comparator Cohort Participants

Table 3.3 compares ICISTEM-0021 to a local cohort of 11 people living with HIV.

Table 3.3: Comparison of major demographic factors between ICISTEM-0021 and a local cohort of people living with HIV. Data are reported as the median with interquartile range or percentage of total. Nadir is the lowest recorded $CD4^+$ T cell count. Viral load control is defined as a viral load of less than 400 copies/mL. Reference ranges were determined by the NSHA Central Zone Laboratory.

	ICISTEM-0021 Pre-transplant	**ICISTEM-0021 9 Months Post-transplant**	**Local Cohort (N=11)**
Age	58	58	56.74 (51.52, 57.98)
% Male	N/A	N/A	100
$CD4^+$ T Cell Count (Normal Range: 300-1500 cells/µL)	836	176	727 (348, 1094)
$CD4^+$ T cell Nadir (cells/µL)	Unknown	Unknown	160 (61, 327)
$CD8^+$ T Cell Count (Normal Range: 140-820 cells/µL)	890	2120	1090 (762, 1389)
CD4:CD8 (Normal Range: 0.66-3.52)	0.94	0.08	0.67 (0.37, 0.83)
% Controlled Viral Load	Yes	Yes	100
% Integrase Inhibitor Use	Yes	Yes	63.6
% Previous Opportunistic Infection	Yes	Yes	54.5
% CMV Seropositive	Yes	Yes	90.9

Prior to transplant, ICISTEM-0021 was similar to the cohort with respect to both $CD4^+$ and $CD8^+$ T cells, however the CD4:CD8 ratio was less skewed than the cohort in general. $CD4^+$ T cell nadir, the lowest recorded $CD4^+$ T cell count, was unknown in ICISTEM, as he was a patient in another country prior to returning to Canada; notably the cohort had a wide range of $CD4^+$ T cell nadirs before initiating ARV therapy, and therefore the ICISTEM-0021 CD4 nadir likely is included in the comparator group. Only half of the cohort and ICISTEM-0021 had a recorded opportunistic infection, as defined

by the CDC (Centers for Disease Control and Prevention, 2009)**.** All individuals maintained controlled HIV viral load, with half having current integrase inhibitor use. As well, all but one person was CMV seropositive. Overall, the cohort was similar pre-transplant to ICISTEM-0021 in the reported immunologic and treatment variables, but ICISTEM-0021 was distinctly different from a $CD4^+$ and CD8+ T cell count perspective in the post-transplant phase.

3.2 Inducible Proviral Reservoir is Reduced Following alloHSCT

To assess the impact of alloHSCT on HIV proviral reservoir, we used the Tat/Rev Limiting Dilution Assay (TILDA) to measure inducible HIV provirus in peripheral $CD4^+$ T cells. ICISTEM-0021 pre-transplant and post-transplant samples were compared to two HIV^+ viral load undetectable individuals. Significant optimization of this assay was required, and is discussed in Appendix A. In keeping with the literature, the pre-transplant number of $CD4^+$ T cells with inducible HIV provirus in ICISTEM-0021 were 19 cells/million $CD4^+$ T cells (Figure 3.2) (Procopio et al., 2015). One comparator individual had similar values to the literature while the other had a slightly lower value at 4 infected cell/million $CD4^+$ T cells. The confidence interval for this individual did fall within the expected frequency in the literature. At 9 months post-transplant, there was no difference in the inducible proviral reservoir of ICISTEM-0021, with an estimated 19 cells/million $CD4^+$ T cells. Overall $CD4^+$ T cell counts were reduced at this time point, as shown in Table 3.1, therefore the total number of infected $CD4^+$ T cells are lower post-transplant than pre-transplant.

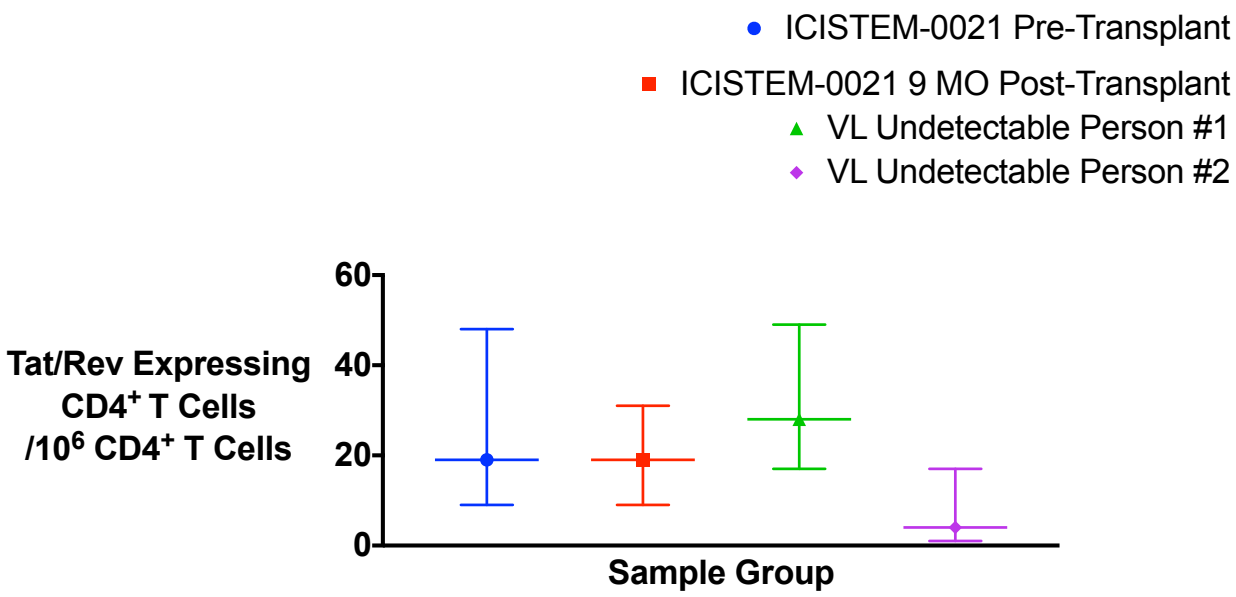


Figure 3.2: Estimated number of provirus-containing $CD4^+$ T cells in ICISTEM-0021 and two matched people living with HIV who have an undetectable HIV viral load. Data are presented as frequency with 95% confidence interval.

3.3 alloHSCT is Associated with a Reduction of Phenotypic Immune Exhaustion in $CD4^+$ T Cells, but not $CD8^+$ T Cells or B cells

Expression of activating and supressing markers on immune cells was assessed by polychromatic flow cytometry. As shown in Figures 3.3, 3.4, and 3.5, the effect of stem cell transplant on exhaustion marker expression and cell subset frequency is cell-type dependent. A sample gating strategy is shown in Appendix B.

3.3.1 $CD4^+$ T Cells

$CD4^+$ T cells help initiate adaptive immune responses, therefore phenotypic exhaustion in this compartment could influence the function of $CD8^+$ T cells and B cells. Within this compartment, there is variation in immune cell types and marker expression that portend function.

Prior to transplant, ICISTEM-0021 had a smaller frequency of activated $CD4^+$ T cells (PD-1+CD28+Tim-3-) to the HIV matched cohort (19.6% and 27.4% respectively), as seen in Figure 3.3A. The frequency of CD28+ cells was similar to that of the cohort, at 92.9% for ICISTEM and 95.7% for the cohort, though trended towards the higher end of the cohort's range with regards to the frequency of activated CD28+ PD-1- Tim-3- (72.5% for ICISTEM and 66.8% for the cohort) and non-activated CD28- PD-1- Tim-3- populations (1.86% for ICISTEM versus 0.99% for the cohort). There was also less frequent expression of the activation/exhaustion marker CD57 (2.06% for ICISTEM versus 8.26% for the cohort), both with (1.71% for ICISTEM versus 5.46% for the cohort) and without (0.35% for ICISTEM versus 2.78% for the cohort) PD-1 co-expression, as seen in in Figure 3.3D. At the same time, ICISTEM cells trended towards the higher end of the cohort in frequency for many exhausted type populations, especially the CD28- PD-1+ Tim-3- population (5.28% for ICISTEM versus 2.39% for the cohort) and the highly exhausted CD28- PD-1+ Tim-3+ (0.01% for ICISTEM versus 0.005% for the cohort) population, as seen in Figure 3.3B. There were also increased frequencies of Tim-3+ cells (0.79% for ICISTEM versus 0.69% for the cohort) and CTLA-4 cells (13.3% for ICISTEM versus 1.05% for the cohort), with the latter being much higher than the cohort as seen in Figure 3.3C and E. Finally, the frequency of perforin expression trended towards the lower end of the cohort prior to transplant (1.25% for ICISTEM versus 2.13% for the cohort).

After transplant, the frequency of many of these populations changed. Both the overall frequency of PD-1 (33.6% post-transplant versus 25.2% pre-transplant), the frequency of activated CD28+ PD-1+ Tim-3- cells (22.9% post-transplant versus 19.6% pre-transplant) and the relative frequency of perforin positive (2.24% post-transplant versus 1.25% pre-transplant) $CD4^+$ T cells increased. Exhausted CD28- PD-1+ Tim-3- (3.43% post-transplant versus 5.25% pre-transplant) and CD28- PD-1+ Tim-3+ (0% post-transplant versus 0.01% pre-transplant) cells were less frequent after transplant. Furthermore CD57+ cells also decreased (0.82% post-transplant versus 2.06% pre-transplant). While the frequency of Tim-3+ cells decreased (0.49% post-transplant versus 0.79% pre-transplant), the frequency of exhausted CD28- PD-1- Tim-3+ cells did increase (0.019% post-transplant versus 0.0067% pre-transplant). Finally, there was a slight decrease in exhausted CTLA-4+ PD-1+ cells (10.9% post-transplant versus 11.9% pre-transplant), though they remained much higher than the comparator cohort.

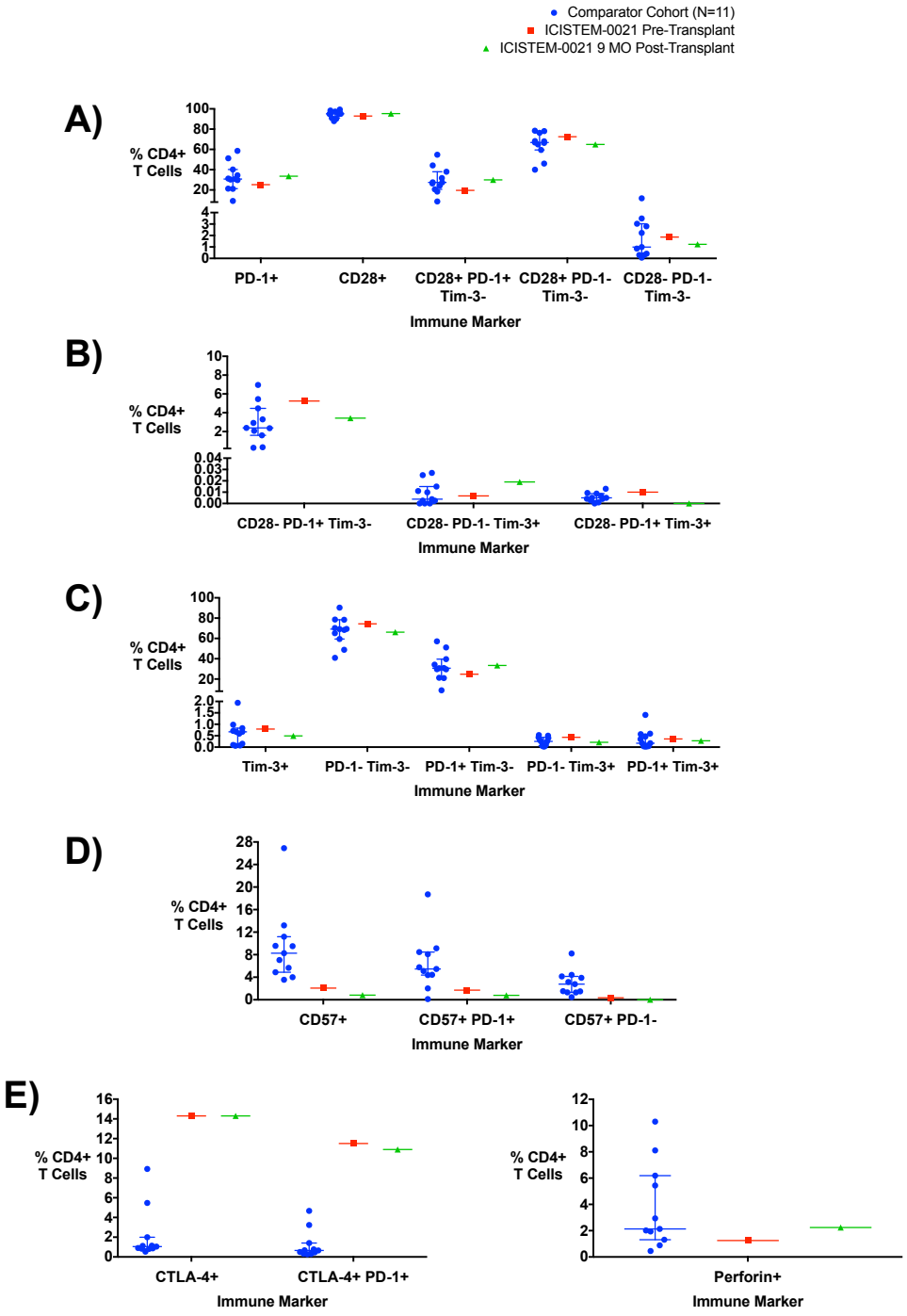


Figure 3.3: Phenotypic characterization of $CD4^+$ T cells in the comparator cohort, ICISTEM-0021 pre-transplant, and ICISTEM-0021 9 months post-transplant. Values are presented as percent of the $CD3^+CD4^+$ T cell population with the median and interquartile range for the comparator cohort. Boolean gating was used to determine co-expression populations. Legend used in (A) was used in all panels.

3.3.2 $CD8^+$ T Cells

As $CD8^+$ T cells are key to clearance of virally infected cells and cancerous cells, we examined changes in immune exhaustion and activation markers in this cell population. Unlike $CD4^+$ T cells, $CD8^+$ T cell populations broadly showed increased frequencies of immune exhaustion marker expressing cells in ICISTEM-0021, as seen in Figure 3.4.

Prior to transplant, ICISTEM-0021 had a high frequency of activated $CD8^+$ T cells. In particular, the frequency of activated CD28+ PD-1- Tim-3- trended towards the higher end of the cohort (62.3% for ICISTEM versus 39.7% for the cohort) and towards the lower end of the cohort for less activated CD28- PD-1- Tim-3- cells (15.6% for ICISTEM versus 24.8% for the cohort), as seen in Figure 3.4A. Lower frequencies of exhausted phenotypes, especially CD28- PD-1+ Tim-3- (2.98% for ICISTEM versus 8.76% for the cohort) and CD28- PD-1+ Tim-3+ (0.018% for ICISTEM versus 0.034% for the cohort), were observed in ICISTEM-0021 compared to the matched cohort, as seen in Figure 3.4B. CD57+ cells were infrequent (7.24% for ICISTEM versus 37.9% for the cohort), regardless of PD-1 co-expression, as seen in Figure 3.4C and D. However, a lower frequency of perforin positive cells was observed in ICISTEM-0021 prior to transplant (12.4% for ICISTEM versus 29.2% for the cohort), as seen in Figure 3.4E. The frequency of CTLA-4 expressing cells were not different than the cohort.

After transplant, several populations differed in frequency. CD28+ cells were substantially less frequent (39.5% post-transplant versus 81.4% pre-transplant), as were activated subgroups such as CD28+ PD-1+ Tim-3- (10.6% post-transplant versus 18.8% pre-transplant) and CD28+ PD-1- Tim-3- (28.5% post-transplant versus 62.3% pre-transplant). More exhausted phenotypes, such as CD28- PD-1+ Tim-3- (11.5% post-transplant versus 2.98% pre-transplant), CD28- PD-1- Tim-3- (48.4% post-transplant versus 15.6% pre-transplant), and CD28- PD-1+ Tim-3+ (0.26% post-transplant versus 0.018% pre-transplant) all increased in frequency after transplant. Overall Tim-3+ (0.93% post-transplant versus 0.36% pre-transplant) and CD57+ (13% post-transplant versus 7.24% pre-transplant) cell frequency also increased, as did the frequency of CD57+ PD-1+ (2.81% post-transplant versus 0.77% pre-transplant) and PD-1+ Tim-3+ (0.52% post-transplant versus 0.17% pre-transplant) populations. There was also a modest increase in

CTLA-4+ PD-1+ cells (2.66% post-transplant versus 1.92% pre-transplant), though no increase in frequency of overall CTLA-4 expressing cells. Finally, there was a substantial increase in frequency of perforin positive cells (53.6% post-transplant versus 12.4% pre-transplant) at the 9 month post-transplant time point.

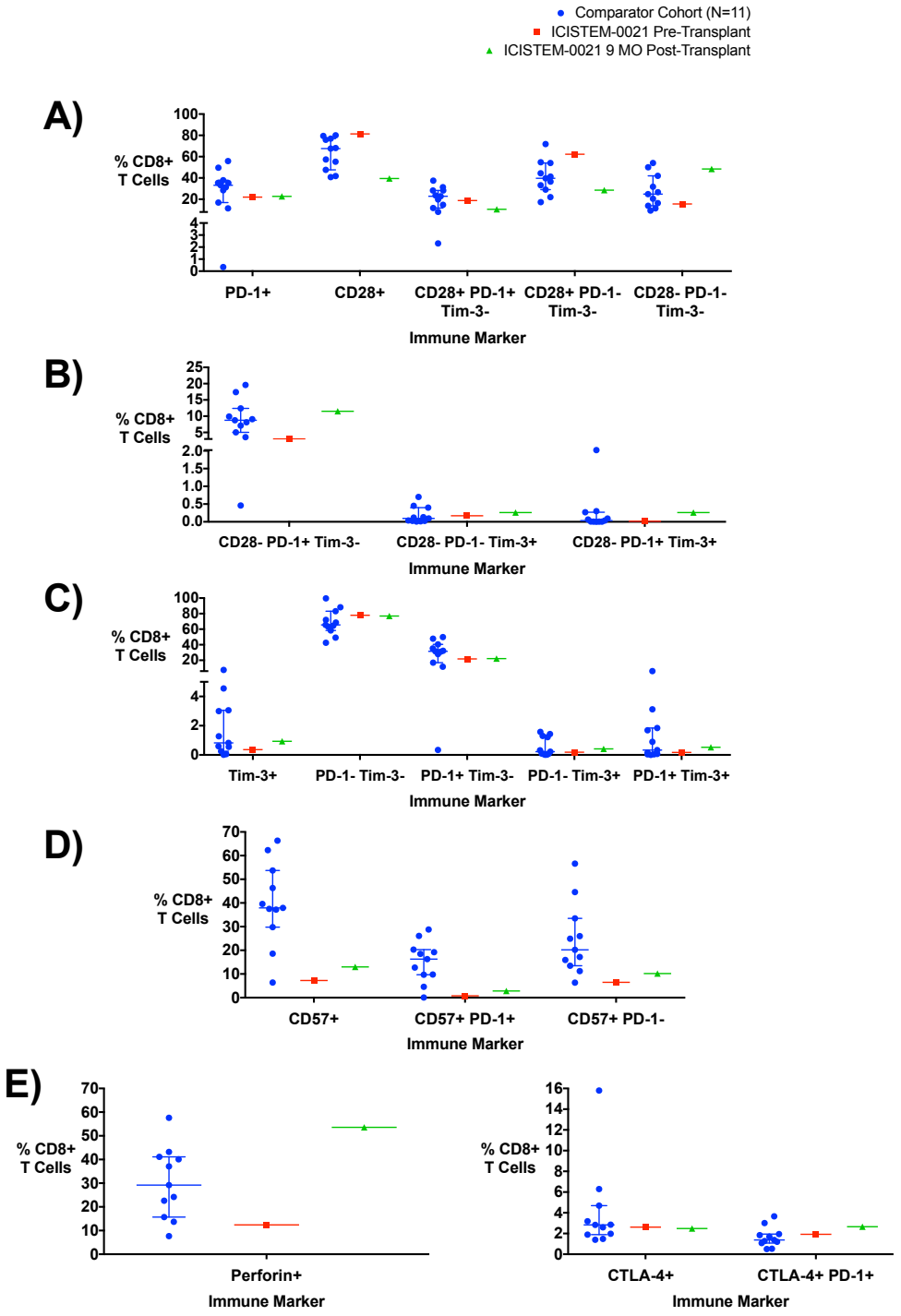


Figure 3.4: Phenotypic characterization of $CD8^+$ T Cells in the comparator cohort, ICISTEM-0021 pre-transplant, and ICISTEM-0021 9 months post-transplant. Values are presented as percent of the $CD3^+CD8^+$ T cell population with the median and interquartile range for the comparator cohort Boolean gating was used to determine the size of the co-expression populations. Legend used in (A) was used in all panels.

3.2.3 B Cells

Phenotypic immune exhaustion in B cells comes from a shift in subpopulations from more functional naïve and resting memory B cells to less functional tissue-like memory, and immature/transitional B cells. Table 3.4 describes the characterization of these populations by immune markers.

ICISTEM-0021 had more exhausted and activated phenotype B cells when compared to the cohort prior to transplant, specifically, a lower frequency of naïve (41.9% for ICISTEM versus 61% for the cohort) and resting memory B cells (8.25% for ICISTEM versus 14.1% for the cohort) and a higher frequency of tissue like memory (18.4% for ICISTEM versus 7.4% for the cohort), activated memory (4.48% for ICISTEM versus 2.88% for the cohort) and immature/transitional B cells (9.4% for ICISTEM versus 7.74% for the cohort). While these were within the range seen in the cohort, the naïve and tissue-like memory B cells trended towards the extremes of this range. Post-transplant, there was a decrease in the frequency of all B cell populations except for immature/transitional B cells. The biggest difference was in the immature/transitional subset (14.82% for post-transplant versus 9.4% for pre-transplant), which increased towards the extreme of the cohort's range (highest cohort frequency was 16.98%) At the same time, the highly exhausted tissue-like memory subset also notably decreased in size (7.95% for post-transplant versus 18.4% for pre-transplant). As such, the stem cell transplant was associated with decrease of all mature B cell populations and an increase in immature/transitional B cells.

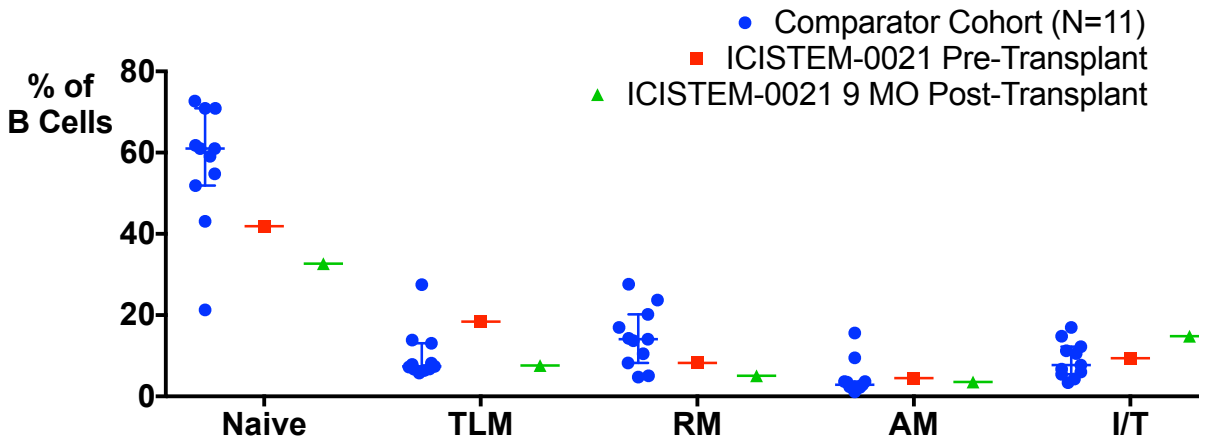


Figure 3.5: Phenotypic characterization of B cells in the comparator cohort, ICISTEM-0021 pre-transplant, and ICISTEM-0021 9 months post-transplant. Values are presented as percent of the $CD19^{+}$ T cell population with the median and interquartile range for the comparator cohort Boolean gating was used to determine the size of the co-expression populations. *TLM = Tissue Like Memory, RM = Resting Memory, AM = Activated Memory, I/T = Immature/Transitional.*

3.4 Anti-HIV T Cell, but not B Cell, Responses are Reduced Post-AlloHSCT

Given the variable changes in phenotypic immune exhaustion, we next examined the functional differences in T and B cell function. As the TILDA identified residual HIV^+ T cells after transplant, we first looked at HIV-specific T and B cell responses by ELISPOT. Figure 3.6A shows the IFN-γ and antibody responses to HIV peptides and HIV surface proteins. While there was variation in the magnitude of HIV-reactive T cell responses in the cohort, ICISTEM-0021 trended towards the lower end of this range before the transplant (250 IFN-γ secreting cells /10^6 PBMC for ICISTEM versus 419 IFN-γ secreting cells/10^6 PBMC for the cohort). After the transplant, these responses were nearly eliminated in ICISTEM (2 IFN-γ secreting cells /10^6 PBMC post-transplant). This is in keeping with ICISTEM-0021 remaining on effective ARV therapy and maintaining an undetectable viral load through transplant to the 9 month period. As such, the burden of any residual HIV should have been minimal and unlike a typical exposure to HIV. B cell responses were also variable within the comparator cohort and ICISTEM-0021 had minimal HIV-reactive antibody secreting cells before the transplant, as seen in Figure 3.6B (19 antibody secreting cells/10^6 PBMC for ICISTEM versus 131 antibody secreting cells/10^6 PBMC for the cohort. Post-transplant, HIV-reactive B cells were elevated (93 antibody secreting cells/10^6 PBMC), but comparable to the cohort.

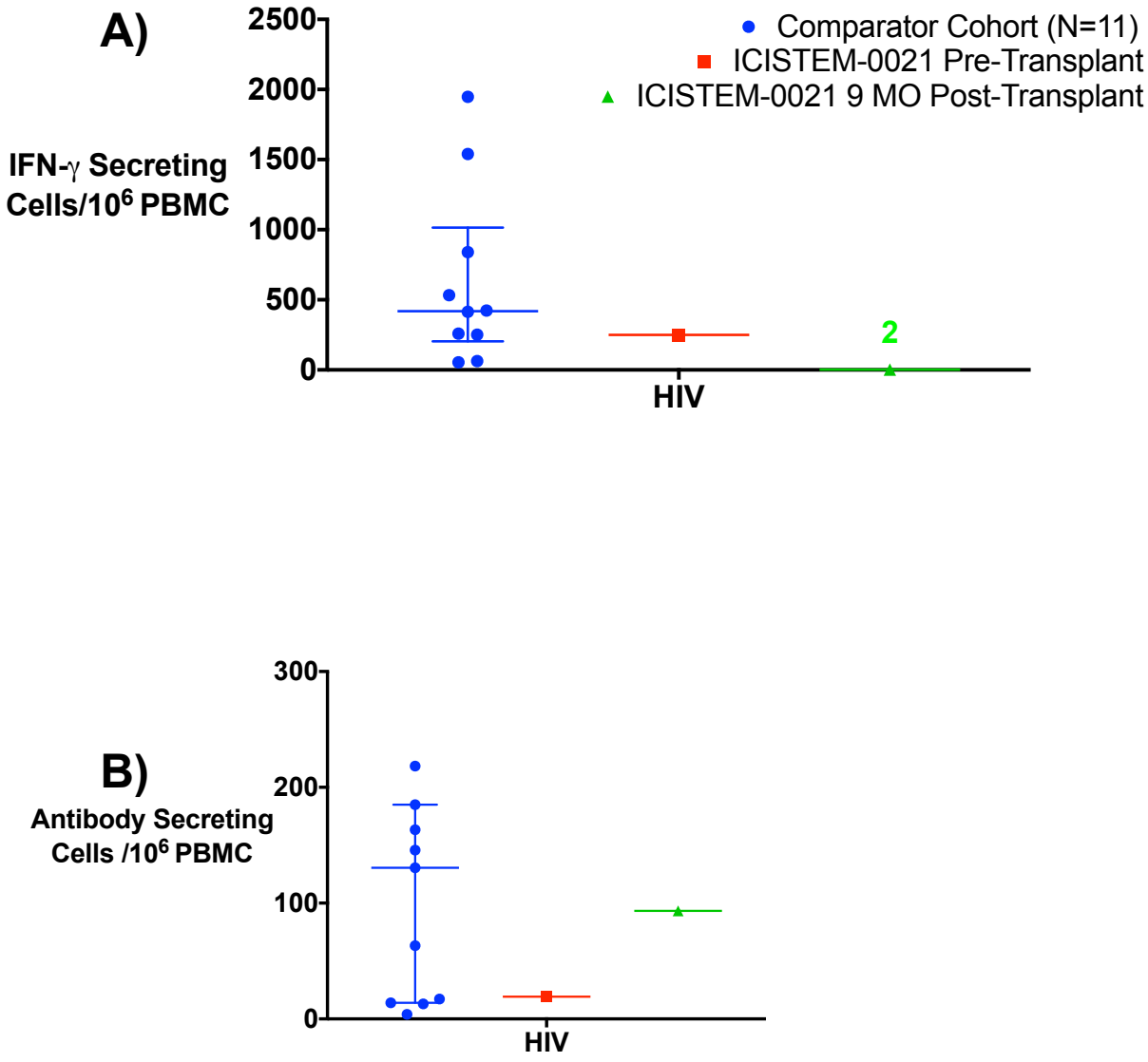


Figure 3.6: Comparison of HIV-reactive responses by ELISPOT. The number of specific spots cells was determined to be the sum of spots seen after *gag* and *env* (A) stimulation or gp120 and gp41 (B) stimulation. Legend seen in (A) was used in both panels. Data is presented as the median with the interquartile range.

As anti-HIV B cell responses remained at 9 months post-transplant, we asked if there were anti-HIV B cell responses earlier than this time point. We evaluated this by using western blot to detect anti-HIV antibodies in plasma immediately after transplant. Western blot analysis showed robust responses to HIV proteins in the pre-transplant period, as seen in Figure 3.7. In the period immediately after the transplant however, anti-HIV B cell responses were still present. Therefore, the stem cell transplant did not lead to a decrease in anti-HIV B cell responses.

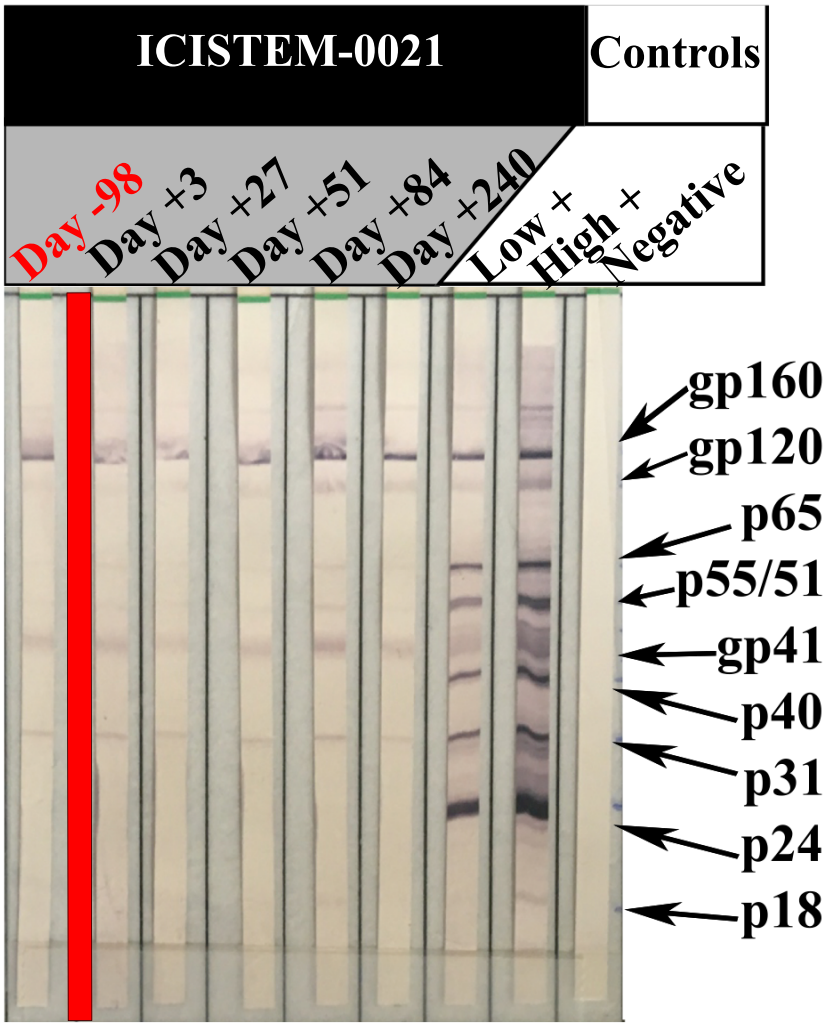


Figure 3.7: Identification of anti-HIV antibodies by Western Blot peri-transplant. Dates relative to transplant are presented above bands. The red text and bar indicate samples from the pre-transplant period. Arrows indicate the location of particular bands on the high positive.

3.5 Anti-CMV T Cell Responses are Elevated Post-Transplant, but Bystander B Cell Responses Remain Diminished

Immune exhaustion within HIV infection may also affect bystander (non-HIV) responses. We examined these changes by measuring IFN-γ and antibody response by ELISPOT and ELISA to two non-HIV viruses. CMV was representative of another chronic viral infection while influenza was chosen as it is an acute infection that people living with HIV are routinely exposed to in the context of vaccination. As seen in Figure 3.8, T cell responses to these antigens vary greatly. Pre-transplant, ICISTEM-0021 trended towards the higher end of the range seen in the cohort for CMV-reactive responses (4842 IFN-γ secreting cells/10^6 PBMC for ICISTEM versus 1400 IFN-γ secreting cells/10^6 PBMC for the cohort). This trend was the same with relation to influenza-reactive responses (113 IFN-γ secreting cells/10^6 PBMC for ICISTEM versus 27 IFN-γ secreting cells/10^6 PBMC for the cohort). At 9 months post-transplant, CMV IFN-gamma responses show no change (4865 IFN-γ secreting cells/10^6 PBMC post-transplant versus 4842 IFN-γ secreting cells/10^6 PBMC pre-transplant, even with the expansion of $CD8^+$ T cells seen in Table 3.1. At the same time points, anti-influenza T cell responses have greatly diminished (4 IFN-γ secreting cells/10^6 PBMC post-transplant versus 113 IFN-γ secreting cells/10^6 PBMC pre-transplant). While the stem cell transplant would have removed memory T cells from previous influenza infections, this reduction remained even after post-transplant influenza vaccination.

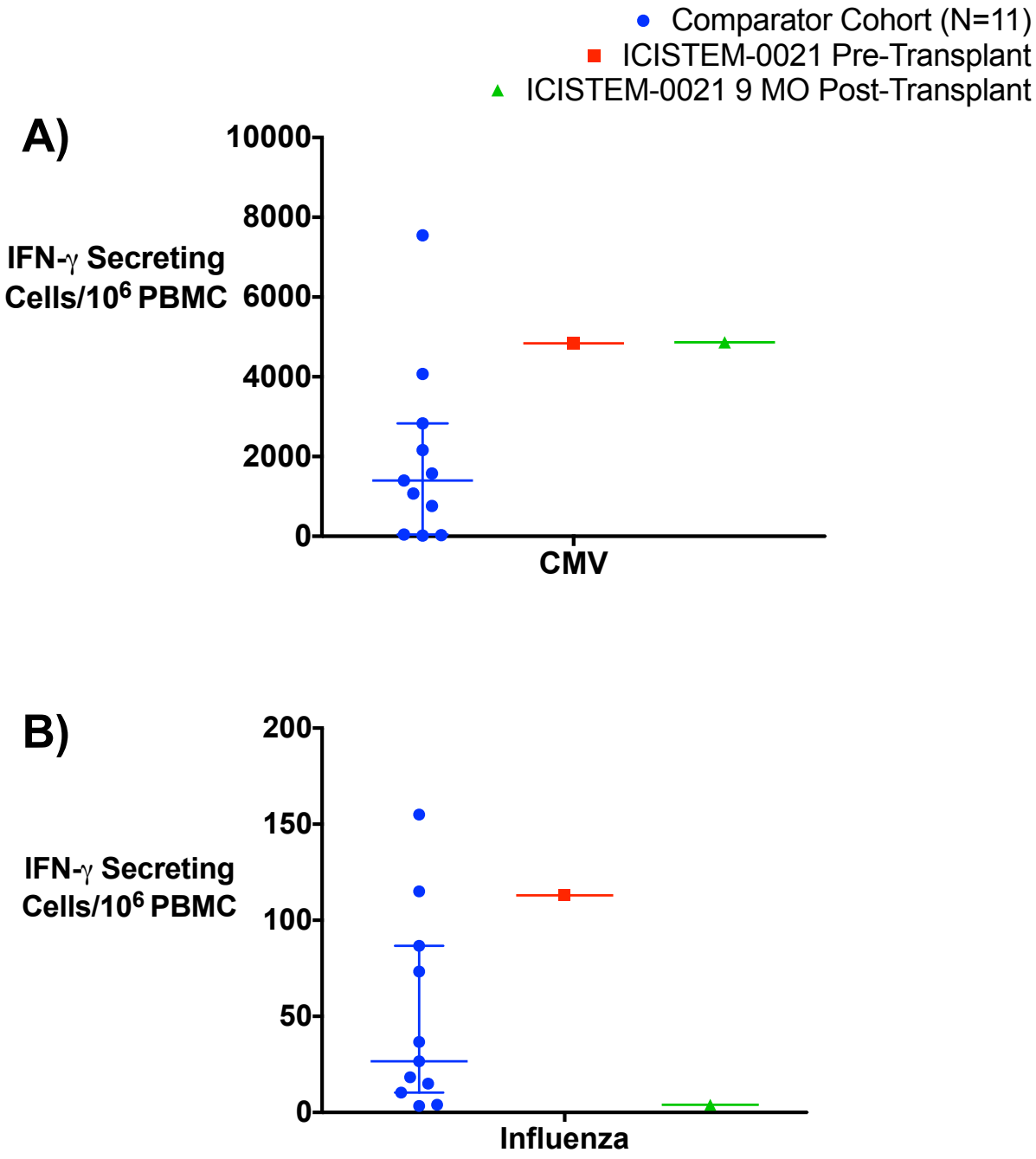


Figure 3.8: Comparison of CMV-reactive (A) and Influenza-reactive (B) responses by T cell ELISPOT. The number of specific spots cells was determined to be the sum of spots seen after IE-1 and pp65 stimulation for CMV (A) or H1N1 hemagglutinin and H1N1 nucleoprotein (B) stimulation. Legend seen in (A) was used in both panels. Data is presented as the median with the interquartile range.

Given the high anti-CMV and low anti-influenza T cell responses, we decided to examine the differences in B cell responses to these viruses by ELISPOT and ELISA. As seen in Figure 3.9A, anti-CMV serum antibody levels were low after the transplant, despite robust T cell responses as previously shown (OD of 0.149. While no plasma was available from before the transplant to compare to, the range of responses seen in the cohort suggest that HIV infection does not preclude producing antibody responses to CMV (Median OD of 0.572 for the cohort). Furthermore, anti-influenza plasma antibody levels trended towards the extreme low end of the cohort, in keeping with the lack of T cell responses to influenza (OD of 0.166 for ICISTEM post-transplant versus median OD of 0.534 for the cohort). Interestingly, antibody secreting cells reacting to influenza could still be detected by B cell ELISPOT, as seen in Figure 3.9B, and these levels post-transplant are comparable to the cohort (130 antibody secreting cells/10^6 PBMC for ICISTEM post-transplant versus 155 antibody secreting cells/10^6 PBMC). ICISTEM-0021 did have a higher number of influenza-reactive B cells pre-transplant when compared to post-transplant however (290 antibody secreting cells/10^6 PBMC for pre-transplant versus 130 antibody secreting cells/10^6 PBMC post-transplant). While they did decrease within ICISTEM-0021 over the course of the transplant, ICISTEM-0021 was able to produce a detectable B cell response to influenza antigens *in vitro.* This response can be detected by ELISPOT even though the number of $CD4^+$ T cells remained very low at the 9 month time point. There are outstanding B cell deficits towards bystander antigens even at 9 months post-transplant, even in the presence of robust T cell responses to some antigens. Overall, a number of bystander response deficits remained in ICISTEM-0021 despite successful stem cell transplant.

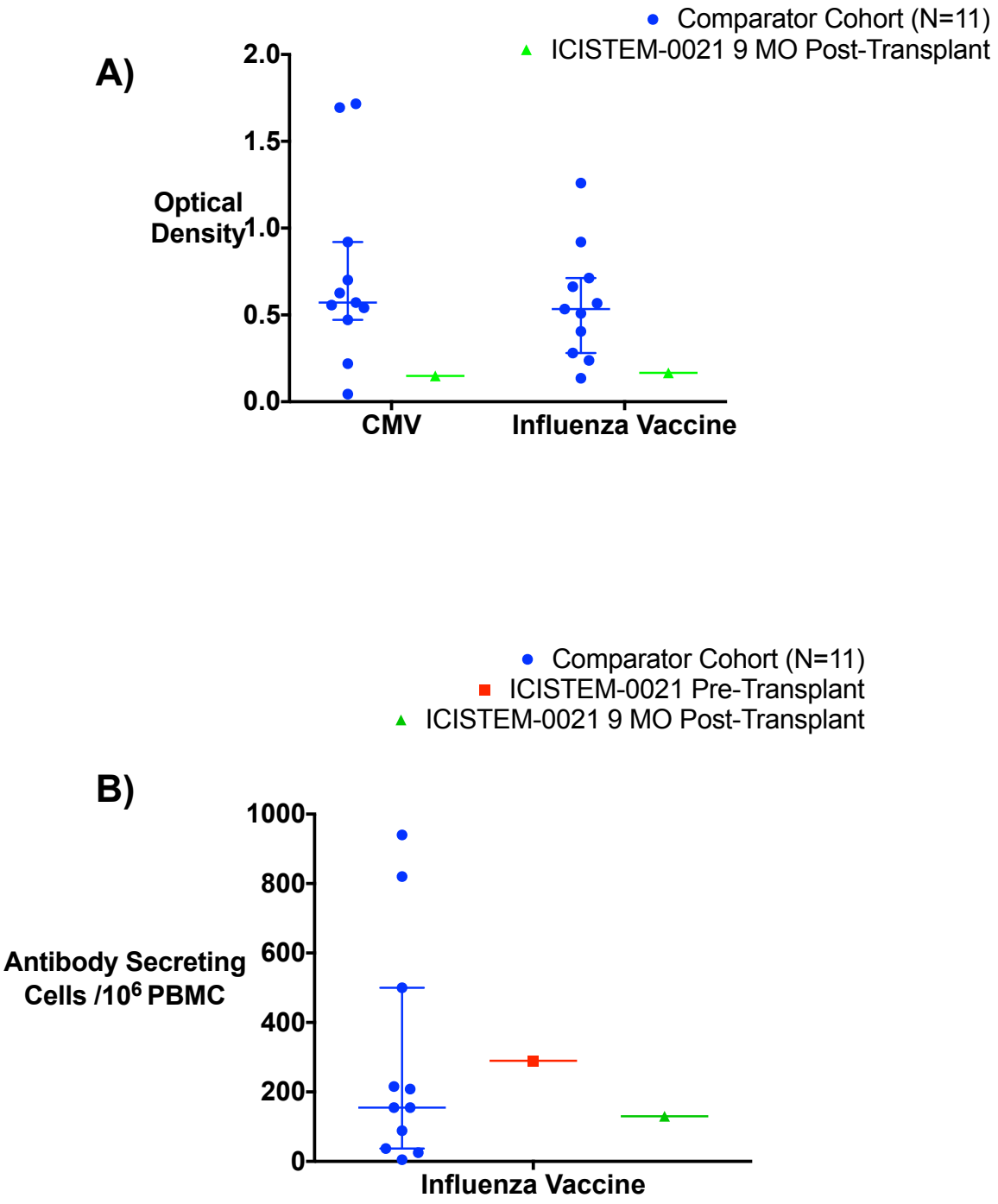


Figure 3.9: Comparison of bystander-specific responses by ELISA (A) and B cell ELISPOT (B). CMV optical density was calculated as the difference between the CMV optical density and the average MRC-5 optical density run in triplicate in the same experiment. Data is presented as the median with the interquartile range.

Chapter 4. Discussion

HIV remains an incurable, chronic viral infection. While much excitement has been generated around the potential for stem cell transplant with HIV-resistant stem cells, we still don't understand the impacts this has on immune exhaustion, one of the primary pathologies of chronic HIV infection. We investigated this by delineating immune responses in a local person living with HIV and CML who was transplanted with CCR5Δ32/Δ32 stem cells. While the HIV proviral reservoir was reduced, immune reconstitution was less favourable, and $CD4^+$ T cell counts remained low even at 9 months after transplant. Phenotypic immune exhaustion varied by cell type, with $CD8^+$ T cells and B cells showing a more exhausted phenotype, while $CD4^+$ T cells showed varied differences in immune exhaustion markers. From a functional standpoint, ICISTEM-0021 showed less HIV-reactive T cells prior to transplant when compared to a local cohort and those responses were not regained after transplant. Anti-HIV B cell responses remained after transplant, and were increased from pre-transplant to 9 months post.

Bystander non-HIV T cell responses were varied, with anti-CMV T cell responses increasing after the transplant, and anti-influenza T cell responses remaining low. The influenza responses, but not the CMV responses, were mirrored in the B cell compartment, with antibody levels against both antigens remaining low, suggesting outstanding B cell dysfunction post-transplant. Interestingly, the number of anti-influenza antibody producing cells increased after transplant. Together, these data show that CCR5Δ32/Δ32 alloHSCT is associated with variable changes in phenotypic and functional immune exhaustion even with reduction in proviral reservoir.

4.1 Reduction of Proviral Reservoir in the Context of Low $CD4^+$ T Cell Counts

The aim of this transplant was twofold: to induce CML remission; and to reduce HIV proviral reservoir size. In contrast to the Berlin patient, this transplant did not fully eradicate the HIV proviral reservoir in peripheral blood $CD4^+$ T cells (Allers et al., 2011; Hütter et al., 2009). As peripheral blood is only a small sample of the total HIV proviral reservoir, it is likely that there are residual reservoir cells elsewhere in the body as well at this time point. Unfortunately, we were unable to obtain ileal biopsy samples after transplant that could help determine this. Such biopsies would allow us to observe the reconstitution of Δ32 homozygous cells in the GALT. While the Berlin patient had full

reconstitution of Δ32 homozygous T cells in the GALT, it is known that the GALT can be resistant to myeloablative conditioning (Allers et al., 2011). Incomplete removal of these recipient cells could provide a source of persistent HIV viral reservoir, even with full chimerisim of the donor cells.

A reduction of the proviral reservoir is an important outcome. A larger proviral reservoir is associated with greater expression of immune exhaustion markers, higher levels of immune activation, and worse neurological and cardiovascular disease (Fromentin et al., 2016; Hatano et al., 2013; Zhao et al., 2009). The assay used to estimate the proviral reservoir is known to overestimate the viral reservoir relative to infective virions (Rosenbloom et al., 2015). How much of the detected viral reservoir is actually capable of infecting new cells is unknown, but the reduction in overall proviral burden is promising given what we know about increased pathogenesis with larger proviral reservoirs (Fromentin et al., 2016).

What is unknown about this participant is the amount of X4-tropic HIV they had in their body. Though most people are infected with R5 tropic HIV, chronic HIV can lead to the development of X4 tropic mutants (Karlsson, Parsmyr, Sandström, Fenyö, & Albert, 1994). Though no X4 HIV was found in our participant's blood pre-transplant, residual recipient-derived $CD4^+$ T cells may be harbouring X4 HIV. As the participant was on ART throughout the transplant, the ability of these mutants to infect new cells would be limited. If a treatment interruption were to take place however, these mutants could reactivate and infect donor-derived cells. Ultrasensitive sequencing from possible reservoir locations, such as the GALT, would provide insight prior to a treatment interruption, but this remains a possibility even with a reduction in overall proviral burden.

4.2 Phenotypic Exhaustion in $CD8^+$ T Cells and B Cells after Allogenic Hematopoietic Stem Cell Transplant

One of the most important findings of this study was that phenotypic immune exhaustion does change after alloHSCT with Δ32/32 stem cells. These changes were not homogenous, especially amongst T cell subsets, suggesting variable impacts of the transplant period on developing immune cells. Exhausted phenotype $CD8^+$ T cells are known to be less functional, and the expansion of highly exhausted subsets (those

expressing two or more immune exhaustion markers with the absence of CD28) are particularly concerning for future control of other infections.

An explanation may lie with CMV infection. CMV is known to lead to profound $CD8^+$ T cell expansion, and drive expression of PD-1, Tim-3, and CD57 on $CD8^+$ T cells (Margolick et al., 2018). The study participant was CMV positive before transplant, and likely had a significant CMV-specific T cell compartment which was depleted (at least in circulation) by the conditioning treatment with anti-thymocyte globulin and chemotherapy. Subsequent responses to CMV would from the newly reconstituted bone marrow, and consist of CMV-naïve donor cells.

ICISTEM-0021 had detectable CMV viremia starting at day 35 post-transplant that was not treated until day 50 post-transplant. $CD8^+$ T cell expansion was detected on day 160, but a previous analysis at day 70 already began to show a divergence between $CD4^+$ T cell and $CD8^+$ T cell counts. Studies from mouse models of CMV suggest that even after viremia has ended, CMV-specific $CD8^+$ T cells retain a higher than normal background proliferation rate, contributing to expansion of this population (Karrer et al., 2003). CMV-specific memory $CD8^+$ T cells are also known to be long lived in large numbers, which is thought to be part of CMV-specific immune exhaustion (Wallace et al., 2011). Given this, it is possible that some of the activation and exhaustion in the $CD8^+$ T cell compartment may be due to CMV, and the expression of the immune exhaustion markers is related to newly activated donor derived CMV-specific $CD8^+$ T cells, and not HIV-specific cells. Assessing the expression of these markers on cells reacting to CMV peptides with flow cytometry, along with assessing $CD8^+$ T cell exhalation markers after transplant but prior to CMV disease, would address this question.

After transplant, B cells subsets remained skewed as well. While there was reduction of exhausted subsets, most notably TLM B cells, there was also reduction in naïve and resting memory B cells, which are thought to be the most functional subsets. This was offset with an increase in I/T B cells, which trended towards the higher end of the cohort. An increase in I/T B cells after alloHSCT has been reported amongst a group of patients with chronic graft versus host disease (Khoder et al., 2017). ICISTEM-0021 did have two bouts of mild graft versus host disease in the 9 months we examined, but did not have chronic graft versus host disease. B cells can take close to a year to normalize in levels

however, so analysis of future time points may reveal a reduction in this population as B cell production starts to regain more function (Park et al., 2015).

An issue with trying to compare the pre and post-transplant periods however is that the two sets of cells come from different people: the recipient pre-transplant and the donor post-transplant. These are functionally two different immune systems with different genetics. Compounding the ability to ascribe clarity to the causality of post-transplant changes is fact that several factors changed at once. This transplant removed 2 different sources of immune exhaustion from the immune system: HIV and CML. It is impossible to delineate the whether the causes of these immune exhaustion differences are the result of curing the cancer or reducing HIV burden.

What is clear is that donor derived cells quickly develop markers of immune exhaustion and activation in the context of persistent CMV infection and reactivation, as well as ongoing pharmacologic immunosuppression during the 9 months post-transplant.

4.3 Anti-HIV T Cell Responses are Reduced After Transplant

To put the phenotypic data in context, we then examined the function of T and B cells *in vitro*. We first examined HIV responsive T cells.

When compared to the cohort, ICISTEM-0021 had fewer HIV-responsive T cells prior to transplant. This may well be because the patient had excellent HIV viral load control for over a decade before the transplant. Increased responsiveness to HIV may indicate better control of viral replication through $CD8^+$ T cell activity in untreated HIV (Klein et al., 1995; Ogg et al., 1999), however during highly effective ART, HIV specific $CD8^+$ T cell responses tend to decline as viral antigen decreases (Casazza, Betts, Picker, & Koup, 2001; Mollet et al., 2000). Post-transplant further decreased the HIV-responsive T cell numbers at the 9 month mark. Any detected donor-derived T cells would be HIV naïve, and therefore, the only HIV specific T cells that should be detected would be from the graft. While we cannot determine definitively if ICISTEM-021 was completely cured of his HIV infection, the dynamics of the T cell response (that is, lack of expansion of the donor derived, naïve HIV specific T cell repertoire up to 9 months post-transplant) suggests that there are aspects of functional immunologic cure.

In addition to reduced HIV T cell responses, there was a reduction in HIV proviral burden and maintenance of an undetectable viral load during the transplant. Reducing the

number of cells with HIV replication potential would be in keeping with lower antigenic burden and therefore limited ability to generate new anti-HIV T cell responses (Stranford et al., 2001). As chronic stimulation by HIV is thought to drive upregulation of immune exhaustion markers, this lack of response to HIV suggests that the phenotypic immune exhaustion seen previously may not be due in large part to HIV (Day et al., 2006). In addition to limited HIV antigenic burden, low anti-HIV $CD8^+$ T cell responses may also be due to a lack of $CD4^+$ T cells. As the donor derived T cells would not have seen HIV prior to transplant, new anti-HIV $CD8^+$ T cell responses would require $CD4^+$ T cell help. During the transplant, $CD4^+$ T cell levels remained very low, suggesting that $CD4^+$ T cells were struggling to reconstitute. Research into the effects of ATG have suggested that it may impair $CD4^+$ T cell recovery following alloHSCT, so this may not be unexpected (Bosch et al., 2012). Such a struggle could lower the amount of $CD4^+$ T cell help and impair formation of new antigen specific responses. The near elimination of HIV-specific T cell responses is promising, as it suggests that, at least in the context of ART, HIV does not undergo sufficient reactivation after the stress of a stem cell transplant to trigger the type of acute HIV infection immune response observed in usual primary HIV infection.

B cell responses on the other hand were not diminished. While the efficacy of antibodies produced against HIV can vary from person to person, most people produce antibodies against prominent proteins such as the envelope proteins gp120 and gp41 (Malaspina et al., 2005; Titanji et al., 2006). Stem cell transplants deplete B cells in addition to T cells, and given the near elimination of anti-HIV T cell responses, we expected to see a decrease in anti-HIV B cell responses as well. This was not the case; instead there was an increase in the number of cells secreting anti-HIV antibodies at 9 months after transplant. Prior to transplant, ICISTEM-0021 was on a tyrosine kinase inhibitor, which is known to impair B cell responses (de Lavallade et al., 2013). This may have dampened the ability for peripheral B cells to respond to activation, even though there were clearly anti-HIV antibodies present prior to transplant in plasma, as detected by Western Blot. As well, the elevated levels of TLM B cells pre-transplant may lower the number of inducible cells, as this population is enriched for anti-HIV B cells and are known for poor proliferative and antibody production ability after stimulation (Amu et al., 2014).

After transplant however, there are multiple routes for the generation of HIV-specific B cells. One is that these are recipient-derived B cells that failed to be ablated during the transplant. Stimulation as part of the B cell ELISPOT protocol would induce production of antibody that would be detectable on the assay. A second option is that the memory cells are donor-derived, but the antigen presenting cells were recipient derived residents of secondary lymphoid organs, and resistant to ablation. It is known that some antigen presenting cells can resist myeloabative treatment and these may represent a cell population able to prime cells for responding to HIV antigens after transplant (Durakovic et al., 2006). While this option would require $CD4^+$ T cells, it may explain an increase in the number of anti-HIV B cells without detectable viremia. Characterization of donor and host anti-HIV antibody allotypes may help to determine if the antibodies produced are of donor or recipient origin (Small, Robinson, & Miklos, 2009). Regardless, anti-HIV B cell responses are maintained after transplant, even though B cell phenotype becomes even more skewed towards a less functional phenotype.

4.4 Functional Bystander Responses Remain Altered, Especially in the B Cell Compartment

Immune exhaustion can impact both HIV-specific and non-HIV-specific adaptive responses. We examined changes in responses to two antigens: CMV and influenza. CMV is a Herpesvirus that causes chronic infection and contributes to persistent immune stimulation, while influenza is an acute viral pathogen that does not cause chronic infection. Both rely on $CD8^+$ T cells for viral control, and while a humorally based vaccine exists for influenza, no such vaccine is available for CMV.

CMV would have been present (but latent) before transplant in the recipient, and CMV-responsive T cells were detected both pre and post-transplant (albeit presumably from the donor derived bone marrow post-transplant). CMV is a potent stimulator of $CD8^+$ T cell expansion and induces strong IFN-γ responses (Margolick et al., 2018). Examination of other alloHSCT patients has shown that anti-CMV $CD8^+$ T cells can be observed shortly after early reactivation of CMV and continue to expand after CMV viremia is no longer detectable (Zabalza, Ciaurriz, Beloki, Ramirez, Gorosquieta, Calvillo, Bandres, Mansilla, & Olavarria, 2013a). Furthermore, this expansion anti-CMV $CD8^+$ T cell responses further strengthens the possibility that the phenotypic $CD8^+$ T cell exhaustion may be due to CMV

reactivation and T cell activation rather than some other source like HIV. Ongoing work will assess immune exhaustion and activation markers on CMV responsive CD8 T cells via a flow cytometric assay to further address this issue.

In contrast to T cell responses, anti-CMV B cell responses remained low after transplant. While there were no pre-transplant levels to compare to, the matched cohort generally showed much higher anti-CMV antibody levels in plasma. Humoral responses take longer to reconstitute in alloHSCT than T cell responses, so these results could be due to the fact that full reconstitution of humoral responses had not happened by 9 months (Park et al., 2015). As well, B cell subsets were skewed towards an immature/transitional phenotype at this point and all classes of memory cells were down, which would reduce the levels of antibodies circulating after viremia has passed. After hematopoietic stem cell transplant, a previous study in HIV negative individuals observed that anti-CMV antibody levels can be limited at 9 months post-transplant when the donor is CMV- and the recipient is CMV+ (Schoppel, Schmidt, Einsele, Hebart, & Mach, 1998). Therefore, B cell responses to CMV remain dysfunctional at 9 months after transplant, and likely are a sequelae of transplant more so than past HIV infection.

Anti-influenza adaptive responses also changed over the transplant period. ICISTEM-0021 showed robust responses to both HA/NP peptides and an HA-based flu vaccine prior to transplant. They trended towards the highest values observed in this cohort, suggesting robust responsiveness in both parts of the adaptive immune system. Yet post-transplant, the donor-derived cells remained unresponsive to influenza derived T cell and B cell antigens. Lower responses to NP, an antigen that would only be seen in active infection with influenza, are not unexpected as they did not have influenza during the 9 months studied. However, the person was vaccinated for influenza using a quadravalent inactivated vaccine 3 months post-transplant, which would help provide anti-HA responses. Influenza vaccines are known to be able to elicit T and B cell responses from stem cell transplant patients, but those who are vaccinated less than 6 months after transplant have much lower rates of seroconversion and lower levels of T cell responses (Avetisyan, Aschan, Hassan, & Ljungman, 2008). ICISTEM-0021 would have been vaccinated prior to the 6 month checkpoint, suggesting that the immune system was not reconstituted enough

to respond to the vaccine. Again, this is most in keeping with previous literature on the impact of stem cell transplant, more so than HIV infection.

4.5 Immunologic Comparison with the Other Functional HIV Cure Patient

This study allowed us to prospectively observe immunologic changes in a CCR5Δ32/Δ32 stem cell transplant that has shown to cure one person of the disease. It is still unknown what factors led to cure of the Berlin Patient, so understanding the differences between clinical trials is important in order to compare results. Table 4.1 provides a brief comparison between the Berlin Patient and ICISTEM-0021.

Table 4.1: Comparison of Immunologic and Clinical Factors between the Berlin Patient and ICISTEM-0021. Berlin Patient data is further expanded on in (Hütter et al., 2009) and (Allers et al., 2011).

Factor	ICISTEM-0021	Berlin Patient
Age at transplant	58	40
Sex	Male	Male
HIV Tropism	R5	R5, with residual X4
Length of HIV infection (years)	22 years	10+ years
Hematologic Malignancy	CML	AML
CCR5 Donor Genotype	Δ32/Δ32	Δ32/Δ32
Conditioning	Myeloablative + ATG	Myeloablative + ATG
Number of Stem Cell Transplants before Cancer Elimination	1	2
ARV use during transplant	Yes	No
CMV Serostatus	Positive	Negative
Graft Versus Host Disease	Yes	Yes
$CD4^+$ T cell count at 9-10 months post-transplant (Cells/μL)	176	100-200, during the second transplant
Elimination of Proviral Reservoir?	No	Residual X4 HIV detected in GALT that has not reactivated. No peripheral reservoir.
HIV Cure	Unknown	Functional Cure

While such rare clinical events make finding a perfect match to the Berlin Patient difficult, ICISTEM-0021 did match to the Berlin Patient on a number of factors. Both received Δ32/Δ32 stem cells that are resistant to their major tropism of HIV, one of the major criticisms with previous stem cell transplants aimed at removing the proviral reservoir (Hütter, 2016). They also both received myeloablative conditioning with ATG for

enhanced removal of $CD4^+$ T cells, which should increase the likelihood that $CD4^+$ T cells are eliminated in the pre-transplant period. As well, both individuals had low $CD4^+$ T cell counts at the 9-10 month post-transplant period. Finally, both also had mild graft-versus-host disease, which is known to help the graft fully eliminate residual host cells (Weiden et al., 1979).

There were some key differences between the two individuals however. First, the Berlin Patient received two rounds of stem cells and two rounds of conditioning due to AML relapse. However, HIV viremia was detected after the first transplant, suggesting that this first transplant did not remove the proviral reservoir. As well, ICISTEM-0021 was CMV seropositive and had reactivation of CMV, which could lead to more immune exhaustion in the post-transplant period and less immune reconstitution (Margolick et al., 2018; Zabalza, Ciaurriz, Beloki, Ramirez, Gorosquieta, Calvillo, Bandres, Mansilla, & Olavarria, 2013b). The Berlin Patient did not have antiretroviral therapy during the transplant, but did shortly between his transplants, but it is unknown what impact this could have on elimination of proviral reservoir. Finally, ICISTEM-0021 was older and had a longer HIV infection duration prior to transplant. This could impact immune function after transplant, as both HIV infection and age are associated with poorer thymic function (Haynes et al., 1999).

Taken together, this comparison suggests that differential outcomes to a Δ32/Δ32 transplant may be less due to HIV related factors and rather due to the stem cell transplant itself. Given that the Berlin Patient had a relapse of HIV after 7 months of not being on ART, it is possible that a single round of myeloablative conditioning with ATG may not be enough to remove the HIV proviral reservoir. The Berlin Patient would then have benefited from a second round of immune destruction and reconstitution that ICISTEM-0021 would not have. This would be further complicated in ICISTEM-0021 due to CMV reactivation potentially driving increased immune exhaustion. Reduced thymic function due to increased age and increased HIV infection could also be playing a role, but both the Berlin Patient and ICISTEM-0021 had similar $CD4^+$ T cell counts at similar time points during the transplant that would cause their cancers to go into remission, so this factor is still unclear. Therefore, the Berlin Patient benefited from a number of non-HIV related factors

surrounding his stem cell transplant that may have contributed to removal of the proviral reservoir.

4.6 Study Limitations

There are a number of limitations in this study. First, there was only one participant in the test group, which limits generalizability of the study findings to others. While more people around the globe are being recruited for CCR5Δ32/Δ32 stem cell transplant trials through the ICISTEM cohort, this number remains small (Hütter, 2016). At the moment, this therapy is only being trialed on people living with HIV who already require stem cell transplant for another indication, such as cancer, and who match to a CCR5Δ32/Δ32 stem cell donor. This makes the potential pool of participants very small, making addition of more participants to this study unfeasible. As well, this study only examined to the 9 month post-transplant time point. We were not able to collect any further samples due to clinical status and participant availability. As well, the participant unfortunately passed away due to a myocardial infarction 1.75 years after transplant, making collection of later samples and treatment interruption impossible. The work presented here is limited to peripheral blood immune cells however some other tissues have been collected for analysis by others in the group.

There were limitations experimentally as well. While the TILDA aims to balance the benefits of viral outgrowth and PCR based assays for proviral reservoir detection, it is not the accepted gold standard for proviral reservoir quantification. The literature suggests that the TILDA may overestimate the size of the reservoir by detecting virus that can produce *tat/rev* without producing virus (Hodel et al., 2016).

While our flow cytometry assessed expression of many immune markers, we did not subset different populations of T cells. There are a number of interactions between HIV and T cell subsets, such as follicular helper T cells, Tregs, and Th17 T cells (Eggena et al., 2005; Jenabian et al., 2013; Lindqvist et al., 2012). These cells are known to regulate the immune system and help form strong functional responses. Given the differences in function seen in ICISTEM-0021 over the transplant, it would be worthwhile to examine these cells more closely to identify differences that may be contributing to the functional profile.

Finally, while our functional assays did test for a number of antigens, we did not know the HLA profile of our cohort apart from ICISTEM-0021. It is well documented that responses to peptides can be influenced by HLA type, so a mismatch may have artificially decreased responsiveness to some antigens (Paul et al., 2015). The influenza T cell ELISPOT also only detected HA and NP responses to the H1N1 strain of influenza, potentially omitting responses to other strains. As well, the B cell ELISPOT and ELISA relied on the flu vaccine to provide the antigen against while antibodies or antibody secreting cells can be tested. However, the flu vaccine used was in circulation the year after the samples were collected for the comparator cohort. While none of the subjects in that cohort had received the flu vaccine for the 2015-2016 season at the time of collection, the formulation of the 2016-2017 vaccine only contained 2 strains used in the either the 2014-2015 or 2013-2014 seasons (A/California pdm09-like for A/H1N1 and B/Brisbane for B/Victoria) (Organization, World Health, 2013; 2014; 2016). A mismatch between the HA used in the assay and the HA used in previous vaccination or previously seen in the environment could lead to a decrease in responsiveness to the assay. This would not have affected ICISTEM-0021 after transplant as they would have been vaccinated with the vaccine used in the assay.

4.7 Future Directions

While this research provides insights into the transplant process in an HIV^+ individual, many questions remain. As the literature evolves, we are beginning to understand the complex relationship between immune cells and their interaction with HIV and immune exhaustion. Our phenotyping data showed noticeable increases in $CD8^+$ T cell exhaustion after transplant, but it is unclear what types of T cells they are and what they are responding to. Further subtyping these cells into different memory classes and using flow cytometry to measure immune exhaustion markers on cells proliferating to particular antigens. Tregs and Th17 T cells have also been identified as having an important role in immunosuppression in HIV infection (Jenabian et al., 2013; Lindqvist et al., 2012). Given the bystander immune dysfunction seen in this study, examining these populations may provide further insight. Finally, examining the different NK cell phenotypes over the transplant may provide insights into the prevalence of dysfunctional subsets within this patient.

While we tested the most prominent effector function of $CD8^+$ T cells and B cells, namely IFN-γ and antibody production, these cells also produce other cytokines that influence immune function, such as IL-2 and TNF- α. Examining the production of these cytokines in relation to antigenic stimulation, by either ELISPOT or flow cytometry, may provide more insight into the polyfunctionality of these cells beyond what we tested. Testing for NK cell functionality, especially if differences in NK cell phenotype are observed, should also be performed to provide a more complete picture of immune function.

4.8 Overall Conclusions

HIV has undergone a major shift within the last three decades, from previously being a death sentence to now being a chronic manageable illness. Despite this, questions remain on how to alter the immune changes seen in HIV that are associated with poor clinical outcomes. We provided insight into some of these questions by resetting the immune system of a person living with HIV via a stem cell transplant with HIV-resistant stem cells. This provided us with the opportunity to examine changes to the immune system after putting cancer in remission and reducing HIV burden. Table 4.2 describes our findings and the impact of contributing factors.

While not possible to know which factor was most important in the post-transplant immune changes, it is likely given Table 4.2 that the stem cell transplant itself is driving most of the associated change, along with subsequent CMV infection. The impact of HIV on the post-transplant individual is very limited and in keeping with much reduced HIV burden. All indicators point to a very limited HIV burden and may actually imply a functional cure would be possible in this individual.

Table 4.2: Summary of Major Study Findings and the Potential Impact of Clinical Factors. Dots represent potential impact of factors on research results.

Immune findings in ICISTEM-0021	HIV	SCT	CML	Age	CMV
Decreased HIV Proviral Reservoir		•			
Increased $CD8^+$ T Cell Exhaustion	•		•	•	•
Decreased Exhausted B Cells		•			
Increased I/T B Cells	•	•	•		
Decreased anti-HIV T Cell Responses		•			
Increased anti-HIV B Cell Responses	•	•	•		
Increased anti-CMV T Cell Responses		•			•
Decreased anti-Influenza T Cell Responses and Low anti-Influenza and anti-CMV B Cell Responses		•		•	•

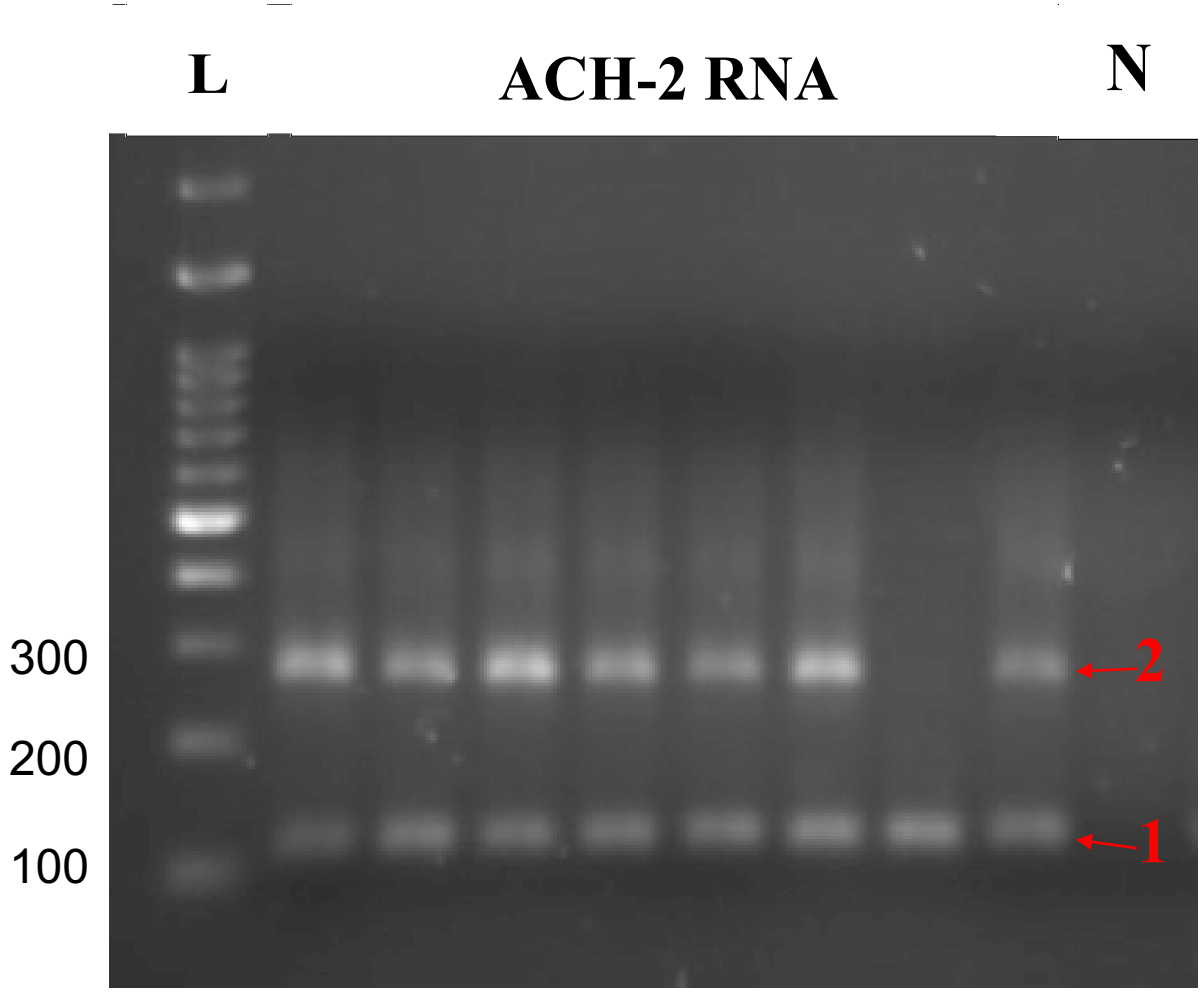
These data demonstrate that the CCR5Δ32/Δ32 alloHSCT was associated with changes to key populations of immune cells known to be impacted in HIV disease, most notably B cells, while also reducing the HIV proviral reservoir size. As such, a CCR5Δ32/Δ32 stem cell transplant may help alleviate some of the immune dysfunction associated with chronic HIV. Furthermore, the donor post-transplant chimeric immune system is in keeping with stem cell transplant complicated by CMV disease, rather than chronic HIV disease. While we will not be able to determine how these changes persist after a treatment interruption and further studies need to be done to examine similarities of our participant to other CCR5Δ32/Δ32 alloHSCT participants, this work has provided important insights into the role of CCR5Δ32/Δ32 alloHSCT to impact reversible immune exhaustion associated with chronic HIV. This has prognostic and potentially therapeutic implications for others living with HIV as they age.

Appendix 1

In the optimization of the TILDA, we performed gel analysis of the amplicons after real time PCR in order to confirm the sequence of expected targets. We tested ACH-2 control RNA that had processed as described in Chapter 2. Agarose gel analysis protocol is described in Appendix 4. Upon resolution of the gel, 2 bands appeared. Appendix 1, Figure 1.1 shows this gel. The smaller band was at the reported size (Procopio et al., 2015). The larger band was nearly double in size between 200 and 300 base pairs. Alterations to the cycling conditions to increase stringency did not cause the second band to disappear.

In order to identify these bands, we performed Sanger sequencing on the fragments that were shown in Appendix 1, Figure 1.1. The Sanger sequencing protocol is described in Appendix 4. We aligned the fragments to the human genome and to the HBX2 HIV reference genome. The results of this analysis are shown in Appendix 1, Table 1.1. All fragments did not align to the human genome. The two smaller fragments, which should be specific for Tat/Rev, did align to the HIV genome as expected in Exon 1 and 2 of Tat/Rev. The larger fragments also aligned to the HIV genome, but they aligned to Vpu or parts of the *Env* polytranscript. The expression of these transcripts is reliant on *tat* and *rev* for efficient transcription and export (Karn & Stoltzfus, 2012). As well, the location of the fragments overlapped with those of the smaller fragments. This suggests that the larger band is a later stage HIV transcript read in a different reading frame than *tat/rev*.

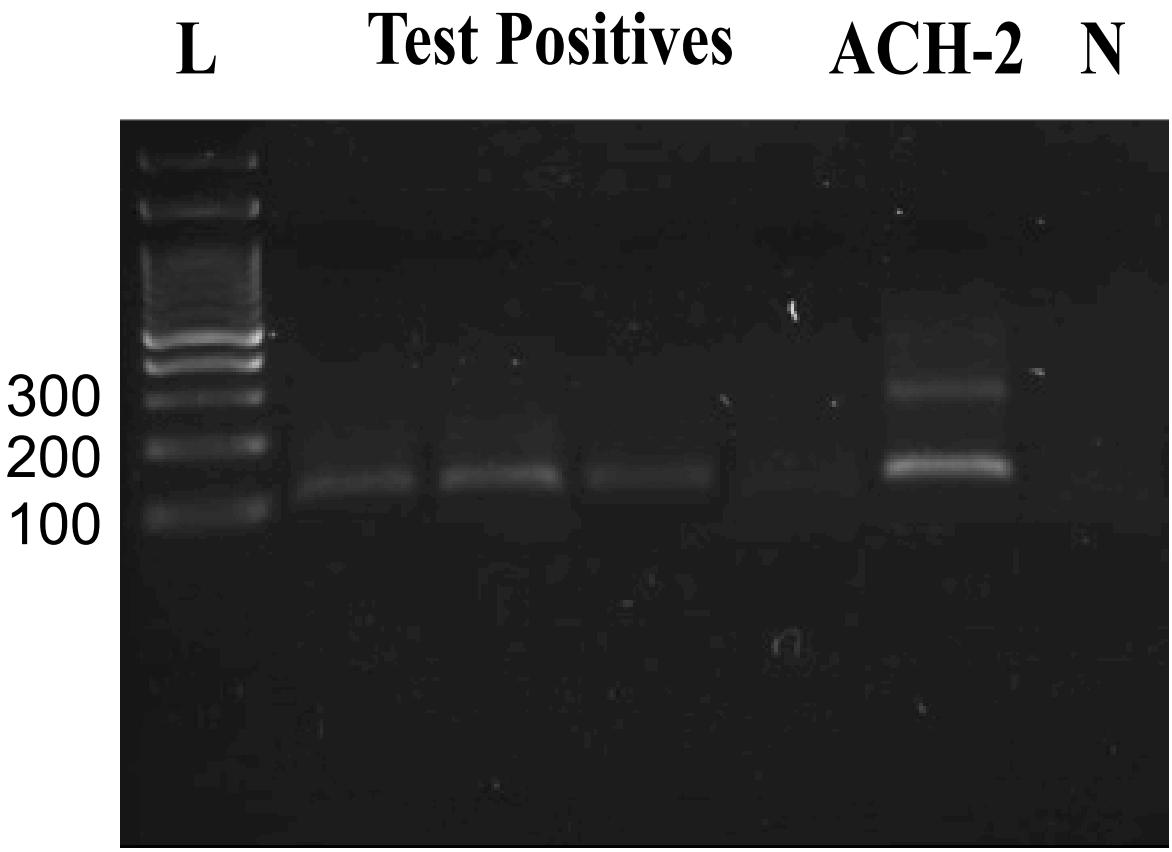
It is known that about 10% of all ACH-2 cells express all HIV proteins constitutively (Clouse et al., 1989; Folks et al., 1989). We hypothesized that the larger band may not be present in clinical samples from individuals with undetectable viral load, as their background HIV replication and viral protein production would be very low. We tested cells from ICISTEM-0021 pre-transplant with the TILDA protocol and ran the positive samples on an agarose gel with a negative and an ACH-2 positive control. Appendix 1, Figure 1.2 shows this gel. The negative control was blank, as expected, and the ACH-2 positive control had 2 bands. The positive test samples only had one band, thereby confirming that the non-specific amplification was limited to the positive controls.



Appendix 1, Figure 1.1: Agarose gel analysis of ACH-2 RNA after Tat/Rev real time PCR. Ladder in bp are annotated on the side. *N= Negative Control, L = Ladder.*

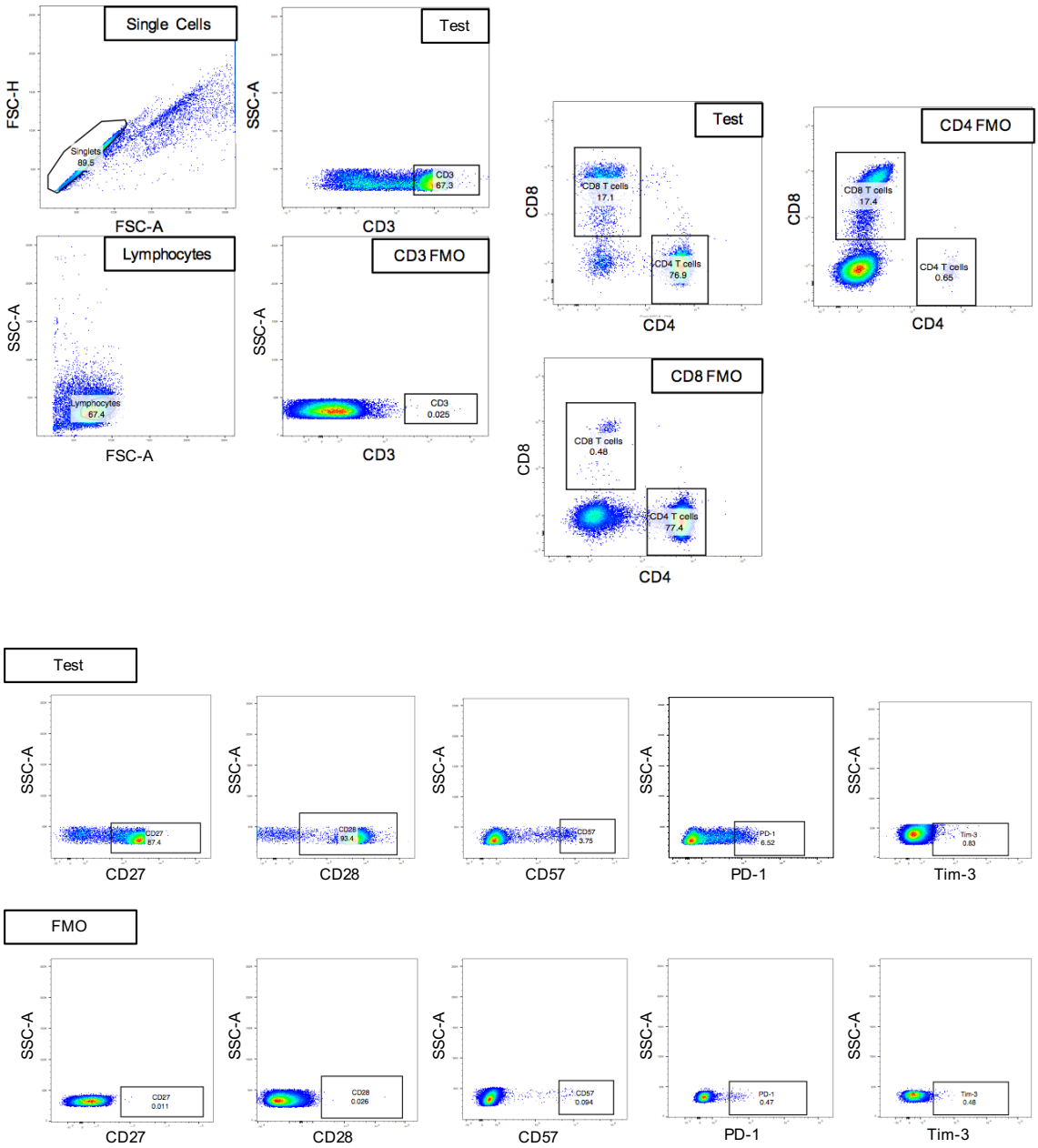
Appendix 1, Table 1.1: Alignment of Band Sequences to the HBX2 Genome. The first and last 10 base pairs are listed for each fragment. Fragments numbers are the same between this table and Appendix 1, Figure 1.1.

	Sequence	**HIV Transcript Alignment**
Fragment 1 Forward	5'-CCGGGGGGAC…AGACAGATCC-3'	Tat/Rev Exon 2, gp41, gp160
Fragment 1 Reverse	3'-TTCGGGCCTG…TCTGACTGTA-5'	Tat/Rev Exon 1, Tat/Rev Exon 2
Fragment 2 Forward	5'-GCCATACAAG…GACAGATCCA-3'	Vpu
Fragment 2 Reverse	3'-GGACTCCCGT…AGTCTGACTG-5'	Tat/Rev Exon 1, Vpu

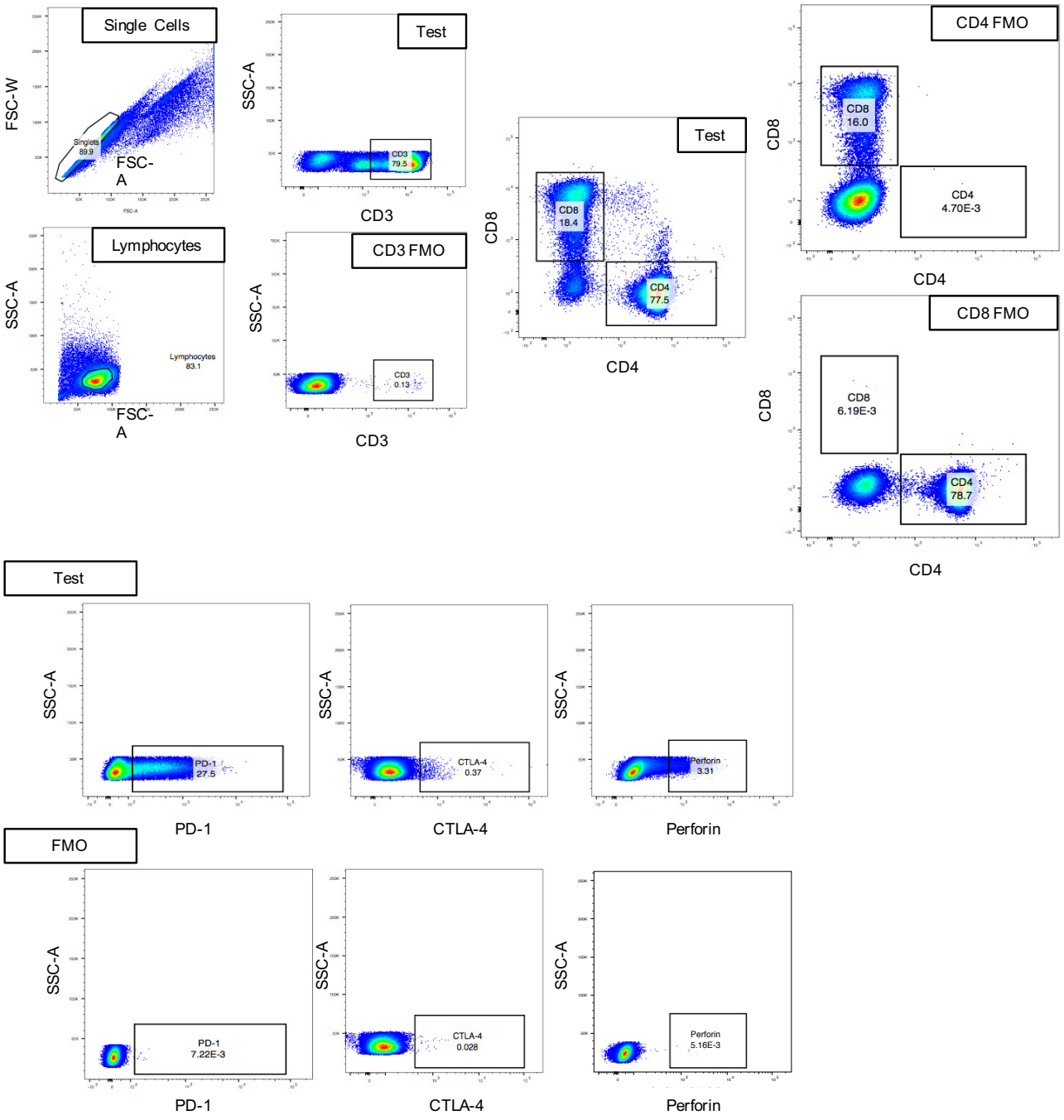


Appendix 1, Figure 1.2: Agarose gel analysis of clinical samples and ACH-2 RNA after Tat/Rev real time PCR. Ladder in bp are annotated on the side. *N = Negative Control, L = Ladder*.

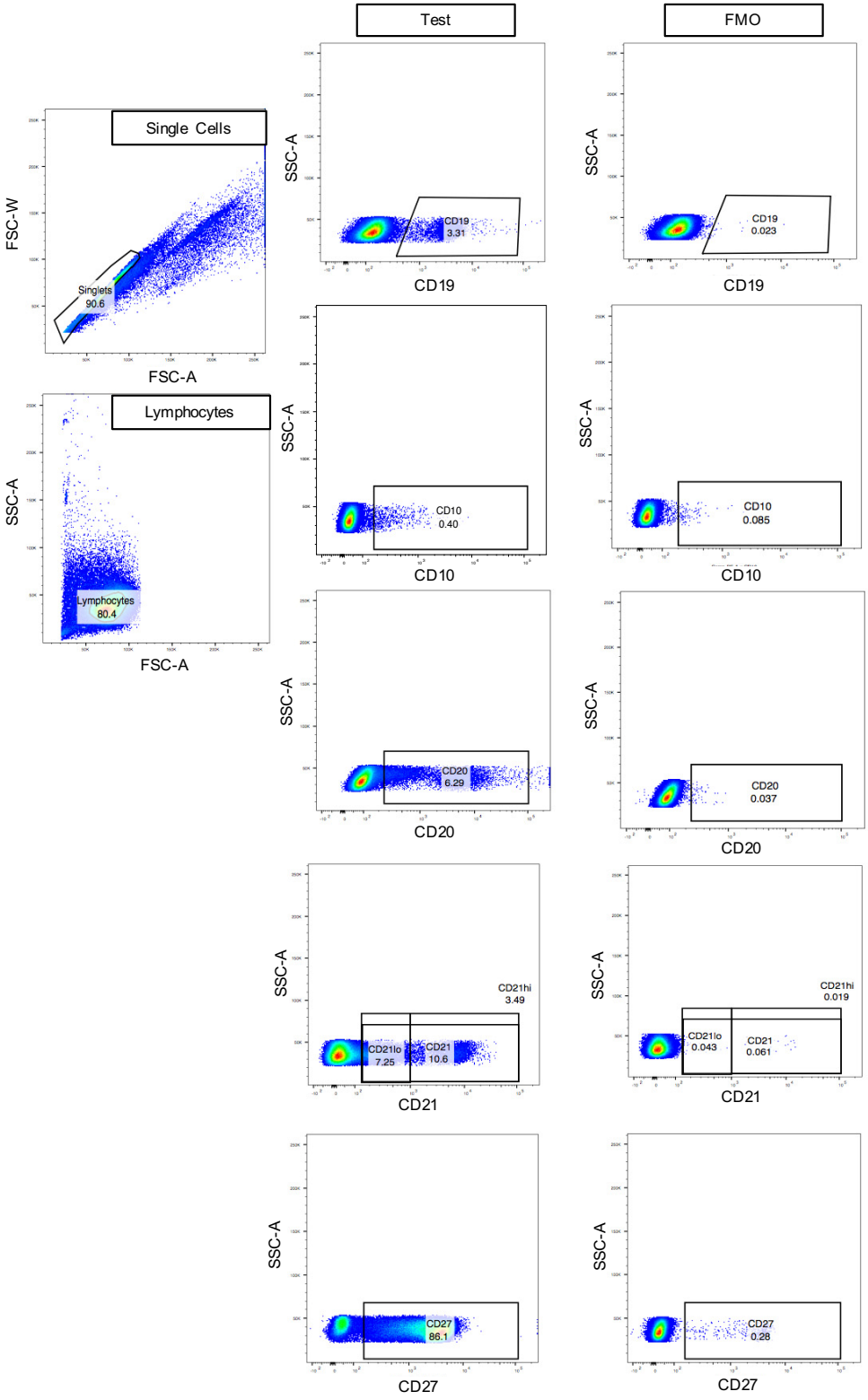
Appendix 2



Appendix 2, Figure 1.1: Sample gating analysis of T cell exhaustion panel. Gates were set based on the FMO for the marker. Boolean gating was done to analyze co-expression populations.



Appendix 2, Figure 1.2: Sample gating analysis of CTLA-4 containing T cell phenotyping panel. Gates were set based on the FMO for the marker. Boolean gating was done to analyze co-expression populations.



Appendix 2, Figure 1.3: Sample gating analysis of B cell panel. Gates were set based on the FMO for the marker. Boolean gating was done to analyze co-expression populations.

Appendix 3

Appendix 3, Table 1.1: Antibodies Used in Flow Cytometry in the T Exhaustion Panel

Target	Clone	Fluorochrome	Supplier
CD3	SK7	APC-H7	BD Pharmingen
CD4	RPA-T4	FITC	BD Pharmingen
CD8	RPA-T8	BV510	BD Horizon
CD27	O323	PerCP-Cy5.5	BioLegend
CD28	CD28.2	PE-Cy7	BD Pharmingen
CD57	TB01	efluor450	eBioscience
CD279 (PD-1)	EH12-2H7	PE	BioLegend
Tim-3	F38-2E2	APC	eBioscience

Appendix 3, Table 1.2: Antibodies Used in Flow Cytometry in the T CLTA-4 Panel

Target	Clone	Fluorochrome	Supplier
CD3	SK7	PerCP-Cy5.5	BioLegend
CD4	RPA-T4	FITC	BD Pharmingen
CD8	RPA-T8	BV510	BD Horizon
CD152 (CTLA-4)	L3D10	APC	BioLegend
CD279 (PD-1)	EH12-2H7	PE	BioLegend

Appendix 3, Table 1.3: Antibodies Used in Flow Cytometry in the B Cell Panel

Target	Clone	Fluorochrome	Supplier
CD10	HI10a	PE	BD Pharmingen
CD19	HIB19	FITC	BD Pharmingen
CD20	2H7	BV421	BioLegend
CD21	B-ly4	PE-Cy7	BD Pharmingen
CD27	M-T271	APC	BD Pharmingen

Appendix 3, Table 1.4: Antibodies Used in Flow Cytometry in Purity Checks for the TILDA

Target	Clone	Fluorochrome	Supplier
CD3	SK7	PerCP-Cy5.5	Biolegend
CD4	RPA-T4	FITC	BD Pharmigen
CD8	RPA-T8	BV510	BD Horizon

Appendix 3, Table 1.5: Primers Used in the TILDA

Primer	Sequence	Source
Tat1.4	5′-TGG CAG GAA GAA GCG GAG A-3′	(Procopio et al., 2015)
Rev	5′-GGA TCT GTC TCT GTC TCT CTC TCC ACC-3′	
Tat2	5′- ACA GTC AGA CTC ATC AAG TTT CTC TAT CAA AGC A -3	
Probe	5′-/56-FAM/TTC CTT CGG /ZEN/GCC TGT CGG GTC CC/3IABkFQ/-3′	

Appendix 4

Appendix 4.1: Production of the CMV Lysate for ELISA

For production of CMV lysate, MRC-5 cells were grown in lymphocyte media to 70% confluency in T75 flasks (VWR). MRC-5 cells are a fibroblast cell line permissive to CMV infection. One flask had the media replenished as normal, while the other was replenished with media containing CMV AD169 at a multiplicity of infection of 0.001. Cultures were allowed to grow for 2-3 days until cytopathic effected reached 100% in the CMV flask. Both flasks were washed with twice with PBS (Gibco) before being scraped with 5 mL PBS. Cells in PBS were added to a 15 mL tube (VWR). 10 mL of PBS + 0.1% Tween-20 was added to each tube and cells were pelleted at 400 g for 8 min. Cells were resuspended in 1 mL cold lysis buffer made from 50 mM Tris (Roche, Basel, Switzerland), 150 mM NaCl (Sigma Aldrich), and 1% Triton X-100 (Sigma Aldrich) at a pH of 7.4. Cells were refrigerated at 4ºC for 45 min, and gently rocked every 10 min. Lysate was then aliquoted at stored at -80ºC until use.

Appendix 4.2: Agarose Gel Analysis of Nucleic Acid Fragments and Sanger Sequencing

To analyze nucleic acid by agarose gel, a 2% agarose gel was prepared from UltraPure Agarose (Thermo Fisher) and TBE Buffer (VWR). Mixture was heated and allowed to cool while swirling. 10X RedSafe was then added in proportion to the volume of the gel, mixed by swirling, and then poured into a prepared gel cast. Combs were added to form wells and the gel was allowed to cool. TBE buffer was added to a Mini-Sub Cell GT (Bio-Rad) and cooled gel was added. Teeth were removed from the gel once submerged in buffer. Samples were thawed and mixed with 2 µL of 6X Loading Buffer (Intron Biotechnology, Burlington, USA)**.** 10 µL of buffered sample was added to appropriate wells. 6 µL of 1kB DNA Ladder (Froggabio, Toronto, Canada) was added to appropriate wells. Gel was run at 75 V for 45 minutes. Gel was analyzed using a Red Imaging System (AlphaInnotech, San Leandro, USA).

Sanger sequencing of fragments was performed by Genewiz (South Plainfield, USA). Briefly, DNA was extracted from the agarose gel with the QIAquick Gel Extraction Kit (Qiagen). DNA concentration was checked on a Nanodrop 2000 (Thermo Fisher) and 10 ng of sample was added to appropriate tubes. 25 pmol of appropriate

primer (Tat2 for forward, Rev for reverse) was added to the tubes. Tubes were then sent to Genewiz for sequencing.

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