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Follicular Regulatory T Cells and Follicular Helper T Cells: Role in HIV Pathogenesis

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Follicular Regulatory T Cells and Follicular Helper T Cells: Role in HIV Pathogenesis

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science
in Microbiology, Immunology, and Molecular Genetics

by

Bradley Salvatore

2018

ABSTRACT OF THE THESIS

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and Follicular Helper T Cells: Role in HIV Pathogenesis

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Master of Science in Microbiology, Immunology, and Molecular Genetics

University of California, Los Angeles, 2018

Professor Christina Uittenbogaart, Chair

The role and function of human T follicular regulatory (TFR) cells in healthy individuals are not completely understood, partly due to its previous grouping with T follicular helper (TFH) cells and partly due to the difficulty in obtaining human secondary lymphoid tissues. A majority of the studies of these two T cell subsets have been done in mice. Up to date, there are only a few studies of human TFR cells in HIV infection in the lymph nodes and the spleen, and peripheral blood TFR cells have not been well defined. The understanding of TFR cells and its phenotypic difference from TFH cells are crucial in the understanding of the humoral response against HIV and other diseases. Also, elucidating TFR cells' contribution to HIV persistence is important in the path of finding a functional cure for HIV infection. In this Master Thesis, I have examined TFH and TFR cells and their surface markers in human peripheral blood. In Chapter 2 ("Characterization of T Follicular Helper Cells and T Follicular Regulatory Cells in HIV-Infected and non-Infected Individuals"), I investigated the phenotypic characteristics of peripheral blood TFH (pTFR) and TFH (pTFH) cells in peripheral blood mononuclear cells (PBMC) of HIV-infected and HIV non-infected individuals from the Multicenter AIDS Cohort Study (MACS).

pTFR cells uniquely expressed the natural T regulatory canonical marker, forkhead box P3 (FOXP3), while pTFH uniquely expressed the TFH cells' master regulatory, B cell lymphoma 6 (BCL6). An effective way to identify pTFH and pTFR in PBMC only using their surface markers would allow for further functional studies. Collectively, this thesis should further advance the field of basic research of T cells and the field of HIV reservoir, bringing the field closer to a functional cure of HIV Infection.

The thesis of Bradley Salvatore is approved.

Benjamin Bonavida

Zoran Galic

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University of California, Los Angeles

2018

Dedication

I would like to dedicate this work to all the individuals within the Lesbian, Gay, Bisexual, Trans, Queer/Questioning, and others (LGBTQ+) community, who have inspired me to go through this master program; and to my mom, who has always been there for me in each and every step of the way.

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CHAPTER 1:

Introduction

I. The history of the Human Immunodeficiency Virus (HIV) and the Acquired Immunodeficiency Syndrome (AIDS)

In 1981, AIDS was first reported in Los Angeles, California, in 4 young men, who experienced pneumonia and other opportunistic infections (1). The cause of AIDS was not identified until two years later. In 1984, the human immunodeficiency virus type 1 (HIV-1) was simultaneously discovered as the etiological agent of AIDS by Dr. Luc Antoine Montagnier of the Pasteur Institute (2) and Dr. Robert Charles Gallo of the National Cancer Institute (3). In 1986, the human immunodeficiency virus type 2 (HIV-2), was isolated from two AIDS patients from West Africa (4) This discovery was soon followed by the identification of simian immunodeficiency viruses (SIVs) and simian relatives of HIV-1 and HIV-2, which trace the origin of HIV-1 and HIV-2 as the zoonotic transfers of viruses infecting primates in Africa (5-7).

HIV/AIDS has claimed millions of lives, especially within the at-risk populations such as men who have sex with men, transgender people, people who inject drugs and sex workers as defined by the World Health Organization (8). The hallmark of AIDS is a progressive depletion of T lymphocytes expressing CD4 (9); thus HIV-infected individuals have a low circulating $CD4^+$ T cell count. Before 1996, monotherapy of HIV-1 specific antiviral drugs was the only available treatment. After 1996, the introduction of highly active antiretroviral therapy (HAART), a combination therapy, was crucial to the reduction of HIV/AIDS related mortality and morbidity (10, 11). Up-to-date, the Food and Drug Administration (FDA) has approved 40 treatments for HIV infection, which are divided mainly into seven classes based on their molecular mechanism: (1) nucleoside reverse transcriptase inhibitors (NRTIs), (2) nonnucleoside reverse transcriptase inhibitors (NNRTIs), (3) protease inhibitors (PIs), (4) fusion inhibitors, (5) entry inhibitors, (6) HIV integrase strand transfer inhibitors, and (7) multi-class combination products (12). Even with the availability with these treatments, HAART only suppresses viral replication and slows down the

progression from HIV infection to AIDS, but HAART cannot cure HIV infection. HIV infection is a chronic infection with no functional cure currently. In 2012, Truvada, a combination of tenofovir and emtricitabine, was approved for pre-exposure prophylaxis (PrEP) by the FDA, and has been shown to reduce chances of contracting HIV more than 90% (13-16).

II. The HIV reservoir as a challenge to a functional cure

Combination antiretroviral therapy (cART) can only maintain HIV viremia below the limits of detection, low-level viremia can persist more than 7 years (17). Even with the intensification of cART, residual HIV-1 viremia cannot be completely eliminated (18-21). $CD4^+$ T cells still harbor HIV-1 integrated genetic materials, allowing the virus to persist indefinitely in a stable latent reservoir (22). An HIV reservoir is defined as any kind of cell type that allows persistence of the virus year after year in patients on cART (23). Thus, the HIV reservoir poses a huge challenge to HIV eradication (24).

Many types of cells can act as an HIV reservoir: T cells, monocytes, macrophages, resident macrophages of the central nervous system (CNS), astrocytes, dendritic cells (DCs), follicular dendritic cells (FDCs), gut associated lymphoid tissue, etc. (23, 25). Notably, HIV can establish latent infection in $CD4^+$ T cells, especially long-lived memory $CD4^+$ T cells (24, 26). $CD4^+$ T cells subsets, displaying the co-receptors CXCR4 and CCR5, are highly susceptible to CXCR4-tropic and CCR5-tropic HIV infection and can thus readily become an HIV reservoir during chronic infection. Within the $CD4^+$ T cell population, T cell subsets that contribute to HIV persistence are: central memory T cells (T_{CM}), transitional memory T cells (T_{TM}), germinal center T follicular cells, resting regulatory T cells and peripheral T follicular helper cells (25, 27-30).

III. T follicular helper (TFH) cells in the germinal center (GC) and peripheral blood (pTFH)

The natural history

In 2000, TFH cells were discovered in the human follicles and germinal center (GC) of secondary lymphoid tissues as CD4+CXCR5+ T cells (31, 32). TFH cells express the master transcription regulator BCL6 (32). In mice, B cell lymphoma 6 (bcl6) and Blimp-1 (or PRDM1, PR domain zinc finger protein 1) were shown to be reciprocal and antagonistic regulators of the differentiation of GC TFH cells, in which bcl6 promotes the GC TFH development, and blimp-1 inhibits GC TFH differentiation (33). Yet, it remains largely unclear which role bcl6 plays in the development of human GC TFH. Since its first discovery in early 2000, the surface markers of GC TFH have been well characterized. GC TFH cells express the chemokine receptor CXCR5 on their surface, allowing them to migrate to the B cell follicles in response to the chemokine CXCL13 (34). Other distinguishable features of GC TFH are the expression of inducible T cell costimulator (ICOS), programmed cell death protein-1 (PD-1), $CXCR4^{high}$, $CCR7^{low}$, and the absence of Blimp-1 (34).

Largely, the definition of TFH cells remains ambiguous (35) as different publications defined TFH with a different set of markers. Commonly, GC TFH cells can be identified by the intracellular marker, BCL6, and/or the combination of the following surface markers: $CD3^{+}$, $CD4^{+,}$ $CXCR5^{+}$, $PD\text{-}1^{+}$, $ICOS^{+}$, $CXCR4^{high}$, $CCR7^{low}$. Within the lymph nodes, CXCR5 can be used in combination with BCL6 or PD-1 to identify extrafollicular TFH and GC TFH in mouse and human (36).

Function

In both mice and humans, bona fide TFH in the GC (GC TFH) are different from Th1, Th2 or Th17, and thus are a distinct subset of $CD4^{+}$ T cells. GC TFH cells migrate to the GC of the B cell follicles, and provide help and survival signals to B cells in order to produce high-affinity

antibodies and generate B-cell memory (37). This specialized subset of T cells is also essential for germinal center formation, affinity maturation, and the development of high-affinity antibodies and memory B cells (36).

HIV reservoir

Human $CXCR5^{+}$ $PD\text{-}1^{+}$ $BCL6^{+}$ GC TFH cells in the lymph nodes were reported to be a major compartment for HIV-1 infection, replication, and production (29). Also, $CXCR5^{intermediate}PD\text{-}1^{intermediate}$ non-GC TFH, $CXCR5^{high}PD\text{-}1^{high}$ GC TFH, and pTFH are responsible for persistent HIV-1 transcription in treated aviremic individuals. In a CCR5- or CXCR4-tropic HIV reporter virus infection model, human tonsillar $CXCR5^{+}$ $PD\text{-}1^{high}$ GC TFH cells were shown to be highly permissive to HIV-1 *ex vivo*, and downregulate PD-1 and CXCR5 expression during productive infection (38).

IV. T follicular regulatory (TFR) cells regulate GC responses

TFR cells in mice

In 2011, T follicular regulatory (TFR) cells with suppressive function in the GC were discovered as a separate and distinct subset of $CXCR5^{+}$ $FOXP3^{+}$ T cells in mice (39-41). Previously, TFR cells were grouped together with TFH cells because both of them share similar phenotypic characteristics such as the TFH transcription factor and master regulator, BCL6. Distinctive from TFH cells, TFR cells express the Treg canonical transcription factor, FOXP3, and were shown to control the GC response (39-41). TFR cells also uniquely express BCL6 and Blimp-1 simultaneously (40). TFR cells do not develop from naïve CD4+ T cells or TFH cells. However, TFR cells are derived from $CXCR5^{-}$ $FOXP3^{+}$ regulatory T cells (Treg) generated in the thymus (39-41). In mice, TFR cells function to limit TFH cells and GC B cell number *in vivo*, thus inhibiting the GC reactions (39-41). The lack of TFR cells leads to greater GC reactions as well

as an outgrowth of non-antigen-specific B cells in the GC (39-41).

In summary, TFR cells share similar phenotypic characteristics with TFH cells and FOXP3+ Tregs (42). Treg-related molecules on TFR cells include: CD25, cytotoxic T-lymphocyte-associated protein 4 (CTLA4), glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR) and granzyme B (42). TFH cell-related molecules on TFR cells include: CXCR5, ICOS, PD-1, and BCL6 (42).

TFR cells in humans

Recently, TFR cells have been identified in humans as well (41, 43, 44). Different from mice, the maintenance of human $CXCR5^+$ $CD57^+$ $CD25^-$ $CD4^+$ TFH cells and $CXCR5^+$ $CD57^+$ $CD127^-$ $CD25^+$ $CD4^+$ TFR cells from iliac lymph nodes do not require GC B cells, as seen in kidney transplant recipients undergoing rituximab (RTX) B cell depletion therapy (44). Compared to TFH cells and extrafollicular $CD4^+$ T cells, tonsillar $CXCR5^+$ $PD\text{-}1^+$ $CD25^+$ $CD127^-$ GC TFR cells express high levels of CCR5 and are more permissive to CCR5-tropic HIV-1 *ex vivo*, (43). Measurements of HIV-1 RNA from the lymph nodes of asymptomatic, chronically HIV-1-infected, untreated individuals show that TFR cells harbor a high concentration of HIV-1 *in vivo* (43). A recent review by Maceiras *et al.* lays out all the TFR cell studies with major findings up to date but there are no reports of peripheral blood pTFR in HIV infection (42). TFR cells reported in the Maceiras *et al.* review were defined by different sets of markers, and there is currently no agreement on the best set of markers to identify TFR cells.

Changes in TFR cells present HIV+ individuals have been reported. The percentage of $FOXP3^+$ $CXCR5^+$ $CD45RA^-$ $CCR7^-$ $CD4^+$ TFR cells increase in the spleens of HIV+ individuals (45). In a study using human and rhesus macaque models, $CD25^+$ $CD127^-$ $CXCR5^+$ $CD3^+$ $CD8^-$TFR cells proportionately and numerically expanded in human and rhesus macaque lymph nodes during

HIV/SIV infection (46). Indoleamine 2,3-dioxygenase (IDO) and TGF-beta production were increased in HIV infection and TFR cells exhibited elevated regulatory phenotypes and impaired the function of TFH cells (46). TFR cells have also been studied in other diseases such as breast cancer, chronic hepatitis B and C, *Schistosoma japonica* infection, food allergy, multiple sclerosis (MS), ankylosing spondylitis (AS), kidney allograft rejection, and myasthenia gravis (42). Similar to how TFH cells' phenotypic characteristics are loosely defined, the characterization of TFR cells in these studies also used different sets of markers. Thus, it remains unclear what would be the best and most comprehensive combination of markers to accurately identify human TFR cells in secondary lymphoid tissues and the blood.

V. The Multicenter AIDS Cohort Study

The Multicenter AIDS Cohort Study (MACS) is an ongoing prospective cohort study of HIV-1 infection in homosexual and bisexual men. The MACS has been conducted over 30 years in 4 different sites: Los Angeles, Chicago, Pittsburgh, and Baltimore. The MACS began in 1984 with 4954 men enrolled initially, followed by a second enrollment of 668 men in 1987 and a third enrollment of 1350 men in 2001. The study screens both HIV-uninfected and HIV-infected men and collects various demographic and health information as well as blood samples for viral load and T cell counts. For more information, please visit aidscohortstudy.org.

VI. Peripheral blood mononuclear cells (PBMC) and their relevance to the lymphoid tissues, a main site of HIV infection

In HIV-1 chronic infection, viral replication mainly concentrates in secondary lymphoid tissues such as lymph nodes and spleens (47, 48). However, the human secondary lymphoid tissues are difficult to obtain and are not always readily available. Thus, most of the research on TFH

and TFR cells has been done in mice (42). TFH cells and TFR cells, with similar function to that of the GC, have been identified in peripheral blood (49). Peripheral blood TFH and TFR cells (pTFH and pTFR) can potentially be used as a proxy measure, mirroring what is occurring in the lymph nodes of patients. We obtained access to viably cryopreserved MACS samples from the Los Angeles site of the MACS, allowing us to study pTFH and pTFR and its contribution to HIV persistence. A panel of CD3, CD4, CXCR5, CD25 and CD127 can be used to determine TFH and TFR cells in human blood (49). There are other ways to gate for pTFH and pTFR cells in the blood such as $CD25^{-/low}$ $CD127^{low/high}$ pTFH cells and $CD25^{high}$ $CD127^{low}$ TFR cells. Whether bona fide GC TFH cells are present in the blood is not clear and controversial (50), but we have found the presence of CXCR5+ BCL6+ TFH-like cells in the peripheral blood (**Chapter 2**). Human blood CXCR5+ CD4+ T cells were shown by Morita *et al.* to be counterparts of T follicular cells and can help B cells to make antibodies (51). Overall, studying pTFH and pTFR cells in viably frozen peripheral blood lymphocytes can provide an alternative and easier way to assess the function of these T cell subsets, which are important in understanding human humoral response to HIV infection.

VII. Conclusion

HIV reservoirs pose a big challenge to the complete eradication of the virus. CD4+ T cells, especially TFH cells, can serve as HIV reservoir and contribute to HIV persistence. Recently TFR cells, with its unique FOXP3 expression, were discovered. TFR were previously grouped together with TFH cells. Thus, TFR cells can potentially contribute to HIV reservoir as well. It is difficult to obtain secondary lymphoid tissues for studying TFH and TFR cells, thus an alternative way would be using PBMC. With the access to the PBMC of HIV-infected and non-infected individuals from the MACS, we aimed to identify these two CD4+ T cell subsets as an alternative way of examining the lymph nodes. Furthermore, little is known about TFR cells in

the human peripheral blood because most studies use mouse models to identify TFR cells. Our study can provide valuable insights into human TFR cells to fill this gap of knowledge and contribute to potential therapy in HIV infection.

CHAPTER 2:

Characterization of T Follicular Helper Cells and T Follicular Regulatory Cells in HIV-Infected and Non-Infected Individuals

Bradley Salvatore, Rachel S. Resop, Ph.D., Brent Gordon, B.S., Marta Epeldegui, Ph.D., Otoniel Martinez-Maza, Ph.D., and Christina Uittenbogaart, M.D.

Abstract

The humoral response is important in fighting bacteria and other intracellular pathogens by the production of specific antibodies by B cells. In the germinal center (GC), T follicular helper (TFH) cells provide important help to B cell antibody production. GC TFH cells have been shown to contribute to HIV persistence and act as an HIV reservoir. HIV reservoirs, which are in cells with integrated DNA of HIV, can persist in the presence of Highly Active Antiretroviral Therapy (HAART) and present major problems in completely eradicating the virus. The recently discovered GC T follicular regulatory (TFR) cells, which inhibit the function of T follicular helper cells, express very similar surface markers as GC TFH cells. The fork head box P3 (FOXP3) is so far the only definitive marker that distinguishes GC TFR cells from GC TFH cells and has not been used to differentiate TFR from TFH in HIV persistence. Thus, it is unknown whether the increase in TFH cells which has been observed in HIV infection is also due an increase in TFR cells and whether TFR cells, like TFH cells, can also contribute to HIV persistence. We used multicolor flow cytometry to detect TFH and TFR cells in cryopreserved peripheral blood mononuclear cells (PBMC) from HIV-infected and non-infected individuals in the UCLA site of the Multicenter AIDS Cohort Study (MACS). In our study, we have characterized and identified the presence of $CD3^+$ $CXCR5^+$ $CD8^-$ $BCL6^+$ peripheral blood T follicular helper (pTFH) cells and $CD3^+$ $CXCR5^+$ $CD8^-$ $FOXP3^+$ peripheral blood T regulatory (pTFR) cells in PBMC. Unlike GC TFR, we found that pTFR cells do not express B cell lymphoma 6 (BCL6), a TFH master regulator. Our results show that the frequency of pTFH cells is higher in HIV-infected than in non-infected individuals. In addition, the proportion of $TIGIT^+$ cells is significantly higher in pTFR than pTFH cells. The frequency of Programmed Cell Death Protein 1 (PD-1) and inducible T-cell costimulator (ICOS) expressing cells show an increasing trend in HIV-infected individuals, but this increase was not statistically significant. ICOS is expressed on activated T cells and its constitutive expression was shown on pTFR cells. In contrast to pTFH, pTFR express more

CCR5 in HIV-infected than non-infected individuals, suggesting their potential to contribute to HIV persistence in pTFR. In summary, our data show that pTFH and pTFR can be detected in peripheral blood of HIV-infected and non-infected individuals and can potentially serve as a representative measure of GC TFH and GC TFR cells inside the lymph nodes. Although the percentage of pTFR is not elevated in HIV-infected individuals, we do not yet know whether their function is affected or whether they harbor HIV and thereby contribute to HIV persistence. Experiments are planned to address these questions. Further understanding of cells contributing to HIV persistence will be beneficial to the discovery of a functional cure for HIV infection.

Introduction

Human Immunodeficiency Virus type 1 (HIV-1) is the etiologic agent of Acquired Immunodeficiency Syndrome (AIDS), which was first described in the United States at UCLA in 1981 (1, 52, 53) and was soon thereafter isolated by Luc Montagnier and Robert C. Gallo (2, 3). With current combination antiretroviral therapies (cART), an HIV-1 infection can be kept under control effectively. However, $CD4^+$ T cells still harbor HIV-1 integrated genetic materials, allowing the virus to persist indefinitely in stable latent reservoirs (22). HIV-infected cells are difficult to eradicate, and researchers have defined HIV reservoir practically as any kind of cell type that allows persistence of the virus year after year in patients on optimal ART (23). Resting memory $CD4^+$ T cells with integrated virus genome are found in individuals in the later stages of HIV-1 infection, are thus considered a latency reservoir (26). There are several potential HIV reservoirs that can contribute to HIV persistence: $CD4^+$ T cells in the lymph nodes, gut-associated lymphoid tissue, the central nervous system, hematopoietic stem cells, macrophages, and regulatory T cells (23, 30).

HIV-infected individuals have low circulating CD4+ T cell counts. CD4+ T cells subsets, displaying the co-receptors CXCR4 and CCR5, are susceptible to HIV infection and can readily act as an HIV reservoir. Previously, the T follicular helper (TFH) cells were shown as a reservoir of HIV (29, 54). TFH cells, different from Th1, Th2 or Th17, are a distinct subset of CD4+ T cells, which were described in the early 2000s (34). These cells localize in the germinal center (GC) of the B cell follicles and provide T cell help to B cells. TFH cells are also essential for GC formation, affinity maturation, and the development of high-affinity antibodies and memory B cells (36). In mice, GC TFH cell differentiation and development are dependent on the master regulator, B cell lymphoma 6 (BCL6) (33, 35, 55). Mouse and human TFH cells express the chemokine receptor CXCR5, which allows their migration to the B cell follicles (34). Additionally, they express inducible T-cell costimulator (ICOS) and programmed death-1 (PD1) (36). In secondary lymphoid tissues, CXCR5 can be used in combination with BCL6 or PD1 to identify TFH cells in mice and human (36). HIV-1 infection of GC TFH cells is dangerous because, without T cell help, B cells will not function properly, thus weakening the immune system. Lastly, TFH cells could act as an HIV reservoir and thus allow HIV to persist in the body, leading to HIV reinfection after cART (23).

Recently, a new $CD4^+$ $CD3^+$ T cell subset, developed from thymus-derived fork head box P3 $(FoxP3)^+$ precursors, was described in mice and named T follicular regulatory T (TFR) cells (39-41). $FOXP3^+$ T regulatory cells maintain immune homeostasis and prevent autoimmunity by inhibiting proliferation and cytokine production of effector T cells (56, 57). In mice, TFR cells have been shown to suppress GC reactions and also play a role in preventing autoimmunity and exaggerated immune responses (39). TFR cells possibly suppress the immune system either by interfering with T cell help to B cells or directly suppressing B cell function. However, the maintenance of TFH and TFR cells is independent of GC in humans (40). TFH and TFR cells both have been found in human tonsil (44). Treatment with CD20 monoclonal antibody rituximab

(RTX), dampening the immune system of renal transplant patients, causes significant depletion of B cells in the GC in the lymph node (44). However, TFH and TFR cells are still present in the absence of B cells, suggesting that the maintenance of TFH and TFR cells is independent of B cell activity in GC (44). If TFR cells can harbor and allow the persistence of HIV, it would be detrimental to the regulation of TFH cells within GC and may even lead to autoimmune diseases. The immunophenotype of TFR cells is similar to that of TFH cells. In the mouse secondary lymphoid tissues, they both express CD3, CD4, CXCR5, PD-1, ICOS on the surface and intracellularly the transcription factor BCL6 (36, 58). Except, TFR cells also express an additional transcription factor FOXP3 (39). Due to their similar phenotypic characteristics, it is possible that the previously identified TFH cell population, acting as HIV reservoir, also includes TFR, and therefore, it is still unknown whether TFR cells can contribute to HIV persistence. To this end we need to examine TFR cells in healthy and HIV-infected individuals in order to determine if they play a role in the maintenance of HIV reservoirs.

Using multi-color flow cytometry, peripheral blood mononuclear cells (PBMC) of HIV-infected and non-infected individuals from the UCLA Multicenter AIDS Cohort Study (MACS) were stained to immunophenotype peripheral blood TFH and TFR (pTFH and pTFR) cells. As a precautionary measure, we initially compared the phenotypic profile of pTFH and pTFR in fresh and frozen healthy PBMC from anonymous donors in order to assess the effect of freezing on the immunophenotype. Our study is the first report of an increase of TFH cells in peripheral blood of HIV-infected individuals. In addition, we found that pTFR cells also expressed a higher level of CCR5 in HIV-infected individuals, thus being more susceptible to CCR5-tropic infection and potentially acting as an HIV reservoir. HIV persistence despite antiretroviral therapy (ART) is a challenge in HIV eradication. The ability to identify certain subsets of T cells, which serve as an HIV reservoir, is an important milestone in developing a cure for HIV-infected individuals. By

eliminating HIV reservoirs, the virus will not be able to persist after ART which may contribute to a functional cure for HIV infection.

Results

The phenotypic characteristics of TFR cells can be altered due to freezing/thawing

Previously, $CXCR5^+$ follicular T cells expressing FOXP3, named T follicular regulatory cells (TFR), have been shown to be present in secondary lymphoid tissues in mice (39). Since then, it remained unclear how to best characterize human TFR cells because previous studies of TFR cells have defined this subset with different sets of markers (42). Here, we set out to find the best way to identify and characterize peripheral blood TFH (pTFH) cells and peripheral blood TFR (pTFR) cells in viably cryopreserved human PBMC from the Multicenter AIDS Cohort Study (MACS), as peripheral blood is easier to obtain than human secondary lymphoid tissues.

The MACS samples have been frozen for 3 to 10 years, and thus it is unclear how the long-term freezing and then thawing would affect the phenotypic characteristics of TFR cells. To determine the effect of freezing/thawing on TFR cells, we compared the profile of TFH and TFR cells within fresh PBMC and short-term frozen (7 days) PBMC. Figure 1A shows the gating strategy of $CD3^+$ $CD4^+$ $CXCR5^+$ $FOXP3^+$ TFR cells within human PBMC in the initial study. The similar percentage of TFR cells in fresh and frozen PBMC from the same donor suggested that the numbers of TFR cells were not affected by freezing/thawing at least after short term storage (Figure 1B, 1C, 2D).

We first examined the markers within the gating strategy of TFR cells. To examine the difference between markers in fresh and frozen PBMC, we set a change in 1-10% as not being

affected and above 10% as being affected by freezing/thawing. The average 2% difference in CD3 expression, the average 5% difference in CD4 expression, and 5% difference in CXCR5 expression indicated that these three surface markers were not affected by freezing (Figure 2A-C, 2J). On average, the 1% difference in BCL6 expression and 2% difference in FOXP3 expression showed that these two intracellular markers were also not affected. Next, the surface markers on $CD3^+$ $CD4^+$ $CXCR5^+$ $FOXP3^+$ pTFR cells were examined. The average 1% difference in CD25 expression and the 6% average difference in CD45RA expression indicated that CD25 and CD45RA were not affected by freezing (Figure 2F, 2G, 2J). The average 27% difference in PD-1 expression and the 33% average difference in CD62L expression indicated that PD-1 and CD62L were affected by freezing (Figure 2H-J). These results prompted an elimination of CD62L from the staining panel but PD-1 was retained because PD-1 expression was crucial to the identification of pTFR cells.

CD4+ T cells expressing CXCR5 can still be detected in long-term frozen human PBMC

We performed a phenotypic comparison of TFR cells population between HIV-infected and non-infected individuals from the MACS. Because CXCR5 expression was slightly affected by freezing and thawing (Figure 2B), we first assessed expression of CXCR5 in the MACS samples. Figure 3A showed a distinct population of $CXCR5^+$ $CD3^+$ T cells within the live lymphocytes singlet in HIV non-infected (left) and HIV-infected (right) individuals. There was an average of 28.63% $CXCR5^+$ $CD3^+$ T cells in HIV non-infected individuals, and there was an average of 19.28% $CXCR5^+$ $CD3^+$ T cells in HIV-infected individuals (Figure 1B). These data showed a decreasing trend of $CXCR5^+$ $CD3^+$ T cells in HIV-infected individuals, but was not statistically significant ($p>0.05$).

Additionally, previous studies showed that CD4 receptor was down-regulated by HIV-1 infection (59). In order to observe the effect of HIV infection on the CD4 receptor, we compared the proportion of $CD4^+$ T cells and $CD8^-$ T cells gated on $CD3^+$ $CXCR5^+$ (Figure 3C, 3D). On average, the proportion of $CD4^+$ T cells decreased from 53.73% in HIV non-infected to 28.71% in HIV-infected individuals ($p^* < 0.05$) (Figure 3C). The average proportion of $CD8^-$ T cells decreased from 64.63% in HIV non-infected to 42.14% in HIV-infected individuals ($p^* < 0.05$) (Figure 3D). The similar decrease in HIV-infected individuals for both $CD4^+$ T cells and $CD8^-$ T cells showed that $CD8^-$ T cells can be used to approximate CD4+ T cells in order to select T cells in which CD4 receptor was down-regulated. Interestingly, the proportion of cytotoxic $CXCR5^+$ $CD8^+$ T cells increased from 34.74% in HIV non-infected to 55.86% in HIV-infected individuals ($p^* < 0.05$) (Figure 3E). Overall, we demonstrated that $CXCR5^+$ $CD3^-$ T cells can be detected in long-term frozen PBMC, and $CD8^-$ gating strategy is an effective way to include T helper cells with down-regulated CD4 receptor due to HIV infection.

In peripheral blood, the frequency of pTFH cells increased in HIV infection, but not that of pTFR cells

First, the standard of gating for pTFH and pTFR in human blood was shown representatively from an HIV non-infected individual and an HIV-infected individual (Figure 4A, 4B). Forward scatter (FSC-A) and side scatter (SSC-A) were used to select for the lymphocytes, and FSC-H and FSC-A were used to exclude cells aggregates from single cells. Then, a ghost dye 510 was used to exclude non-viable cells. From there, $CD3^+$ $CXCR5^+$ $CD8^-$ T cells were selected. Lastly, BCL6 and FOXP3 were used to select for pTFH and pTFR cells, which expressed CD3, CXCR5, CD4, and no CD8. In human PBMC, pTFH cells uniquely expressed BCL6, and pTFR uniquely expressed FOXP3 (Figure 4A, 4B, last plot). These results showed that pTFH and pTFR cells are phenotypically different in BCL6 expression than GC TFH and GC TFR.

It is also unknown whether the increase in TFH cells during HIV infection is due to an increase in TFR cells. Thus, we investigated whether HIV infection had an effect on the frequency of pTFH and pTFR cells. The proportion of $BCL6^+$ $CD3^+$ $CXCR5^+$ $CD8^-$ pTFH cells was higher in HIV-infected individuals than in non-infected individuals (Figure 4C). The proportion of $FOXP3^+$ $CD3^+$ $CXCR5^+$ $CD8^-$ pTFR cells was similar in PBMC from HIV-infected and non-infected individuals (Figure 4D). In order to minimize the effect of individual variation in the number of $CD4^+$ T cells, the frequency of pTFH or pTFR cells was assessed by the ratio of pTFH and pTFR divided by total $CD4^+$ T cells. Relatively to all CD4+ cells, the frequency of pTFH cells was significantly higher in HIV-infected than in HIV non-infected individuals ($p<0.05$) (Figure 4E).). An increase in GC TFH has been reported in lymph nodes (60) and spleens (45) of chronically HIV infected individuals and may contribute to the dysregulation of B cell function such as hyper-gammaglobulinemia, polyclonal activation and a decrease in antigen-specific antibody production. In addition, the frequency of pTFR cells relatively to all CD4+ cells remains similar in HIV-infected and non-infected individuals (Figure 4E), which is in contrast to the increase in TFR cells observed in spleen and lymph nodes of HIV-infected patients (45, 46). Based on these results, an increase in pTFH cells in peripheral blood of HIV-infected individuals was not due to the increase in pTFR cells.

HIV co-receptor CCR5 expression on pTFR increases in HIV-infected individuals

We aimed to identify the contribution of pTFR to HIV persistence by comparing the phenotypic characteristics of pTFR cells between HIV-infected and non-infected individuals. GC TFH has been shown to be highly susceptible to HIV infection and can contribute to HIV persistence (29, 36). Both pTFH and pTFR cells expressed CCR5, indicating that they are susceptible to CCR5-tropic HIV infection. In pTFR cells, the proportion of CCR5 expression was significantly higher in

HIV-infected individuals (p*<0.05) while in pTFH cells there was a not statistically significant decrease in CCR5 expression in HIV-infected individuals (p>0.05) (Figure 5A).

Changes in surface markers on pTFH and pTFR cells

The presence of bona fide TFH cells and TFR cells in human blood are controversial, partly due to its varied phenotypic definition from studies to studies (48). In our study, pTFH cells were defined as $CXCR5^+$ $CD3^+$ $CD4^+$ $BCL6^+$ and pTFR cells were defined as $CXCR5^+$ $CD3^+$ $CD4^+$ $FOXP3^+$. In order to effectively sort pTFH and pTFR by its surface markers for functional studies in the future, we aimed to further characterize pTFR and pTFR cells with PD-1, TIGIT, ICOS, CD25, and CD45RA. We also aimed to determine the phenotypic differences between pTFH and pTFR cells in HIV-infected and non-infected individuals.

Both pTFH and pTFR cells expressed PD-1, TIGIT, ICOS, CD25, and CD45RA. The proportion of TIGIT expression is significantly higher in pTFR than in pTFR cells (p**<0.001) (Figure 5B), agreeing with data in mice indicating that TFR expressed two-fold higher TIGIT than TFH cells (61). We found that expression of TIGIT on TFR cells was similar in HIV-infected and non-infected individual (Figure 5B). Distinctly, the expression of PD-1 and ICOS in pTFH and pTFR cells showed an increasing trend in HIV-infected individuals, but this was not statistically significant (Figure 5C, 5D). As shown, we found a wide range of PD-1 and ICOS expression in HIV-infected individuals which may be due to a different stage of HIV progression. Previous studies characterized pTFH and pTFR cells in the lymph nodes by using just the surface markers CXCR5 and PD-1 or CXCR5 and CD25 (42). In peripheral blood, we cannot use PD-1 or any other surface marker to reliably distinguish pTFH and pTFR without staining for their intracellular markers.

Discussion

TFR cells were first identified in the GC of the mouse lymphoid tissue (39) and then in human tonsils (44). There is little known of TFR cells and their function in humans. In this study, we aimed to characterize TFR cells in PBMC of HIV-infected and non-infected individuals from the frozen peripheral blood lymphocytes from the MACS. Upon freezing, we found that the surface markers PD-1 and CD62L were destabilized in cells from the same PBMC samples because of the stress occurring during the freezing/thawing procedure. CD62L, a homing receptor to lymph nodes, was not crucial in the identification of TFR cells, and thus was not included in later staining of the MACS samples. However, we have to keep PD-1 on the staining panel because this marker has been commonly used to in combination of CXCR5 and ICOS identify TFH and TFR cells (42). Other markers such as CD3, CD4, FOXP3, BCL6, and especially CXCR5 remained unaffected by freezing/thawing.

Similar to TFH cells, the precise identification of TFR cells has been controversial and difficult because of its varied surface phenotypic definition in different reports (42). It was expected that pTFR cells are different from their counterpart in the secondary lymphoid tissues. In this study, we showed that $CD3^+$ $CD4^+$ $CXCR5^+$ follicular T cells in peripheral blood express FOXP3, along with other surface markers such as PD1, CD45RA, ICOS, TIGIT, and CD25. Human pTFR cells, unlike their counterpart GC TFR, do not express BCL6 in peripheral blood, indicating a potential difference in function between pTFR and GC TFR; or pTFR may even be a completely novel population. The lack of BCL6 in pTFR cells reinforced the finding of the precursor of GC TFR cells which is reported to be thymic-derived FOXP3+ Treg (39-41). Without BCL6 expression in the blood, $FOXP3^+$ $BCL6^-$ pTFR cells are more Treg-like and less TFH-like because BCL6 is essential for the differentiation of TFH cells (39). Additionally, even though pTFR cells express CXCR5 which distinguishes them from natural Treg, pTFR cells potentially could lose CXCR5

upon activation (62). We need to perform a functional assay for TFR cells in order to confirm their suppressive function. If pTFR have the suppressive properties of lymphoid-tissue TFR (34), pTFR could be the precursor of lymphoid-tissue TFR.

Most importantly, we hypothesized that TFR cells are a potential candidate for HIV reservoirs because TFH cells, which previously included TFR cells, contribute to HIV persistence (63). A viral reservoir is a challenge in finding a functional cure for HIV infection. After an individual has a successful ART treatment and thereby undetectable viremia, HIV can still persist in certain tissues and resurface in the blood because of latent infection. Our findings suggest that pTFR cells can potentially harbor HIV and contribute to HIV persistence because they express the increased levels of HIV co-receptor CCR5. However, based on the fluidity of blood and minimal cell interactions, there might not be enough contacts for the virus to infect the pTFR cells. HIV infection occurs mostly in the lymph nodes due to a higher cell density and a closer cell-to-cell contact. Further characterization, testing for the presence of HIV gag protein, will allow for further interpretation of this data. The presence of pTFH and pTFR cells in the blood can be a proxy measure for the condition of the lymph nodes during HIV infection, allowing for potential therapeutic application. Thus, understanding the immunophenotype and function of pTFR and their connection to lymphoid-tissue TFR can yield insight into HIV persistence in HIV-infected individuals.

Lastly, we have yet to find a surface marker that can substitute for the intracellular marker FOXP3 in order to identify pTFR cells for cell sorting. Nevertheless, our data showed that HIV infection affects the phenotypic characteristics of pTFH and pTFR cells. First CCR5, an activation marker and HIV co-receptor, was upregulated in HIV infection, similar to high level of CCR5 expression in tonsillar TFR (43). Secondly, TIGIT expression on pTFR cells was similar in both HIV-infected and non-infected individuals, suggesting that these cells are not exhausted

and can potentially suppress pTFH cells in an *ex vivo* suppression assay. Thirdly, PD-1 expression on frozen cells was reduced significantly compared to the fresh cells. Although freezing/thawing did affect PD-1 dramatically, with the detected PD-1, the frequency of PD-1^{+} pTFH and pTFR cells showed a trend of increasing in HIV infection. In mice, PD-1 signals suppressed TFR cells in peripheral blood (64). This suggested that more PD-1 expression in HIV infection can affect the function of TFR cells. Fourthly, 5% of TFR expressed ICOS constitutively (as compared to CD3+CXCR5+ T cells). In influenza infection, ICOS stimulation is needed to maintain FOXP3 expression in the absence of IL-2 (65). In mice, ICOS-expressing T cells produced IL-2, and a distinct population of memory phenotype CD4+ T cells constitutively expresses ICOS (66). In human pTFR cells, ICOS may play a similar role in producing IL-2 in order to maintain FOXP3 expression. It is also possible that this small population of $ICOS^{+}$ TFR cells represents memory cells, which came from the lymph nodes. Additionally, the increasing trend of ICOS expression in HIV-infected individuals suggested that pTFR and pTFH cells are activated due to HIV infection because ICOS is expressed on activated CD4 and CD8 cells (67).

Overall, we showed that pTFR only express FOXP3 and that they do not express BCL6 using our current protocol for intracellular staining, in contrast to data from our laboratory showing $FOXP3^{+}$ $BCL6^{+}$ TFR in the tonsils (data not shown). These pTFR cells are uniquely different from the $CD25^{+}$ $CXCR5^{-}$ Treg by their expression of CXCR5. Because of high levels of CCR5 expression TFR could potentially be infected by HIV and contribute to HIV persistence. However, we need to further our research to delineate the difference in cell surface markers on pTFR in HIV- and HIV+ individuals by using the Helios Mass Cytometer (CyTOF based system). The CyTOF allows the identification of cells with more than 40 parameters using antibodies conjugated to metal isotopes. Understanding TFR characteristics and function upon HIV infection can be a valuable step in the path of devising a functional cure for HIV.

Materials and Methods

Experimental Design

Our experimental aims are designed to immunophenotype pTFR cells expressing uniquely FOXP3 intracellular marker and differentiate this newly found population from the well-known pTFH cells. We conducted cytometric analyses of PBMC using multi-color flow cytometry to detect the combination of extracellular markers: CD3, CD4, CD8, CXCR5, CD25, CCR5, PD-1, ICOS, and TIGIT. We also stained for intracellular expression of FOXP3 and BCL6 to differentiate TFR and TFH. We defined pTFH cells as $BCL6^{+}$ $CD3^{+}$ $CXCR5^{+}$ $CD8^{-}$, and pTFR cells as $FOXP3^{+}$ $CD3^{+}$ $CXCR5^{+}$ $CD8^{-}$. We first assessed the integrity of surface markers after freezing/thawing by comparing fresh and short-term (7 days) frozen peripheral blood samples from the same donor. This short-term freezing and then thawing would mimic the conditions that the MACS samples experienced and could give an overview of the freezing and thawing effect.

PBMC

Peripheral blood mononuclear cells (PMBC) were obtained from anonymous healthy donors from the UCLA Center for AIDS Research (CFAR), Virology Core. Since secondary lymphoid tissues are harder to obtain, we chose to use viably cryopreserved PBMC from the Multicenter AIDS Cohort Study (MACS) for affordability and easy accessibility. The MACS HIV infected samples were at various stages of HIV infection, but were not diagnosed with AIDS in order to not confound the effect of cancer and other opportunistic infections.

Flow cytometry

This intracellular staining procedure was modified from our previously published paper (68). Intracellular protein staining was combined with surface protein staining as a way to identify pTFH and pTFR cells. First, 4-5 x 10^{6} cells were washed with FACS staining buffer and then

incubated with human AB serum along with all surface staining antibodies. Surface staining antibodies included: CD3, CD4, CD8, CXCR5, CD25, CCR5, PD-1, ICOS, and TIGIT. Details of antibodies sources can be found in Table 1. Extra antibodies were washed off with FACS staining buffer. The cells were then fixed and permeabilized with FOXP3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent (eBioscience, Cat. No. 00-5521-00) for 30 minutes at room temperature. The cells were washed twice with Permeabilization buffer (eBioscience, Cat. No. 00-8333-56). Next, cells were stained intracellularly with FOXP3, BLC6 and appropriate IgG control (antibodies sources in Table 1). Extra antibodies are washed off with FACS staining buffer. Finally, cells were re-suspended in FACS buffer before acquisition on a SORP HTLSRII Analytic Flow Cytometer (BD Immunocytometry Systems). Compensation tubes were not permeabilized, but were fixed with 1% Paraformaldehyde.

Statistical Analysis

All statistical analyses were conducted with GraphPad Prism 5.02 (Company: GraphPad Software). All variables were shown as means with standard error of the mean (SEM). Unpaired t-test and Man-Whitney test were used when appropriate to compare the mean between different populations. A significant p-value is equal to or less than 0.05. (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$)

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Flow cytometry was performed in the UCLA Jonsson Comprehensive Cancer Center (JCCC) and Center for AIDS Research Flow Cytometry Core Facility that is supported by National Institutes of Health awards P30 CA016042 and P30 AI028697.

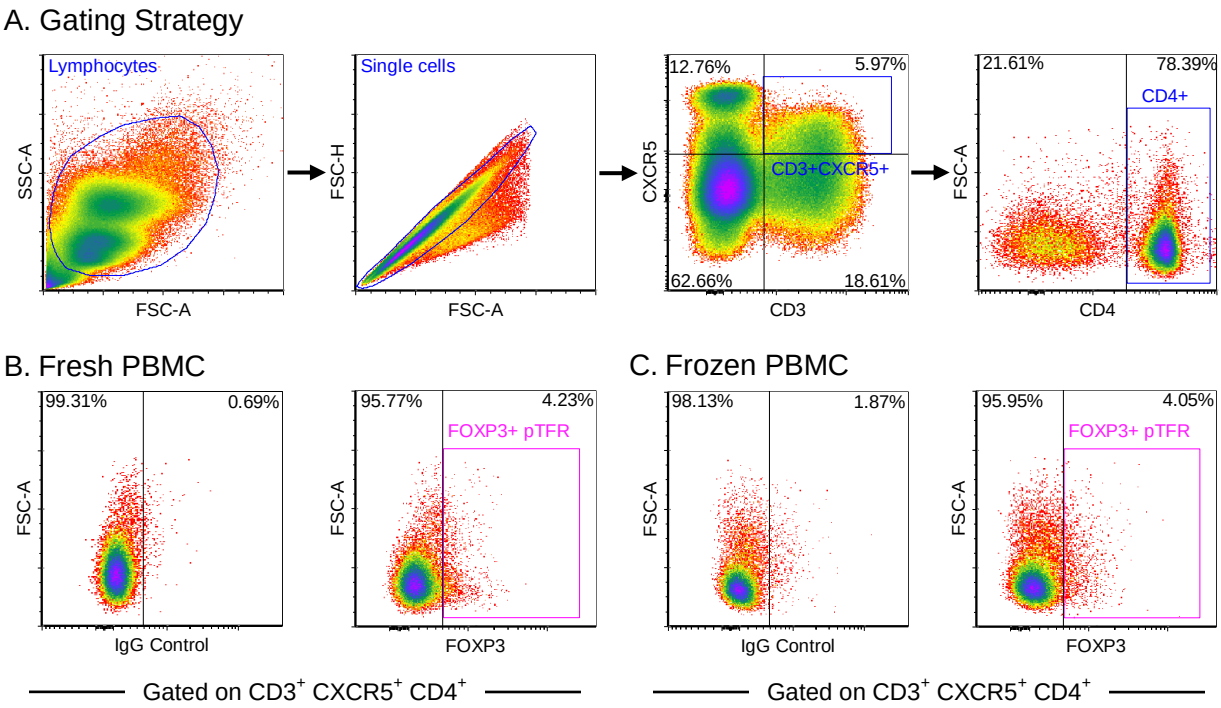

Figure 1: Comparison of TFR cells in *fresh* and *frozen* human peripheral blood. Freezing and thawing does not alter the expression of FOXP3 in TFR cells. **(A)** In our initial study, this series of the density plots shows the gating strategy of TFR cells in human PBMC from healthy donors. **(B)** Representative flow plot of CD3+ CXCR5+ CD4+ TFR cells expressing FOXP3+ in fresh PBMC from a healthy donor. **(C)** Representative flow plot of CD3+ CXCR5+ CD4+ TFR cells expressing FOXP3+ in frozen PBMC from the same healthy donor.

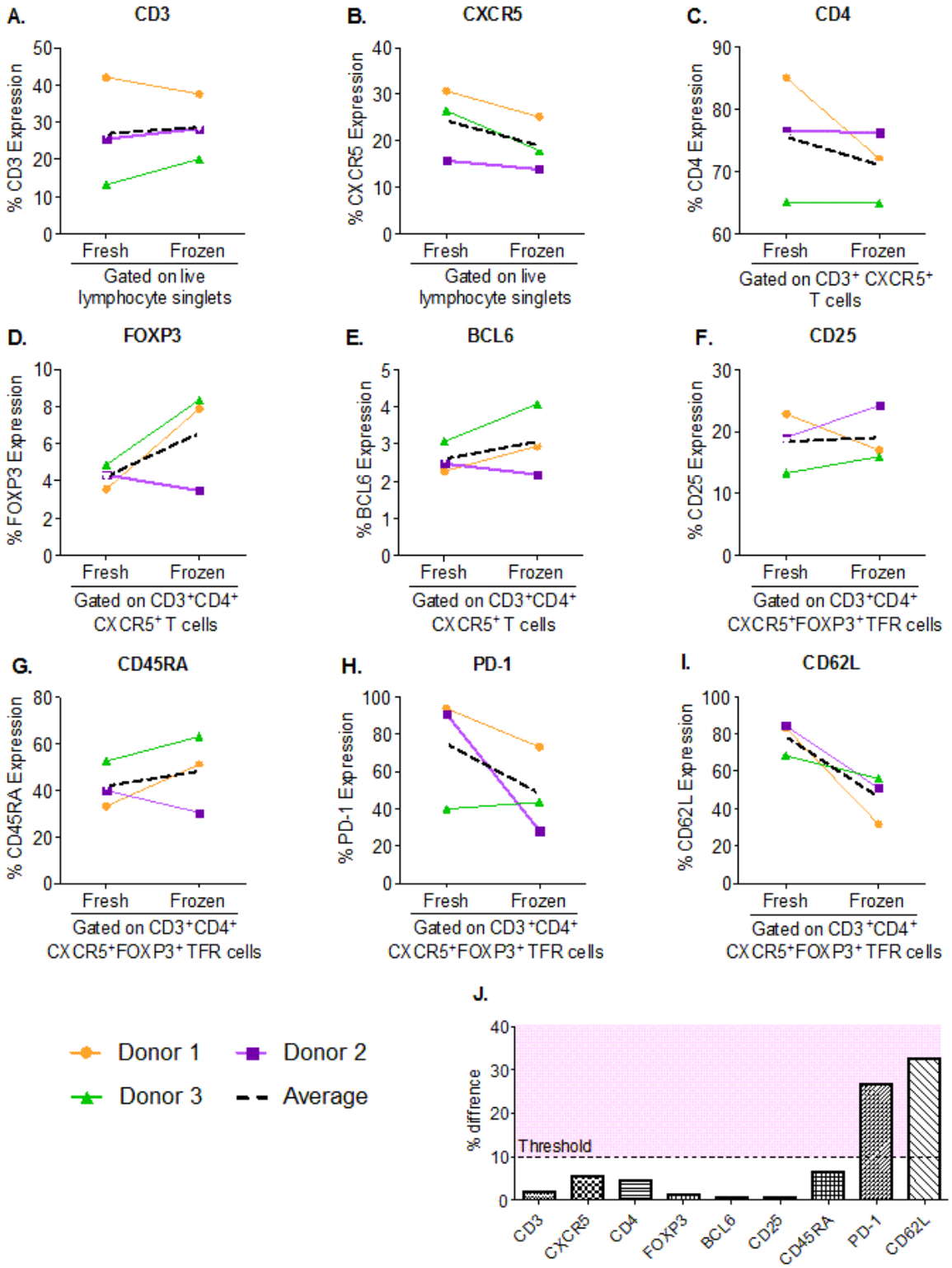

Figure 2: Comparison of cellular markers in fresh and frozen PBMC from healthy donors. PBMC from human blood were stained without freezing (fresh) or 7 days after freezing (frozen). **(A-I)** Respectively, the expression of CD3, CXCR5, CD4, FOXP3, BCL6, CD25, CD45RA, PD-1 and CD62L were compared in fresh and frozen PBMC from healthy donors. Fresh (n=3), frozen (n=3), average of three donors (dotted line). **(J)** The difference of expression of each cellular marker in fresh and frozen PBMC.

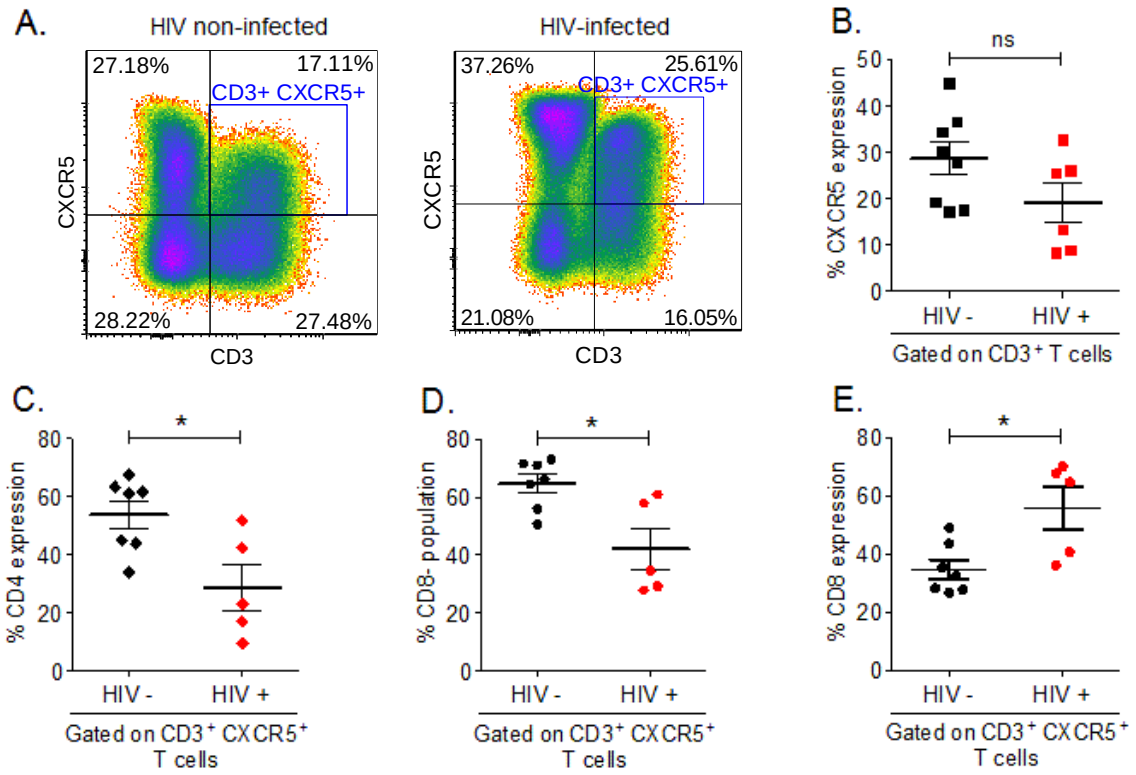

Figure 3: CD4$^+$ CXCR5$^+$ T cells can be detected in long-term frozen human PBMC. Previously frozen and then thawed PBMC samples from the MACS were stained with antibodies against CD3, CXCR5, CD4, and CD8. **(A)** Representative flow plots from an HIV non-infected individual (left) and an HIV-infected individual (right), showing CXCR5$^+$ CD3$^+$ population within total human PBMC. **(B)** Quantification of CXCR5 expression within the CD3$^+$ T cells. HIV– (n=8, mean ± SEM), HIV+ (n=6, in red, mean ± SEM), Mann-Whitney U test, (p = 1.079, ns). **(C)** Quantification of CD4 expression within the CXCR5$^+$ CD3$^+$ T cells. **(D)** Quantification of CD8$^-$ population within the CXCR5$^+$ CD3$^+$ T cells. **(E)** Quantification of CD8 expression within the CXCR5$^+$ CD3$^+$ T cells. **(C, D, E)** HIV– (n=8, mean ± SEM), HIV+ (n=5, in red, mean ± SEM), Mann-Whitney U test (*p<0.05).

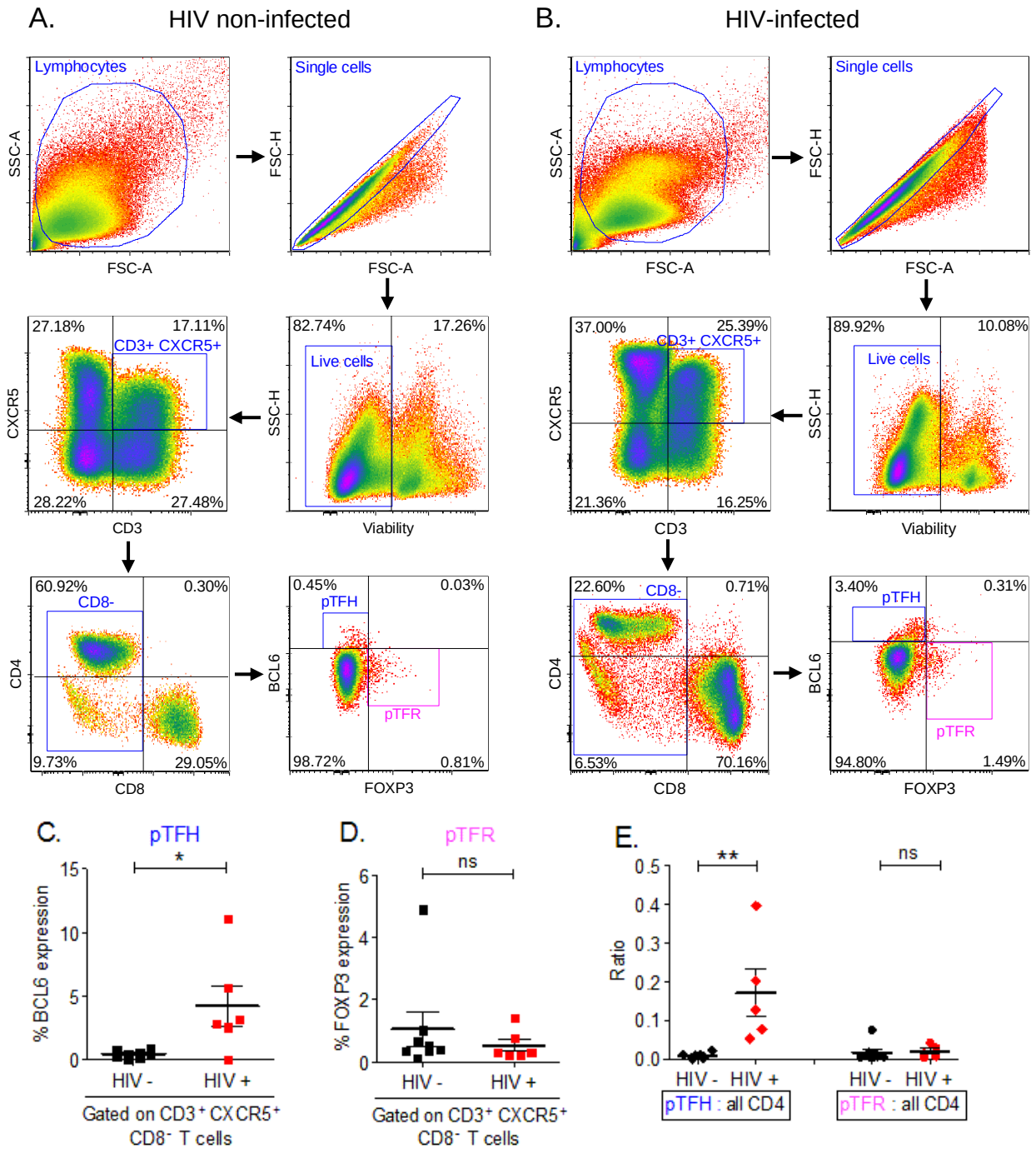

Figure 4: In peripheral blood, the frequency of pTFH cells increased in HIV infection, but not the frequency of pTFR cells

PBMC samples from the MACS were stained with antibodies against CD3, CXCR5, CD4, CD8, FOXP3, and BCL6. **(A, B)** The comparison of pTFH and pTFR cells in HIV-infected and non-infected individuals. **(C)** Quantification of pTFH percentage in human PBMC (*p < 0.05). **(D)** Quantification of pTFR percentage in human PBMC (p > 0.05, ns). **(E)** The ratio of pTFH to all $CD4^+$ T cells (, **p < 0.005) and pTFR to all $CD4^+$ T cells (p > 0.05, ns). **(C, D, E)** HIV– (n=8, mean ± SEM). HIV+ (in red, n=6, mean ± SEM). Statistical analyses were conducted using Mann-Whitney U test.

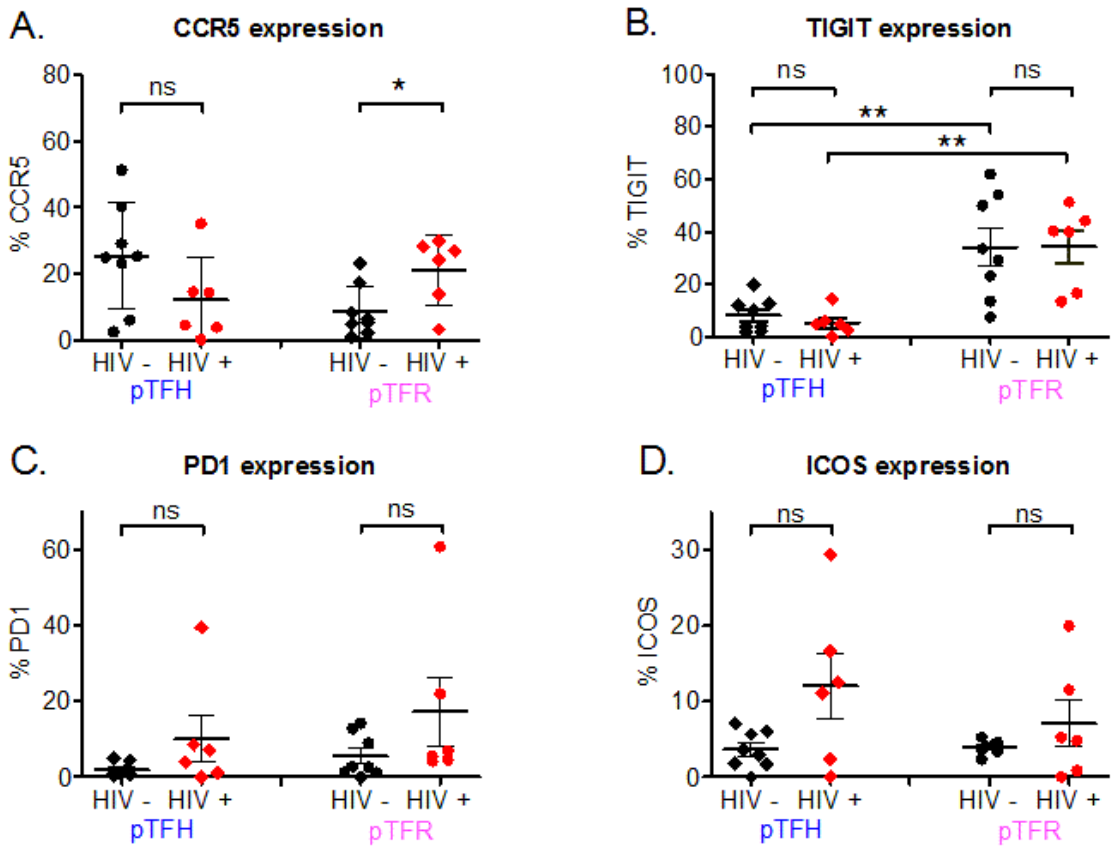

Figure 5: Expression of surface markers of pTFH and pTFR. (A, B, C, D) Quantification of the percentage of cells expressing CCR5, TIGIT, PD1, or ICOS on pTFR and pTFH, respectively. HIV- (n=8, mean ± SEM). HIV+ (in red, n=6, mean ± SEM) (Mann-Whitney U test). **(A) (B)** In both pTFH and pTFR, the percentage of TIGIT expressing cells is similar in HIV-infected and non-infected individuals. In HIV-infected and non-infected individuals, the expression of TIGIT is higher in pTFR than in pTFH. **(C, D)** In both pTFH and pTFR, the percentage of cells expressing PD1/ICOS is similar in HIV-infected and non-infected individuals.

ANTIGEN	FLUOROCHROME	COMPANY	CLONE	CAT NO.
FOXP3	FITC	eBioscience	236A/E7	11-4777-42
IgG1	Alexa Fluor® 488	Invitrogen	P3.6.2.8.1	53-4714-42
BCL6	PE	BD Bioscience	K112-91	561 522
IgG1	PE	BD Bioscience	X40	349 043
CCR5 (CD195)	PE- CF594	BD Bioscience	2D7/CCR5	562 456
IgG2a	PE-CF594	BD Bioscience	MOPC-173	563 489
TIGIT	APC	eBioscience	MBSA43	17-9500-42
Mouse IgG1	APC	BD Bioscience	-	340 442
CD3	eVolve™ 605	Invitrogen	OKT3	83-0037-42
CD4	APC-Cy7	BD Bioscience	RPA-T4	557 871
CD8a	eVolve™ 655			
CD25	eFluor® 450	eBioscience	BC96	48-0259-42
CD45RA	PerCP Cy5.5	eBioscience	HI100	45-0458-42
CXCR5 (CD185)	PE-Cy7	eBioscience	MU5UBEE	25-9185-42
ICOS (CD278)	APC	eBioscience	MBSA43	17-9948-42
PD1 (CD279)	APC	eBioscience	eBioJ105	17-2799-42
Viability Dye	Violet ™510	Tonbo	n/a	13-0870-T100

Table 1: Antibodies and Their Sources
Table 1 showed the list of antibodies, their conjugated fluorochrome, and the sources of each antibody.

CHAPTER 3:

Concluding Remarks

Research Relevance

T follicular helper (TFH) cells are an important part of the humoral arm of the immune system, and T follicular regulatory (TFR) cells keep TFH cells in check and prevent autoimmunity. Both of these cell populations are affected upon HIV infection, and can serve as potential HIV reservoir thereby contributing to HIV persistence. In this Master Thesis, I laid out a comprehensive way to characterize TFH and TFR cells in peripheral blood and their phenotype in non-infected and HIV-infected individuals. The major findings of **Chapter 2** of the Master Thesis are: 1. freezing/thawing affected the markers PD-1 and CD62L; 2. TFR cells expressed higher CCR5 in HIV infection; 3. the immunophenotype of TFH and TFR cells are both altered in HIV infection. 4. The increase in pTFH during HIV infection is not due to the increase in pTFR cells. This was different from our original hypothesis that TFR cells were increased in HIV infection. TFR were previously grouped together with TFH cells because of their similarity in phenotype, and the frequency of TFH cells was reported to increase in HIV infection. Surprisingly, since GC TFR cells express both FOXP3 and BCL6 in humans and mice, we observed that pTFR did not express BCL6. Nevertheless, further research regarding the function of pTFR needs to be conducted in order to confirm it suppressive function.

Future Experiments

Our current challenge is that we cannot yet identify pTFH and pTFR cells using only surface markers, without also using intracellular markers. Ultimately, we need to sort pTFR to identify the function of pTFR cells and how HIV infection affects its ability to suppress pTFH or GC TFH cells, as observed in lymphoid tissues of mice. In future experiments, the Helios Mass Cytometer (CyTOF based system by Fluidigm) would be utilized, as it allows for 40+ parameter cell analysis. PMBC from healthy individuals (from the UCLA CFAR Virology Core) will be

immunophenotyped for the expression of the following surface markers: CD3, CD4, CD8, CXCR5, PD-1, ICOS, CCR5, CXCR4, CD31, CD45RA, CCR7, LAG3, TIGIT, CTLA4, CD127, CCR3, GITR, and intracellular markers: BCL6, FOXP3, Ki67, Blimp-1. We need to identify cell surface markers to distinguish on $FOXP3^+$ TFR cells so that they can be sorted with the Aria II cell sorter for functional assays and determination of HIV-DNA and HIV-RNA

Furthermore, we want to identify the ability of this pTFR population to harbor HIV by staining for HIV gag protein using a combination of T cell surface markers. After optimizing TFR cells identification, we will use the CyTOF and the new imaging technique (ImageStream) to examine the MACS samples. We will compare the $CD4^+$ TFR in healthy individuals to those of HIV-infected individuals in order to assess the effect of HIV infection on peripheral blood TFR cells. A recent publication shows that HIV gag protein could be detected in a subset of CD4/CD8 double negative T cells in peripheral blood using flow cytometry combined with fiber optic imaging technology (69). We expect that a small subset of $FOXP3^+$ TFR cells in HIV-infected individuals will be positive for HIV gag protein, and will continue to exist and contribute to HIV persistence. We also expect that HIV infection will alter the number and function of TFR in a way that they can no longer regulate TFH, thereby contributing to the dysregulation of the humoral immune responses.

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