THE EFFECT OF CHRONIC HIV-1 INFECTION ON CIRCULATING B CELLS AND PERIPHERAL T FOLLICULAR HELPER CELLS

By

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To my Creator and Savior, for giving me the strength and peace I needed to get through this journey.

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HIV	Human immunodeficiency virus
AIDS	Acquired immune deficiency syndrome
HAART	Highly active anti-retroviral therapy
T_{FH}	T follicular helper
pT_{FH}	Peripheral T follicular helper
CXCR5	CX chemokine receptor type 5
GC	Germinal center
Bcl-6	B cell lymphoma 6 protein
IL	Interleukin
CD40L	CD40 ligand
ICOS	Inducible T cell costimulator
SAP	SLAM associated protein
PD-1	Programmed death 1
RNA	Ribonuclease acid
HLA	Human leukocyte antigen
IFN-γ	Interferon gamma
TNF-α	Tumor necrosis factor alpha
MIP-1β	Macrophage inflammatory protein-1 beta
BNAbs	Broadly neutralizing antibodies
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
SIV	Simian immunodeficiency virus
Tim-3	T Cell Ig- and mucin-domain-containing molecule-3
Ig	Immunoglobulin
Lag-3	Lymphocyte activation gene 3
FBS	Fetal bovine serum
DMSO	Dimethyl sulfoxide

AT-2	Aldrithiol-2
MFI	Mean fluorescence intensity
CCR6	CC chemokine receptor type 6
TIGIT	T cell immunoreceptor with Ig and ITIM domains
viSNE	Visualization of t-distributed stochastic neighbor embedding algorithm
FCS	Flow cytometry standard
FCAs	Fluorochrome-conjugated antibodies
MCAs	Metal-conjugated antibodies
MMI	Mean mass intensity
MMO	Mass minus one

CHAPTER I

INTRODUCTION

Significance and overview

Left untreated, the mortality rate of human immunodeficiency virus (HIV) is higher than 95%, making it one of the most fatal diseases in existence. During HIV infection, only 5% of CD4+ T cells are productively infected with virus, yet extensive decline of CD4+ T cells is the hallmark of progression to AIDS (Doitsh et al., 2010). Highly active anti-retroviral therapy (HAART) can delay progression to acquired immune deficiency syndrome (AIDS) but is not curative and is not used in over 60% of cases worldwide due to cost, availability, and knowledge of HIV+ status (WHO, 2015). Despite extensive efforts to create an efficacious vaccine to prevent HIV infection, no vaccine for HIV is currently available. Vaccines for HIV will need to induce both cellular and humoral responses to prevent HIV infection.

T follicular helper-like cells (T_{FH} cells) are a type of CD4+ T cells whose enhanced capabilities at collaborating with both B cells (Breitfeld et al., 2000; Crotty, 2011; Schaerli et al., 2000; Vinuesa, Tangye, Moser, & Mackay, 2005) and CD8+ T cells (Elsaesser, Sauer, & Brooks, 2009; Yi, Du, & Zajac, 2009) make them an attractive vaccine target to elicit both cytotoxic CD8+ T cell responses and broadly neutralizing antibody responses from B cells (Burton et al., 2012; Kwong, Mascola, & Nabel, 2013; Picker, Hansen, & Lifson, 2012; Streeck, D'Souza, Littman, & Crotty, 2013). Peripheral T_{FH} cells (pT_{FH} cells) are a putative counterpart to T_{FH} cells in the germinal center (GC T_{FH} cells) that can serve as immune correlates to vaccination (Bentebibel et al., 2013; Bentebibel, Schmitt, Banchereau, & Ueno, 2011; N. Chevalier et al., 2011; He et al., 2013; Herati et al., 2014; Locci et al., 2013; Morita et al., 2011; Pallikkuth et al., 2012; Schultz et al., 2016). To better understand the cooperative efforts of pT_{FH} cells and B cells we extensively studied the frequency,

phenotype, and responses to antigen stimulation of pT_{FH} and B cells circulating in chronically infected HIV+ individuals.

T follicular helper cells

Germinal center T_{FH} cells

Studies of the T and B cell collaborative processes have only emerged over the past decade with the discovery of T_{FH} cells and have largely been preformed in mice (Crotty, 2011). Follicular B helper T cells (T_{FH}) are the best model for T cell-B cell help. They were first discovered as an abundant population of CD4+ T cells in tonsils, distinguished from other T helper cells by their surface expression of CX chemokine receptor type 5 (CXCR5) (Breitfeld et al., 2000; Kim et al., 2001; Schaerli et al., 2000). CXCR5 is a chemokine receptor that binds to its ligand, CX chemokine ligand type 13 (CXCL13), ensuring proper migration of the cells that express it through germinal centers (GC) to the B cell-containing follicles (Ansel, McHeyzer-Williams, Ngo, McHeyzer-Williams, & Cyster, 1999; R. Forster et al., 1996; Kim et al., 2001). T_{FH} cells are thus able to home to follicles, where they directly interact with B cells. CXCR5 expression alone is insufficient evidence that T_{FH} cells are of a lineage distinct from the well-established CD4+ T cells subsets T helper 1, T helper 2, T helper 17, and T regulatory cells. However, the discovery of B-cell lymphoma 6 protein (Bcl-6) in 2009 as the master transcription factor required for T_{FH} differentiation established T_{FH} cells as a distinct CD4+ T cell lineage (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). Since then a vast amount of research efforts have focused on better understanding the phenotype, function, and differentiation of T_{FH} cells in humans.

T_{FH} cells have various collaborative functions with several immune cell subsets including other T_{FH} cells (Eto et al., 2011; Nurieva et al., 2008; Vogelzang et al., 2008) and

CD8+ T cells via interleukin (IL)-21 secretion (Elsaesser et al., 2009; Yi et al., 2009). Their most important and well-studied function, however, is their interaction with B cells, which is critical for the generation and maintenance of protective immunity via antibody responses. T_{FH} cell help to B cells is multifaceted: they help form and maintain germinal centers (Allen, Okada, & Cyster, 2007; Linterman et al., 2010; Qi, Cannons, Klauschen, Schwartzberg, & Germain, 2008) and provide signals to B cells to initiate hypermutation, affinity maturation, and differentiation into long-lived plasmablasts and memory B cells (N. Chevalier et al., 2011; MacLeod et al., 2011; Vinuesa et al., 2005). To interact with B cells, T_{FH} cells utilize surface proteins and cytokines that are critical for productive interactions with B cells including (but not limited to) CD40 ligand (CD40L), inducible T-cell costimulator (ICOS), (SLAM-associated protein) SAP, and IL-21 (Figure 1-1). Each protein is a critical contributor to the magnitude and quality of T-B cell help and Table 1-1 summarizes each of them and their ligands, function, and effects/diseases resulting from their absence or mutation.

Along with CXCR5, programmed death 1 (PD-1) is a canonical marker of GC T_{FH} cells and is highly expressed on these cells (Dorfman, Brown, Shahsafaei, & Freeman, 2006; Good-Jacobson et al., 2010; N. M. Haynes et al., 2007). PD-1 is upregulated on the surface of lymphocytes upon extended T cell receptor signaling, and attenuates T cell activation, proliferation, and cytokine production (Day et al., 2006; Freeman et al., 2000; J.L. Riley, 2009; Sharpe, Wherry, Ahmed, & Freeman, 2007; Trautmann et al., 2006). It acts by blocking the activation of phosphatidylinositol-3-kinase early in the T cell receptor signaling pathway, which prevents Akt activation (Figure 1-2) (Parry et al., 2005; J.L. Riley, 2009; K. A. Sheppard et al., 2004). It is hypothesized that PD-1 is so highly expressed on T_{FH} cells because within germinal centers they are repeatedly stimulated through T-B cell interactions. In this thesis, CXCR5 and PD-1 were regularly used to identify circulating T_{FH} cells.

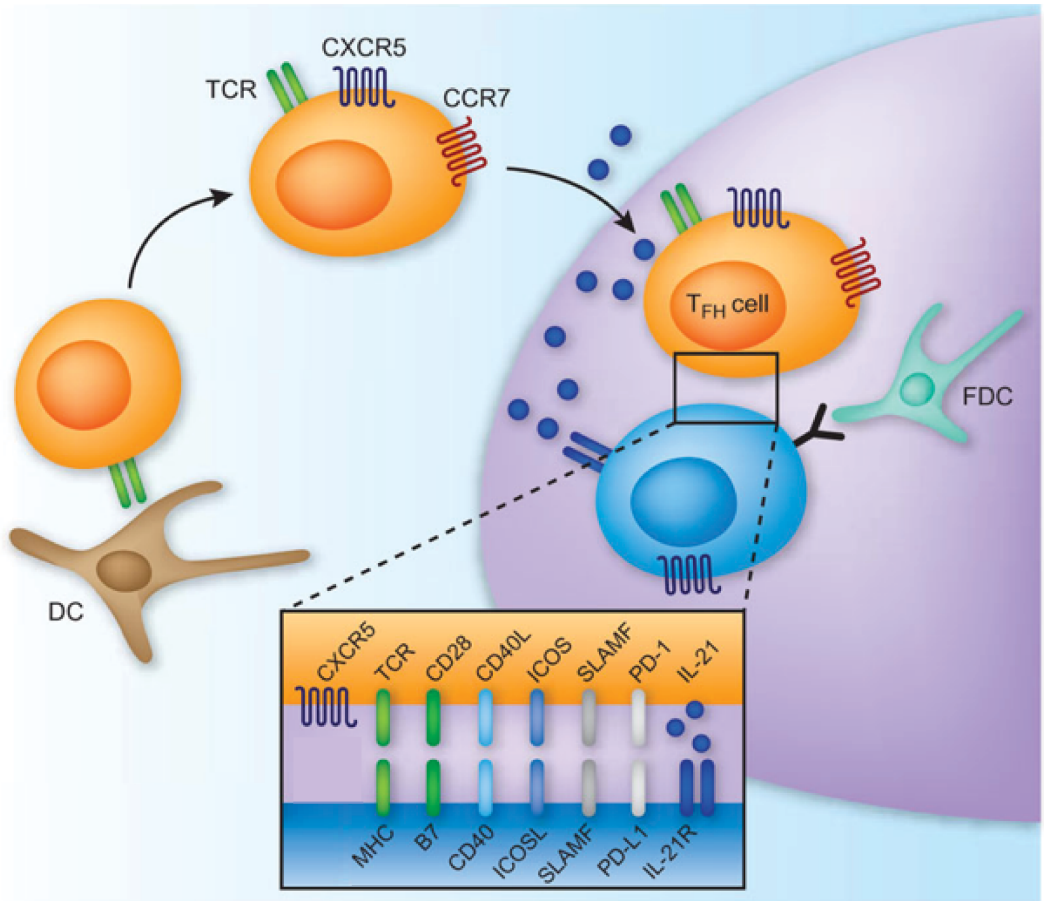


Figure 1-1: Germinal center interactions between T_{FH} and B cells. Antigen-specific T cells, primed on dendritic cells (DC) in the T cell areas, upregulate ICOS, PD-1 and CXCR5 and migrate toward the B cell follicles. After interacting with their cognate B cells, these T cells mature into T_{FH} cells that express Bcl-6 and abundant PD-1. In the GC, T_{FH} cells interact with GC B cells through an array of molecular pairings. These interactions culminate in the secretion of cytokines by T cells, particularly IL-21, which is received by the B cells to influence their subsequent activity. From Nutt and Tarlinton 2011 (Nutt & Tarlinton, 2011).

Table 1-2: Proteins associated with T_{FH} cells.

Protein *(Ligand)*	*Main function*	*Result of absence/deletion/mutation*	*Associated diseases*
CD40L *(CD40)*	Induces signaling in B cells via CD40 engagement	Absolute block of GC formation; blocks plasma cell development	Hyper-IgM syndrome
ICOS *(ICOSL)*	Necessary for maintenance and differentiation of T_{FH} cells	Severe loss of memory B cells; complete absence of antigen-specific IgG responses	Common variable immunodeficiency
SAP *(SLAM family proteins)*	Required for sustained T-B cell physical interactions	Impaired help to B cells due to lack of extended T-B conjugations; no sustained antibody responses to pathogens	X-linked lymphoproliferation
IL-21 *(IL-21R)*	Maintains Bcl-6 expression in B cells	Decreased/impaired antigen-specific B cells; decreased B cell proliferation	Common variable immunodeficiency
PD-1 *(PD-L1, PD-L2)*	Prevents excess T_{FH} proliferation	Higher frequency of T_{FH} cells; worsened B cell responses; abnormal GC formation	Autoimmunity
CXCR5 *(CXCL13)*	Trafficks T_{FH} cells to B cell follicles	T and B cells do not colocalize properly/efficiently	?

Major proteins associated with T_{FH} cells, their ligands, functions, and what happens when they are absent or mutated.

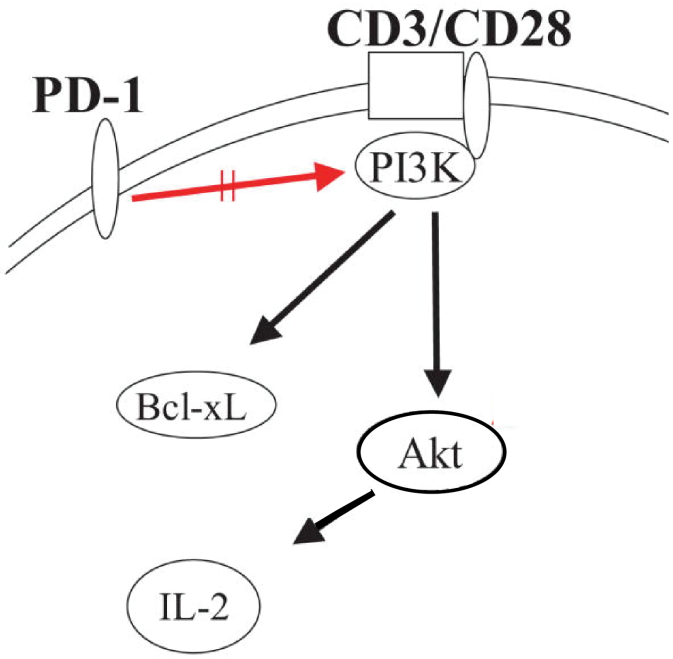


Figure 1-2: PD-1 targets early signaling events in T cell receptor signaling. PD-1 signaling inhibits Akt activation by targeting a membrane proximal step: phosphatidylinositol-3-kinase activation. Adapted from Riley 2009 (J. L. Riley, 2009).

Peripheral T_{FH} cells

At first, CXCR5 expression was only associated with T_{FH} cells in the lymph nodes. But circulating CD4+ T cells expressing CXCR5 were discovered that appeared to share functional properties with GC T_{FH} cells. Initial experiments on circulating CXCR5+CD4+ cells sought to identify their relationship to other T helper subsets and several gene expression analyses suggest that circulating CXCR5+CD4+ T cells are a new subset of T helper cells, with only a few similarities to T helper 2 and T helper 17 subsets (Bentebibel et al., 2011; N. Chevalier et al., 2011; Morita et al., 2011). Subsequent studies showed that pT_{FH} cells secrete more IL-21 and stimulate B cells to mature to immunoglobulin-secreting plasmablasts more efficiently than $CXCR5^{-}CD4^{+}$ cells (Bentebibel et al., 2011; N. Chevalier et al., 2011; Morita et al., 2011). Accordingly, CXCR5+CD4+ T helper cells in the periphery are hypothesized to be a distinct T helper subset that can act similarly to T_{FH} cells in the lymph nodes, and so have been termed pT_{FH} cells. Circulating CXCR5+ cells also express other T_{FH} surface molecules important for B cell helper function, including CD40L, ICOS, and PD-1 (Bentebibel et al., 2011; N. Chevalier et al., 2011; Morita et al., 2011). Compared to their GC counterparts, pT_{FH} cells express lower levels of these markers suggesting they are in a resting state, but upon stimulation they are capable of expression levels similar to GC T_{FH} cells (Locci et al., 2013; Morita et al., 2011). The canonical markers of pT_{FH} are CXCR5 and PD-1 and their combination was used throughout my studies described here to identify pT_{FH} cells.

HIV infection

Background information

HIV is the causative agent of AIDS (Barre-Sinoussi et al., 1983; Gallo et al., 1984; Popovic, Sarngadharan, Read, & Gallo, 1984). 60 million infections have caused more than 34 million deaths worldwide since its discovery (WHO, 2015). HIV currently infects

over 35 million people, and between 2 and 3 million new cases emerged each year over the past ten years (WHO, 2015). In the United States it is estimated that almost 1.5 million people are infected with HIV, with roughly 50,000 new cases each year (CDC, 2015). It is estimated that over 50% of individuals infected with HIV worldwide are unaware of their infection while in the United States the frequency is closer to 12% (CDC, 2015; WHO, 2015). Thus, three decades after its discovery, HIV represents a significant burden on the health and economies of countries worldwide.

HIV can be transmitted sexually across mucosal surfaces, during childbirth and breastfeeding, and by percutaneous inoculation (G. M. Shaw & Hunter, 2012). HIV specifically targets the host immune response by infecting CD4+ T cells and less often cells expressing low levels of CD4, including macrophages. During HIV infection, only 5% of CD4+ T cells are productively infected with virus, yet extensive decline of CD4+ T cells is the hallmark of progression to AIDS (Doitsh et al., 2010). The half-life of most CD4+ T cells infected with HIV is short but some cells become latently infected with HIV, representing a barrier to a cure (Finzi et al., 1999; Hellerstein et al., 1999; Ho et al., 1995; Perelson et al., 1997; J. D. Siliciano & Siliciano, 2006; R. F. Siliciano & Greene, 2012). Human immunodeficiency virus (HIV) is a 10 kilobase retrovirus and its genome encodes for a variety of structural, regulatory, and accessory proteins. To study responses to HIV stimulation in my thesis, I regularly used forms of the structural precursor Gag protein and a cleaved Gag protein, p24, that forms the viral capsid.

Clinical stages of HIV infection

Following transmission, the 'acute stage' of HIV infection where the virus establishes a reservoir as it replicates to high titers ($\leq 10^7$ ribonuclease acid (RNA) copies/mL plasma) and rapidly depletes the host's CD4+ T cell population (Brenchley et al., 2004; Guadalupe et al., 2003; Q. Li et al., 2005; Mattapallil et al., 2005; Mehandru et al., 2004; Ribeiro et al., 2010; Veazey et al., 1998). Within a year of acquiring HIV

infection, a viral 'set point' is reached defined by the clinical characteristics of a viral load between 10^1 and 10^5 copies/mL and a CD4+ T cell count around 10^3 cells/uL (Katzenstein et al., 1996; Lefrere et al., 1998; Mellors et al., 1995; Pedersen, Katzenstein, Nielsen, Lundgren, & Gerstoft, 1997; Schacker, Hughes, Shea, Coombs, & Corey, 1998). This is followed by chronic infection, also known as the 'clinical latency stage', which can last from 1-20 years and is defined by fairly stable viral loads and slowly declining CD4+ T cell counts. Most patients experience no symptoms during this stage of chronic infection. All of the individuals studied in this thesis were in the clinical stage of infection except one who we identified during acute infection.

Progression to AIDS begins when the virus depletes the CD4+ T cell population to the point where the host can no longer combat foreign pathogens. Since the early 1990s AIDS has been defined as having less than 200 CD4+ T cells per uL of blood (CDC, 1993). Symptoms can include rapid weight loss, prolonged swollen lymph nodes, skin discoloration, and extreme fatigue. If untreated, the mortality rate of AIDS is greater than 95%, usually from opportunistic infections, such as pneumonia. A summary of clinical stages of HIV infection are illustrated Figure 1-3.

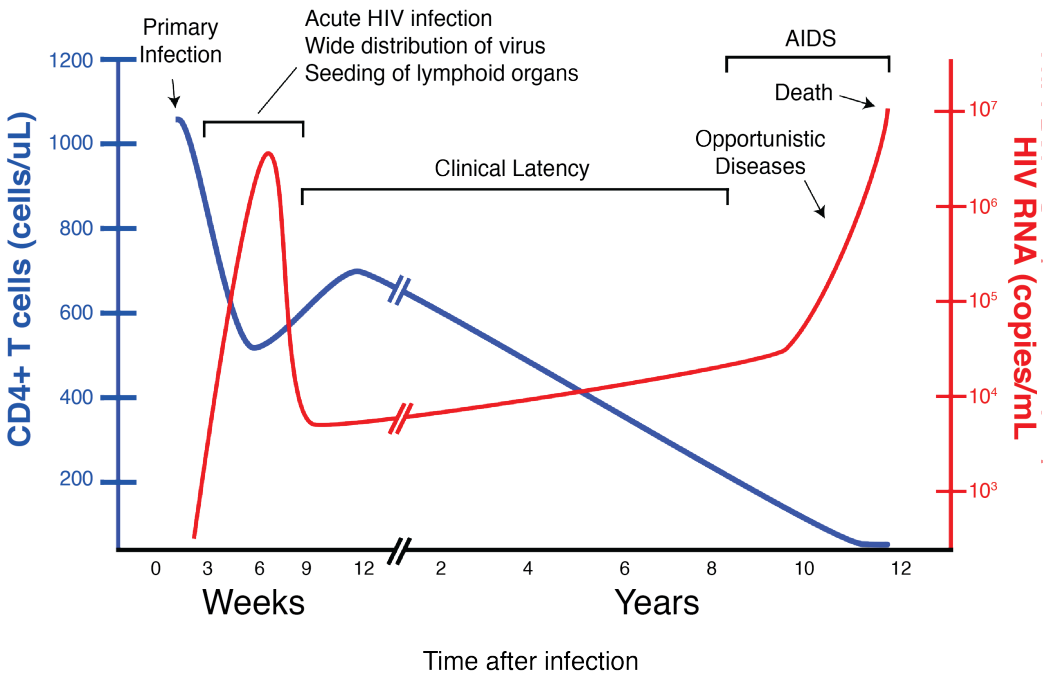


Figure 1-3: Time course of typical HIV infection. Patterns of CD4+ T cells (blue) and HIV RNA (red) during HIV infection. Modeled after Fauci and Desrosiers 1997 (Fauci & Desrosiers, 1997).

HIV Controllers

A decade into the AIDS epidemic rare patients were identified that were HIV-infected but did not progress to AIDS (Easterbrook, 1994; Hardy, 1991; Levy, 1993; Lifson et al., 1991). A substantial effort was then made to characterize the immune systems of these subjects termed 'long-term nonprogressors' (LNTP) (Cao, Qin, Zhang, Safrit, & Ho, 1995; Pantaleo et al., 1995). With the advent of more sensitive HIV RNA detection methods it was observed that these individuals were heterogenous in the low level of virus they maintained. Two prospective French studies of large HIV cohorts helped classify 'elite controllers' as having a viral load below 50 copies/mL while 'viremic controllers' maintain viral loads below 2000 copies/mL in the absence of therapy (Grabar et al., 2009; Hubert et al., 2000). Long-term studies of HIV controllers demonstrate that viral control can be maintained over significant periods of time in the absence of HAART. Elite controllers can maintain control for >20 years, but control in viremic controllers appears to be transient with most subjects eventually progressing and requiring therapy (Grabar et al., 2009; Lefrere et al., 1997; Okulicz & Lambotte, 2011; Okulicz et al., 2009).

Studies of the immune systems of HIV controllers demonstrate they maintain CD4+ counts similar to HIV-uninfected controls (Cao et al., 1995; Okulicz & Lambotte, 2011; Okulicz et al., 2009; Pantaleo et al., 1995; H. W. Sheppard, Lang, Ascher, Vittinghoff, & Winkelstein, 1993). Identifying what regulates control has been of great interest to the field with the idea that there may be a mechanism that could be harnessed in vaccine strategies (Blankson, 2011; Picker et al., 2012; B. D. Walker, 2007; B. D. Walker, Ahmed, & Plotkin, 2011). Elucidating whether HIV control is due to the virus or the immune system has been the focus of many research studies over the past 15 years (Deeks & Walker, 2007; Pereyra et al., 2008; Saag & Deeks, 2010; Saez-Cirion et al., 2007). Immune system studies have specifically sought to determine whether control is genetic or inducible (Lambotte et al., 2005). While the human leukocyte antigen (HLA) B*57 allele is the strongest correlate of protection (Migueles et al., 2000), a wide variety of immune phenotypes have also been

associated with control such as IL-21 production by CD4+ and CD8+ T cells (M. F. Chevalier et al., 2011; Williams et al., 2011; Yue et al., 2010), HIV-specific T cell responses to Gag (Ferrando-Martinez et al., 2012; Kiepiela et al., 2007; Saez-Cirion et al., 2009), and elevated frequency of regulatory T cells (Hunt et al., 2011; Schulze Zur Wiesch et al., 2011; J. M. Shaw et al., 2011). The finding that IL-21 has been shown to correlate with control proposes the idea that T_{FH} cells may play a role in HIV control. To potentially address this, I chose to study HIV+ individuals with a variety of control in this thesis work.

Elite controllers are rare and comprise less than 0.5% of HIV-infected persons while roughly 10% of HIV-infected individuals are viremic controllers (Grabar et al., 2009; Hubert et al., 2000). Dr. Kalams has spent over 15 years identifying and creating a cohort of HIV controllers for laboratory studies (Conrad et al., 2012a; Conrad et al., 2011; Meyer-Olson et al., 2006; Meyer-Olson et al., 2010; K. J. Nicholas et al., 2013; Simons et al., 2008). The individuals studied in my thesis work include a mix of elite controllers, viremic controllers, and HIV progressors before therapy was initiated (Table 1-2). By choosing to study individuals with a variety of control and thus viremia, I was able to correlate my findings with clinical aspects of disease throughout this thesis.

Table 1-2: Characteristics and definitions of HIV controllers

	Viral load (copies/mL)	*Duration of control (years)*	*Frequency in HIV+ population*	*CD4+ T cell count (cells/uL)*	*Require therapy?*
Elite controllers	<50	>20	<0.5%	Normal	No
Viremic controllers	<2000	~10	10%	Normal/below average	Eventually
Non-controllers	>2000	<2	90%	Low	Yes

HIV controllers are defined based on their viral load in this thesis (Grabar et al., 2009; Hubert et al., 2000). Duration of control and frequency in population are averages based on the literature.

HIV and the immune system

Adaptive immune response to HIV

The immune response to HIV exists at both the innate and adaptive levels. There is evidence that the innate immune response during acute infection influences viral control and the quality of the adaptive immune response during chronic infection (Pantaleo et al., 1997). Although the innate immune response significantly reduces viral load, it is unsuccessful at stopping HIV's depletion of CD4+ T cells. This leaves the adaptive immune system to fight a lifelong chronic infection missing a portion of its CD4+ T cells. This is highly detrimental as these cells help to coordinate responses between the other two major players in the adaptive response: CD8+ T cells and B cells. Inducing strong CD4+ T cell responses cells can initiate and maintain both cytotoxic CD8+ cell responses and antibody responses are of interest to the HIV vaccine field.

CD8+ T cells can inhibit the replication of HIV by noncytolytic mechanisms as well as by secreting soluble factors that can destroy HIV-infected cells (Barber, Wherry, & Ahmed, 2003; Betts et al., 2006; Streeck et al., 2008). Early studies of HIV's effects on the immune system focused on CD8+ T cells since HIV does not deplete them. However, these studies discovered that CD8+ T cells in HIV-infected persons had robust effector functions yet these persons were still progressing to AIDS (Nixon et al., 1988; Plata et al., 1987; B. D. Walker et al., 1987). The explanation for this finding is two-fold. First, due to chronic antigen stimulation in which the CD8+ T cells respond to constantly evolving HIV, they become severely impaired—or 'exhausted'—during chronic infection and are unable to clear infection (Barber et al., 2006; Trautmann et al., 2006; Wherry et al., 2007). Second, in the absence of adequate CD4+ T cell help, CD8+ T cell functionality is comprised both in early and chronic infection (Aubert et al., 2011; Nakanishi, Lu, Gerard, & Iwasaki, 2009).

While CD4+ T cell function is often significantly impaired in HIV infection, they can still provide critical help for CD8+ T cell and B cell responses. In elite controllers, HIV-specific CD4+ T helper cell proliferation and secretion of cytokines—including interferon gamma (IFN-γ), IL-2, tumor necrosis factor alpha (TNF-α), and macrophage inflammatory protein 1 beta (MIP-1β)—appear to be preserved (Emu et al., 2005; Ferre et al., 2010; Harari, Petitpierre, Vallelian, & Pantaleo, 2004). While in the majority of HIV-infected individuals CD4+ T cell responses are rare and weak, higher levels of HIV-specific CD4+ T cell help are associated with more robust CD8 + T cells responses (Kalams et al., 1999; Pitcher et al., 1999). CD4+ T cell responses target the HIV proteins Gag and Nef more frequently than other viral proteins, and multiple HLA class II molecules can be used for peptide presentation (Kaufmann et al., 2004). Many of these studies focused on CD4+ T cells as a whole, but I decided to specifically study CD4+ HIV responses in pT_{FH} cells since they provide the best help to CD8+ T cells and B cells.

In HIV infection, the idea that B cells can produce antibodies that neutralize HIV was first proposed in 1985 (Weiss et al., 1985). These neutralizing antibodies can bind HIV and prevent entry and replication in CD4+ T cells (Kwong et al., 1998; Overbaugh & Morris, 2012; Richman, Wrin, Little, & Petropoulos, 2003; Wei et al., 2003). Unfortunately, these antibodies also drive HIV envelope evolution (Albert et al., 1990; Frost et al., 2005; Richman et al., 2003; Wei et al., 2003). In what has been described as the "clash of evolutionary titans" B cells constantly evolve to produce neutralizing antibodies to HIV but cannot keep up with the quickly mutating HIV envelope protein (Burton, Stanfield, & Wilson, 2005). In fact, studies have shown that while serum from HIV+ individuals cannot neutralize the virus they are currently infected with, their current serum can neutralize virus they were infected with previously (Deeks et al., 2006; Mahalanabis et al., 2009; Moog, Fleury, Pellegrin, Kirn, & Aubertin, 1997). The research described in this thesis studies both circulating B cells and CD4+ pT_{FH} cells since B cell responses to HIV are dependent on CD4+ T cell help.

Broadly neutralizing antibodies

Despite immune cell dysregulation in HIV-1 infection, 20-30% of HIV+ subjects produce antibodies that can neutralize heterologous HIV-1 strains across clades A, B and C (Mikell et al., 2011; Sather et al., 2009). These antibodies are termed broadly neutralizing antibodies (BNAbs) and they target various locations of the HIV-1 envelope protein: the CD4-binding site, glycans, and the membrane-proximal external region (Kwong & Mascola, 2012; Pantophlet & Burton, 2006). Figure 1-4 illustrates the four main targets of BNAbs. By binding to conserved epitopes in these regions on the surface of the HIV virion, BNAbs prevent infection of host target cells. Immunogenetic studies indicate that extensive rounds of somatic hypermutation—and thus divergence from germline sequences—are required for the development of BNAbs (Klein et al., 2013; L. M. Walker et al., 2011; Wu et al., 2010; Wu et al., 2011). BNAbs develop early in HIV infection (within 2-3 years) (Gray et al., 2011; Mikell et al., 2011), and it has been shown that breadth found in the plasma of subjects can come for either single or multiple specificities (Gray et al., 2009; Scheid et al., 2009; L. M. Walker et al., 2010).

It is important to note that individuals who naturally make BNAbs do not benefit from them (Doria-Rose et al., 2010; Gray et al., 2011; Sather et al., 2009; Stamatatos, Morris, Burton, & Mascola, 2009). In fact, broadly neutralizing antibodies in natural infection are associated with increased immune activation and viremia (Gray et al., 2011). However, when BNAbs have been administered passively in animal models they can completely prevent HIV infection (Hessell, Poignard, et al., 2009; Hessell, Rakasz, et al., 2009; Mascola et al., 2000; Moldt et al., 2012; Shibata et al., 1999). The quest to elicit broadly neutralizing antibodies to HIV in uninfected individuals has become a major focus of HIV vaccine research (Kwong et al., 2013; Stamatatos et al., 2009).

As a result, for the majority of individuals studied in this thesis, we have BNAb data ranging from 0-100%. It was important to study these individuals so that as I collected data on B cell pT_{FH} cell frequency and function I could correlate my findings to BNAb production.

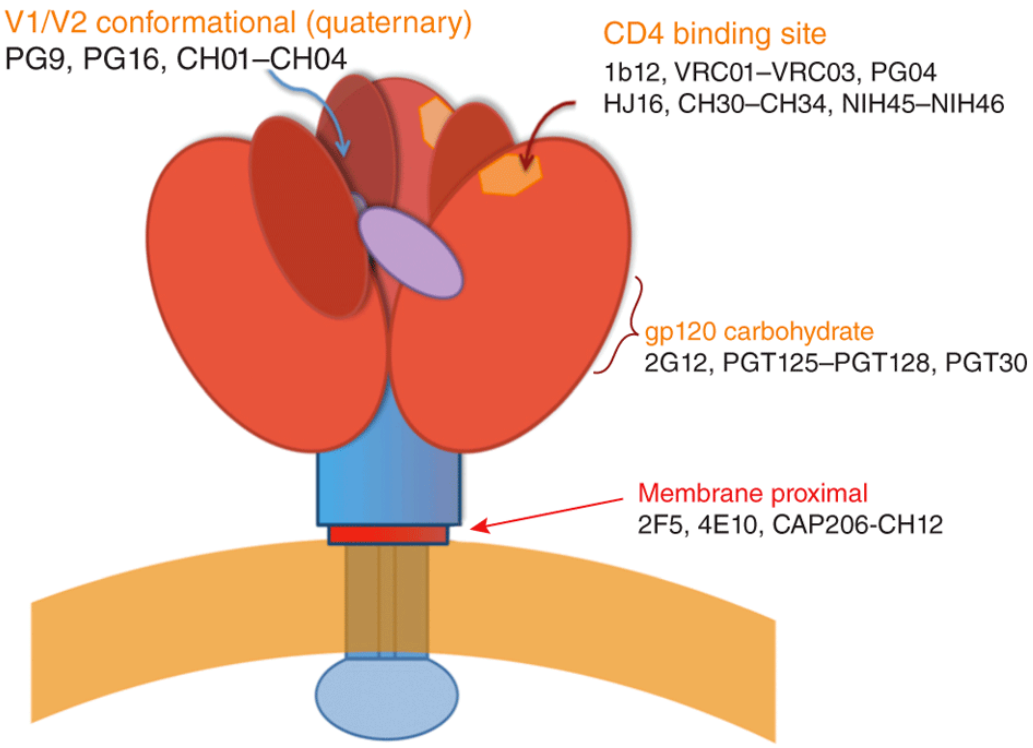


Figure 1-4: Schematic diagram of trimeric HIV-1 Envelope with sites of epitopes for broadly neutralizing antibodies. The four general specificities for BNAbs detected to date are: the CD4 binding site, the V1/V2 variable loops, certain exposed glycans and the membrane-proximal external region. Red ovals, gp120 core; dark red ovals, V1/V2 loops; magenta ovals, V3 loop; blue and red squares, gp41; bright red stripe, MPER of gp41; light brown curved stripe, viral membrane bilayer. PGT antibodies and 2G12 depend on Env N-linked glycans for binding gp120, as do V1/V2-directed conformational antibodies. From Haynes et al. 2012 (B. F. Haynes, Kelsoe, Harrison, & Kepler, 2012).

B cell dysfunction

As discussed above, during HIV infection immune responses are not absent but are instead dysregulated leading to suboptimal function. Persistent high-level viremia is associated with increased expression of activation markers on B cells (R Forster et al., 1997; Martinez-Maza, Crabb, Mitsuyasu, Fahey, & Giorgi, 1987), hypergammaglobulinemia (H. Lane et al., 1983; L Morris et al., 1998; R. Pahwa, R. Good, & S. Pahwa, 1987; A. Shirai, M. Cosentino, S. Leitman-Klinman, & D. Klinman, 1992), and decreased antibody responses to *in vivo* vaccination (Klatt et al., 2011; Lange et al., 2003; Steinhoff et al., 1991; Valdez et al., 2000). In addition to antibody production, B cell antigen presenting function is also impaired after HIV infection (A. Malaspina et al., 2003). It has been proposed that B cell function is deficient as a result of a lack of CD4+ T cell help (Bussmann et al., 2010), and that there also are intrinsic B cell defects in HIV infection (S Moir et al., 2003).

B cells in chronic viral infection have a phenotype consistent with immune exhaustion and terminal differentiation (Figure 1-5) (Moir et al., 2008; Moir et al., 2001; Moir, Malaspina, Pickeral, Donoghue, Vasquez, Miller, Krishnan, Planta, Turney, & Justement, 2004). In HIV-infected individuals, expression of the IL-2 receptor, CD25, on B cells in response to *in vitro* stimulation is lower than in uninfected individuals. This is despite the fact that their CD4+ T cells express CD40L which can stimulate B cells, and this defect persists even after the addition of supplemental IL-2 (S Moir et al., 2003). The bidirectional interaction between CD80 and CD86, ligands of the B7 family, and their receptor, CD28 on CD4+ T cells, is also critical for an effective humoral response. In HIV infection, B cells of viremic subjects not only have decreased ability to increase expression of CD80 and CD86 in response to *in vitro* B cell receptor and CD40L stimulation, but they also are ineffective at stimulating CD4+ T cells, suggesting impairment in both directions of the interaction (A Malaspina et al., 2003). In Chapter II of this thesis we studied expression of CD86 and CD25 on B cells with and without various types of stimulation.

The decreased responsiveness of B cells may be due to impaired help they receive from exhausted CD4+ T helper cells in HIV infection (Day et al., 2006; C. Petrovas et al., 2006; Trautmann et al., 2006; V. Velu et al., 2007). Exhausted CD4 and CD8 T cells exhibit decreased responses to antigen and often express high levels of inhibitory receptors such as PD-1 and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) on their surface. Studies have likewise termed B cells "exhausted" due to their poor proliferative capacity that is only partially restored with the addition of stimulatory cytokines and soluble CD40L (Moir et al., 2008; Moir, Malaspina, Pickeral, Donoghue, Vasquez, Miller, Krishnan, Planta, Turney, & Justement, 2004). B cells from HIV-infected individuals have increased expression of several inhibitory receptors, and siRNA downregulation of these receptors increases memory B cell proliferation and increases the number of antibody-secreting B cells (Kardava et al., 2011). In a simian immunodeficiency virus (SIV) study in macaques, *in vivo* blockade of the inhibitory receptor PD-1 increased the proliferative capacity and frequency of B cells and the production of SIV-specific binding antibody (V Velu et al., 2008). Thus, in Chapter II I studied B cell PD-1 expression *ex vivo* and also measured B cell responses to antigen stimulation with PD-1 blockades.

CD4+ T cell exhaustion

In humans, fine-tuned regulation of the immune response is imperative to control the quality of response and to contract the response when appropriate (Thaventhiran T, 2012). Regulation of T cell responses is carried out in part by 'inhibitory receptors', and these receptors can return the cell to homeostasis from an activation state via multiple pathways (Long, 1999; Ravetch & Lanier, 2000). Specifically, they interfere with the T cell signaling pathway by preventing its initiation at the cell surface and by intracellularly preventing completion of the signal by binding to signaling molecules in the pathway (Jones et al., 2008; Parry et al., 2005; K. A. Sheppard et al., 2004). Altered regulation that is too strong or too weak can have devastating effects on the immune system. Lack of

regulation by inhibitory receptors can lead to autoimmunity (Ansari et al., 2003; Nielsen, Hansen, Husby, Jacobsen, & Lillevang, 2003; Ueda et al., 2003) while improperly heightened regulation can prevent necessary activation of the cells needed for the immune system response as seen in cancer (Hamid et al., 2013; Hirano et al., 2005; Pardoll, 2012) as well as chronic viral infection (Day et al., 2006; Khaitan & Unutmaz, 2011; Wherry, 2011).

Over the course of HIV infection T cells are persistently stimulated with antigen, leading cells to be improperly regulated and termed 'exhausted'. Exhausted T cells express high levels of inhibitory receptors that dampen activation and over time lead to a step-wise decrease in effector function accompanied by a loss of proliferative capacity (Figure 1-6) (Bennett et al., 2003; Blackburn et al., 2009; Khaitan & Unutmaz, 2011; Wherry, 2011; Wherry et al., 2007; Yamamoto et al., 2011). Exhausted CD4+ T cells are unable to collaborate properly with B cells and CD8+ T cells, which contributes to dysregulated immune responses in HIV infection in the absence of treatment.

The transcriptional and differentiation programs of exhausted CD4+ T cells are distinct compared to exhausted CD8+ T cells as well as 'non-exhausted' CD4+ T cells (Crawford et al., 2014). The expression of many inhibitory receptors on the surface of CD4+ T cells is associated with exhaustion of CD4+ T cells in chronic viral infection including PD-1, CTLA-4, T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), lymphocyte-activation gene 3 (LAG-3), and 2B4 (Figure 1-7) (Crawford et al., 2014; Jones et al., 2008; Kassu et al., 2010). These receptors function by mechanisms that both redundant and distinct. However, the exact function of all the inhibitory receptors in CD4+ T cell exhaustion has been less studied compared to CD8+ T cells. It is important to note that throughout this thesis PD-1 is used both as a marker of immune cell exhaustion and as a canonical marker to identify pT_{FH} cells.

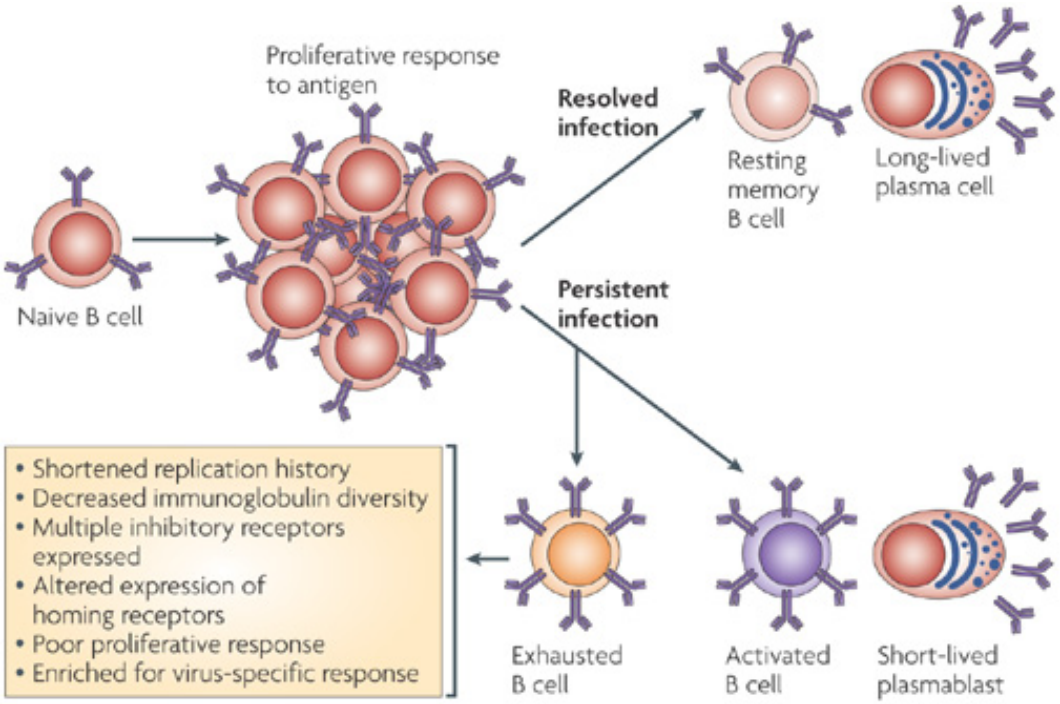


Figure 1-5: B cell exhaustion induced by persistent HIV infection. In response to HIV infection, naive B cells respond to antigen by migrating to lymph nodes and initiating a germinal center reaction that selects for B cells with improved antigen binding. When they exit the germinal center they are either long-lived resting memory B cells or plasma cells. In the context of persistent HIV infection, however, chronic immune activation increases the frequency of antigen-experienced B cells that are short-lived and have undergone several cell divisions. This chronic immune activation also gives rise to exhausted B cells. From Moir and Fauci, 2009 (Moir & Fauci, 2009).

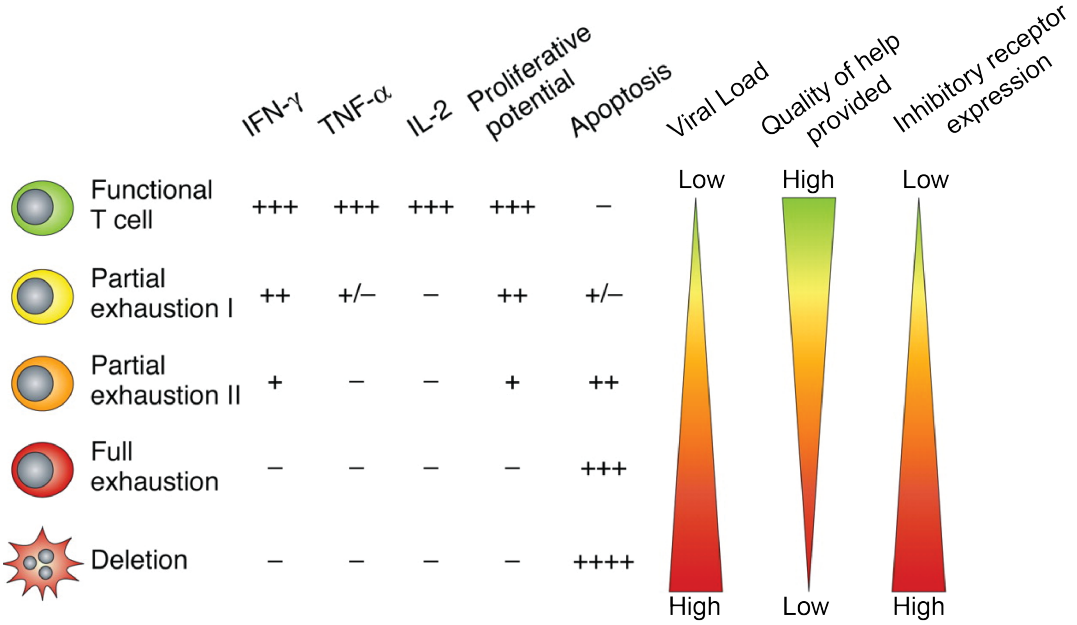


Figure 1-6: CD4+ T cell exhaustion during HIV infection. Virus-specific CD4+ T cells posses multiple functions including production of IFN-γ, TNF-α, IL-2, cytotoxicity, antigen-driven proliferation, and resistance to apoptosis. During chronic HIV infection, however, CD4+ T cells lose these effector functions in a step-wise fashion. Exhaustion ranges from mild to severe, ending in physical deletion (apoptosis) of T cells. Progressively exhausted CD4+ T cells correlate with viral load, inhibitory receptor expression, and inversely correlate with the quality of help CD4+ T cells provide. Adapted from Freeman et al. 2006 (Freeman, Wherry, Ahmed, & Sharpe, 2006).

Increased surface expression of PD-1 on T cells is sustained over the course of chronic viral infection (Barber et al., 2006; Freeman et al., 2006). Fortunately, however, exhaustion appears to be a reversible impairment of T cell function in HIV and other chronic viral infections (Golden-Mason et al., 2009; Hams et al., 2011; Kaufmann et al., 2007; McMahan et al., 2010; Nakamoto et al., 2009; K. J. Nicholas et al., 2013; Porichis et al., 2011; Trautmann et al., 2006; V. Velu et al., 2009). It has been proposed that reinvigoration of T cell responses—by administering antibodies to inhibitory receptors—could boost historically poor vaccine responses in HIV-infected individuals (Ha et al., 2008). The function of T cells from HIV-infected individuals can be partially restored by *in vitro* blockade of the PD-1/PD-L1 interaction (Day et al., 2006; Freeman et al., 2006; Porichis et al., 2011; Shetty, 2012; Trautmann et al., 2006). Blockades of other inhibitory receptors alone or in combination with PD-1 also have been shown to reverse exhaustion phenotypes of T cells in a variety of chronic viral infections (Golden-Mason et al., 2009; Kaufmann et al., 2007; McMahan et al., 2010; Nakamoto et al., 2009). More of these studies focused on CD8+ T cells than CD4+ T cells. However, in the lymphocytic choriomeningitis virus model of chronic viral infection, rescuing CD4+ T cells alone improved CD8+ T cell responses (Aubert et al., 2011). This underscores the importance of a better understanding of the exhaustion and its reversibility of CD4+ T cells in HIV infection. In this thesis I describe PD-1 frequencies on CD8+ T cells, B cells, CD4+ T cells in general, and pT_{FH} cells and how PD-1 blockade improves antigen responses.

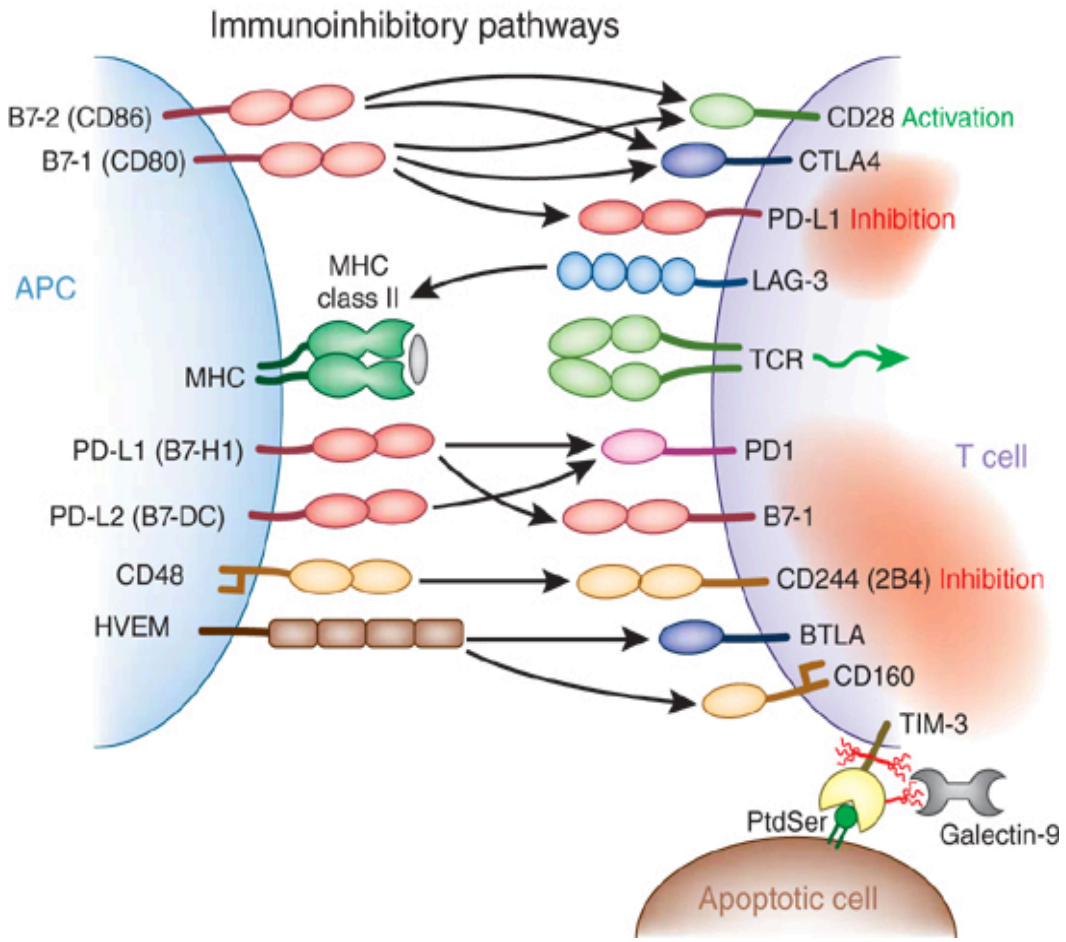


Figure 1-7: Inhibitory receptors on CD4+ T cells. Exhausted T cells can express multiple inhibitory receptors, which modify the outcome of a T cell antigen receptor signal and limit the population expansion, functional activity and survival of T cells. PtdSer, phosphatidylserine. Adapted from Freeman and Sharpe 2012 (Freeman & Sharpe, 2012).

Research objectives

This thesis provides an overview of circulating B and pT_{FH} cells in untreated chronic HIV+ infection. In Chapter I, I include background information on T_{FH} cells, pT_{FH} cells, clinical aspects of HIV disease, and B and T cell exhaustion during HIV infection. To make accurate conclusions from the data presented in this thesis it is necessary to understand the specific cell type studied, the clinical stage of HIV studied, and the state of the cells during chronic HIV infection. The goal of this project was to determine how chronic HIV-1 infection affected the frequency, phenotype, and antigen responses of circulating B and pT_{FH} cells.

In Chapter II, I present my findings on B cell impairment during chronic HIV infection. I found that higher levels of B cell activation were strongly correlated with viremia. High baseline B cell activation also correlated with decreased ability of B cells to respond to stimulation with inactivated HIV-1, but this was improved with PD-1 blockade. Finally, I demonstrate in a series of cell isolation experiments that antigen-specific responses of B cells are greatly enhanced in the presence of autologous CD4+ T cells.

In Chapter III, I describe my findings on pT_{FH} cells in chronic HIV infection. I found that there were similar frequencies of pT_{FH} cells in HIV- and HIV+ individuals. However, the pT_{FH} cells from HIV+ subjects were impaired in their ICOS and CD40L expression after maximal stimulation. Despite a decreased maximal response, pT_{FH} cells in HIV+ subjects maintained recall antigen-specific responses.

In Chapter IV, I describe my studies to evaluate a new technology, mass cytometry, which will drastically increase the depth in which we can explore cellular phenotypes. I found that data generated from mass cytometry was highly comparable with data gathered from the current standard technology in the field, fluorescence cytometry. It was also determined that a new unsupervised analysis program, viSNE, provides valuable insight into single cell data, regardless of the instrumentation used to collect that data.

In Chapter V, I summarize my findings of alterations in the frequencies and phenotypes of circulating B cells and pT_{FH} cells in chronic HIV infection. I propose that future studies should focus on in-depth characterization of circulating B cells and pT_{FH} cells and how their exhaustion may be linked to impaired responses to superantigen and antigen stimulation. This can primarily be accomplished using the approaches described in Chapter IV: a powerful new technology (mass cytometry) and analysis tool (viSNE) for immunologic studies. I also propose that pT_{FH} and B cell surface and transcriptional phenotypes be used in vaccines studies to help identify immune correlates of protection, help predict and identify individuals that will not respond to standard vaccinations, and help direct further research towards adjuvants that optimize T_{FH} cell responses.

CHAPTER II

B CELL RESPONSES TO HIV ANTIGEN CORRELATE WITH VIREMIA AND IMPROVE WITH PD-1 BLOCKADE

Introduction

Infection with HIV-1 induces defects of both cellular and humoral immune responses, inhibiting the immune system from mounting an effective response against infection. Since shortly after AIDS was identified, abnormalities of both B cell and T cell function have been described in HIV-infected individuals (H. C. Lane et al., 1983). Chronic antigen stimulation due to high viral loads is associated with increased expression of activation markers on T and B cells (R Forster et al., 1997; Martinez-Maza et al., 1987), hypergammaglobulinemia (H. Lane et al., 1983; L Morris et al., 1998; R Pahwa et al., 1987; A Shirai et al., 1992), and decreased antibody responses to *in vivo* vaccination (Klatt et al., 2011; Lange et al., 2003; Steinhoff et al., 1991; Valdez et al., 2000). In addition to antibody production, B cell antigen presenting function is also impaired after HIV infection (A. Malaspina et al., 2003). While it has been suggested that B cell function may be deficient as a result of a lack of CD4+ T cell help (Bussmann et al., 2010), there also may be intrinsic B cell defects in HIV infection (S Moir et al., 2003).

In chronic viral infection, B cells become exhausted and terminally differentiated (Moir et al., 2008; Moir et al., 2001; Moir, Malaspina, Pickeral, Donoghue, Vasquez, Miller, Krishnan, Planta, Turney, & Justement, 2004). Surface expression of CD25 and CD86 on B cells are altered in HIV-infected individuals and this persistent phenotype is associated with decreased *in vitro* responses to stimulation. The decreased responsiveness of B cells may be due to impaired CD4+ T cell help they receive due to lymphocyte exhaustion in HIV infection (Day et al., 2006; C. Petrovas et al., 2006; Trautmann et al., 2006; V. Velu et al., 2007). Exhausted CD4+ and CD8+ T cells exhibit decreased responses to antigen and

often express high levels of inhibitory receptors such as PD-1 on their surface. Subsequent studies termed B cells “exhausted” due to their poor proliferative capacity that can only be partially restored with the supplemental stimulatory cytokines and soluble CD40L (Moir et al., 2008; Moir, Malaspina, Pickeral, Donoghue, Vasquez, Miller, Krishnan, Planta, Turney, & Justement, 2004). Increased surface expression of PD-1 on T cells is sustained over the course of chronic viral infection (Barber et al., 2006; Freeman et al., 2006) and may define a reversible impairment of HIV-specific T cell function (Bennett et al., 2003; Day et al., 2006; Hokey et al., 2008; C. Petrovas et al., 2006; Trautmann et al., 2006). The function of T cells from HIV-infected individuals can be partially restored by *in vitro* blockade of the PD-1/PD-L1 interaction (Day et al., 2006; Kaufmann et al., 2007; Porichis et al., 2011). After acute SIV infection, *in vivo* blockade of PD-1 has been shown to increase the proliferative capacity and frequency of B cells and the production of SIV-specific binding antibody (V Velu et al., 2008). B cells from HIV-infected individuals have increased expression of several inhibitory receptors, and small interfering RNA downregulation of these receptors increases memory B cell proliferation and increases the number of antibody-secreting B cells (Kardava et al., 2011). While blocking these inhibitory pathways may provide opportunities to restore CD4+ T cell help for B cells, these interactions have not yet been directly evaluated.

We measured B cell activation markers CD25 and CD86 in the setting of chronic HIV-1 infection after *in vitro* culture with and without stimulation of peripheral blood mononuclear cells (PBMCs) by a variety of antigens. We found high frequencies of CD86+ B cells in HIV-infected individuals, and their frequency correlated with the level of viremia. B cell responsiveness to inactivated HIV, however, negatively correlated with viral load. We also performed a series of co-culture experiments with purified B cells and autologous CD4+ T cells, as well as *in vitro* blockade of PD-1 to investigate the requirements for CD4+ T cell help and the role of inhibitory molecules for inducing B cell activation. We provide

evidence that lack of HIV-specific CD4+ T cell helper responses and high PD-1 expression on B cells in the setting of HIV-1 infection both contribute to B cell dysfunction.

Materials and methods

Study subjects

Seven HIV-negative controls and 21 HIV-infected individuals were studied (Table 2-1). PBMCs were separated from blood samples using a Ficoll-Paque™ Plus density gradient, cryopreserved in fetal bovine serum (FBS) with 10% dimethyl sulfoxide (DMSO), and stored in liquid nitrogen until thawed for immediate use. HIV-infected subjects were ART-naïve with wide ranges of CD4 T cell numbers (132 to 1374 cells/uL), viral load (50 to 76,427 copies/mL), and duration of infection (<1 to 23 years post-diagnosis). The Vanderbilt University School of Medicine Institutional Review Board approved this study, and all individuals provided written informed consent. All HIV-infected individuals were recruited from the Vanderbilt Comprehensive Care Center, and HIV negative individuals were recruited from Vanderbilt University Medical Center.

Table 2-1: HIV+ subject characteristics

Subject ID	Time post-infection (years)	Viral Load (copies/mL)	CD4+ T cells (per mm^3 blood)
10031	11	50	856
10071	14	50	950
10040	14	50	1,161
10067	17	199	814
10004	19	253	360
10060	4	722	688
10070	12	750	915
20018	2	1,886	1,374
20011	<1	2,655	512
10023	6	7,178	390
10027	14	7,340	378
10054	3	7,522	350
10035	20	7,750	464
10026	6	8,865	414
10108	3	9,698	960
20028	<1	15,800	515
10042	23	20,211	331
10114	17	20,574	966
10076	6	21,339	700
20017	1	23,317	435
10086	17	76,427	132

Organized by increasing viral load.

In vitro cell stimulation

PBMC from each subject were cultured at 10 x 10^6 cells/mL in 48-well plates (2 x 10^6 cells/well) in R10 medium (RPMI 1640 containing 10% heat inactivated FBS, 2 mM L-glutamine, 50 ug/mL penicillin, 50 ug/mL streptomycin, and 10 mM Hepes) and co-stimulated with anti-CD28 (1 ug/mL, BD Biosciences) and anti-CD49d (1 ug/mL, BD). Cells were non-specifically stimulated with plate-bound OKT3 (1 ug/mL, ATCC) or Staphylococcal Enterotoxin B (SEB) (1 ug/mL, Sigma). HIV-specific stimulation was performed with AT-2 inactivated HIV-1 MN particles (0.53 ug/mL p24, Lot# P3964, generously provided by Dr. Jeff Lifson) (Rossio et al., 1998; Rutebemberwa et al., 2007), or HIV-1 p24 core protein (1 ug/mL, Protein Sciences). As controls, PBMCs were incubated with media alone, or HIV-1 MN control, containing AT-2 treated microvesicles prepared from matched uninfected cultures, used at a comparable total protein concentration (Lot# P3914) (Rossio et al., 1998; Rutebemberwa et al., 2007). At 24 hours post-stimulation, PBMCs were recovered, washed, and stained with the appropriate combination of surface marker antibodies. For experiments evaluating PD-1 blockade, PBMCs were incubated overnight with 10 ug/ml anti-PD-1 (EH12.2H7, BioLegend).

Flow cytometric evaluation of lymphocyte surface markers

B and T cell surface markers were analyzed by flow cytometry using a combination of an amine-reactive viability dye (LIVE/DEAD Aqua, Invitrogen), CD3-AF700 (UCHT1, BD), CD4-PETR (S3.5, Invitrogen), CD8-V450 (RPA-T8, BD), CD19-PECy-7 (SJ25C1, BD), CD25-FITC (2A3, BD), CD86-PE (2331, BD) and PD-1-PE (EH12.2H7, BioLegend) (Table 2-2). PBMC were stained with Panel 1 after overnight *in vitro* incubation, while PBMC were stained with Panel 2 *ex vivo* (Table 2-2). In prior studies, we used an indirect staining method for detection of PD-1 with purified anti-PD-1 (Mouse IgG1, clone EH12.2H7, BioLegend) followed by goat-anti-mouse IgG-Pacific Blue (Molecular Probes) (Conrad et al., 2012a; Conrad et al., 2011). However, this lead to very high background

staining of CD19+ B cells (i.e. the goat anti-mouse antibody labeled B cells even in the absence of a primary antibody) that could not be overcome by incubation of cells with human serum. The anti-PD-1-PE directly conjugated antibody yielded frequencies of PD-1 expression on CD4+ T cells similar to the indirect method. Panel 3 utilized CD4v4-PE (L120, BD) to stain CD4 cells after they had been purified using methods described below (Table 2-2). Lymphocytes were discriminated based on cell size and granularity using forward and side-scatter properties. Non-viable cells were excluded from analysis.

In vitro stimulation after B cell and CD4+ T cell isolation

CD19+ B cells were purified using EasySep Human CD19 Positive Selection Kit and Robosep (StemCell Technologies). CD4+ T cells were purified from the CD19-depleted fraction using EasySep Human CD4 Positive Selection Kit and Robosep (StemCell Technologies). Purity was assessed after each selection step by flow cytometry: B cell purity ranged from 92-99% of live cells and CD4+ cell purity ranged from 70-90% (depending on the subject's monocyte population). Total PBMCs, positively selected B cells, or positively selected B cells combined with positively selected CD4+ T cells were cultured overnight in a 48-well plate. Cells were left unstimulated or were stimulated with p24 antigen or SEB. As a positive control for B cell stimulation, a subset of cells from each condition was stimulated with soluble CD40L (10 ng/mL, GIBCO) and purified anti-human IgM (10 ug/mL, BioLegend). After 24 hours cells were washed and stained with the appropriate surface marker antibodies.

Table 2-2: Fluorescence cytometry instrument and antibody panel information

Instrument characteristics			*Reagent panels*		
Laser emission and output power	Bandwidth transmitted to PMT	Fluorochrome	**1**	**2**	**3**
639nm (40mW)	705-750	AF700	CD3	CD3	CD3
488nm (50mW)	505 - 550	FITC	CD25		
561nm (150mW)	600 - 620	PETR	CD4	CD4	
	750 - 810	PE-Cy7	CD19	CD19	CD19
	575 - 590	PE	CD86	PD-1	CD4v4
404nm (100mW)	430 - 470	V450	CD8	CD8	CD8
	505 - 550	Aqua	Live/dead	Live/dead	Live/dead

Three fluorescence cytometry panels were designed to identify T and B cells. Panel 1 was used to measure activation of lymphocytes. Panel 2 measured PD-1 on T and B lymphocytes. Panel 3 was designed to assess the purity of magnetically isolated T and B cells.

Statistical analysis

All statistical analysis was done using GraphPad Software (GraphPad, La Jolla, CA, USA). Correlations were performed using the Spearman rank method. All paired comparisons were analyzed with the Wilcoxon matched pairs t-test while unpaired comparisons were analyzed using the Mann Whitney t-test.

Results

B cell responsiveness correlates with HIV viremia

The baseline expression of the activation markers CD86 and CD25 was measured on B cells from a cohort of HIV-infected individuals with a wide range of viremia (Table 2-1) and seven HIV-negative controls. The baseline frequency of CD86-expressing B cells in HIV-infected individuals was higher than that of uninfected control subjects (medians of 38% compared to 26%, $p=0.03$) and positively correlated with viral load (Figure 2-1; $r=0.63$, $p=0.003$). Baseline expression of CD25 on B cells ($p=0.48$) or CD4+ T cells ($p=0.97$) did not correlate with viral load.

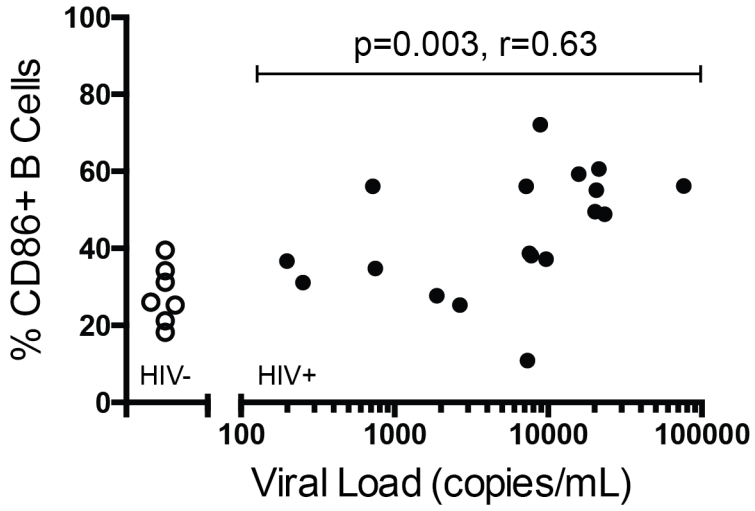


Figure 2-1: CD86+ B cells are more frequent in HIV+ than HIV- subjects and correlate with viremia. PBMCs from HIV- (open circles) or HIV+ (closed circles) subjects were incubated overnight without stimulation and evaluated for surface level CD86 expression on B cells. HIV+ subjects had a higher frequency of CD86+ B cells compared to HIV- subjects (unpaired t-test not shown on graph, p=0.03). The frequency of CD19+CD86+ B cells in HIV+ individuals correlates with the level of viremia (r=0.63; p=0.003). Correlation statistics shown are derived from HIV+ subject data only and do not include data from HIV- subjects.

It has previously been shown that B cells from HIV-infected individuals have a diminished ability to express CD25 after *in vitro* stimulation, and this contributes to low proliferative capacity (Akinsiku, Bansal, Sabbaj, Heath, & Goepfert). We measured the expression of CD25 on both CD4+ T cells and B cells after direct stimulation of PBMCs with anti-CD3 antibody in 14 HIV-infected individuals off anti-retroviral therapy with a range of viral loads (Figure 2-2A). CD25 expression on CD4+ T cells increased after anti-CD3 stimulation, and there was a weak negative correlation between the change in expression of CD25 and viral load (Figure 2-2B, r=-0.53, p=0.056). There was a strong inverse correlation between the change in expression of CD25 on B cells (Figure 2-2C, r=-0.63, p=0.02) and viral load. The change in expression of CD86 on B cells in response to anti-CD3 did not correlate with viral load (Figure 2-2D, r=-0.44, p=0.11).

To measure antigen-specific responses, we next evaluated the ability of B cells to respond to *in vitro* stimulation with aldrithiol-2 (AT-2) inactivated HIV-1 MN in the presence of CD4+ T cells. Bidirectional activation of B cells and CD4+ T cells requires co-stimulatory interactions between CD80/CD86 on B cells and CD28 on CD4+ T cells. We stimulated PBMC with AT-2 inactivated HIV-1 MN or HIV-1 MN control (Figure 2-3A). Responses to treatment by HIV-1 MN control were not above baseline levels. In HIV-negative control subjects, we observed minor differences between expression of CD86 on B cells to inactivated HIV antigen compared to the control (median of -0.3%). However, HIV-infected individuals responded to HIV antigen, and the degree of CD86 expression inversely correlated with the level of viremia (median of 4.95%, r=-0.60, p=0.006) (Figure 2-3B). Responsiveness of CD4+ T Cells to HIV-1 MN as measured by increased expression of CD25 was significantly higher in HIV infected individuals compared to negative controls (p=0.02), but the degree of responsiveness to HIV antigen did not correlate with viral load (p=0.4, data not shown). These results demonstrate that in chronic HIV-1 infection, the activation state and responsiveness of B cells to both HIV and non-HIV antigens are much better correlates of control of viremia than similar parameters on CD4+ T cells.

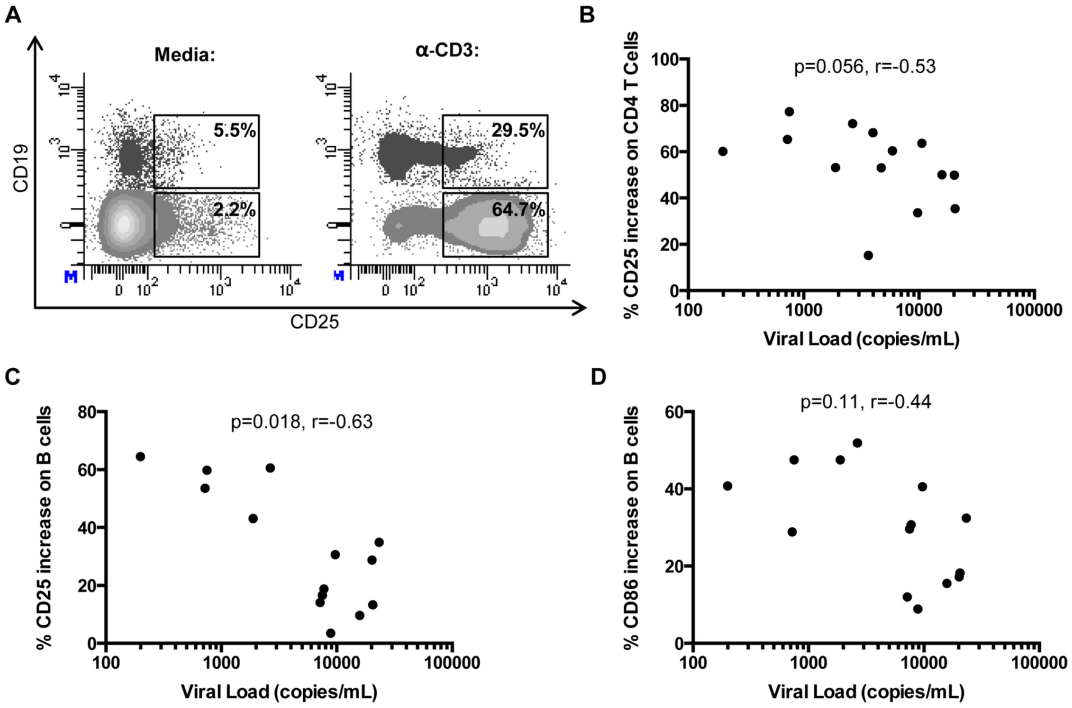


Figure 2-2: Change in CD25 expression on B and CD4+ T cells negatively correlates with viral load. PBMCs were cultured overnight with or without anti-CD3 stimulation. Change in CD25 or CD86 expression was determined by subtracting the frequency of expression before stimulation from the frequency of expression after stimulation. (A) Representative plots of CD25 expression on CD4+ T cells and B cells with (bottom) and without (top) anti-CD3 stimulation. CD4+ T cell population shown is CD3+CD4+CD19- and B cell population shown is CD3-CD4-CD19+. (B-D) Change in expression of CD25 on CD4+ T cells (r=-0.53; p=0.056) (B), CD25 on B cells (r=-0.63; p=0.018) (C), and CD86 on B cells (r=-0.44; p=0.11) (D) correlates negatively with viral load.

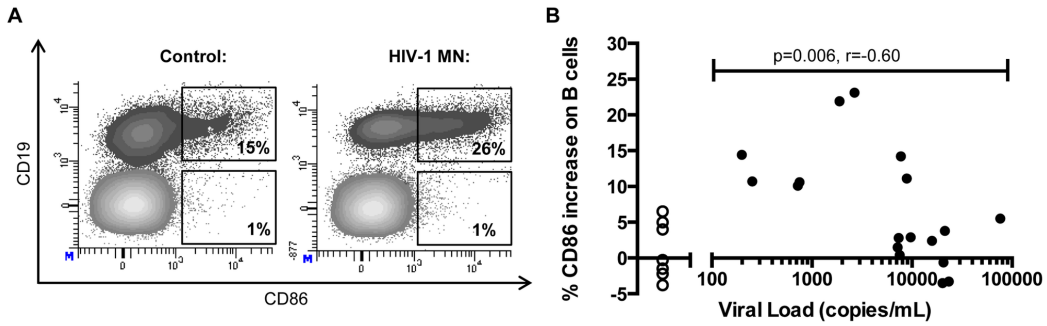


Figure 2-3: Magnitude of B cell responses to inactivated HIV-1 inversely correlates with viral load. (A) Expression of CD86 on B cells after stimulation of PBMC with HIV-1 MN control (left column) or HIV-1 MN (right column). Shown are representative plots from one individual (subject 10071). (B) PBMCs from 21 HIV-infected individuals (closed circles) and 7 HIV-negative control subjects (open circles) were incubated with inactivated HIV-1 MN, and changes in CD86 expression on B cells were measured. Change was calculated by subtracting the frequency of CD86 expression from stimulation with the HIV-1 MN control (containing no HIV proteins) from stimulation with HIV-1 MN. CD86 expression on B cells in response to HIV-antigen in HIV infected individuals is negatively correlated with viral load (r=-0.6; p=0.006). Correlation statistics are only applied to HIV-infected individuals and do not include data from uninfected subjects.

Autologous CD4+ T cells enhance B cell responses to HIV antigen

We next evaluated the contribution of CD4+ T cells to B cell responses to antigenic stimulation. We purified B cells from 7 HIV-infected individuals who demonstrated responses to HIV antigen and who had sufficient numbers of PBMCs available for analysis, and measured CD86 expression after *in vitro* stimulation in the presence or absence of CD4+ T cells. We observed that CD4+ T cells respond more robustly to p24 protein stimulation compared to inactivated HIV-1 MN stimulation (data not shown). Since we sought to measure the CD4+ T cell contribution to B cell activation, in this series of cell isolation experiments we stimulated cultures with HIV p24 antigen. We found that in the absence of CD4+ T cells, purified B cell populations had a markedly reduced ability to respond to the superantigen SEB (Figure 2-4A; p=0.02) or HIV p24 antigen (Figure 2-4B; p=0.02). The ability of B cells to respond to antigen was restored after addition of autologous CD4+ T cells back to the cultures. As a positive control, B cells were able to directly respond to anti-IgM and soluble CD40L to a similar extent whether CD4+ T cells were present or not (data not shown). These data suggest that in this system, B cells have an enhanced response to HIV antigen when they receive help from antigen-specific CD4+ T cells.

PD-1 blockade improves B cell responses to HIV antigen

We next investigated whether PD-1 expression on B or T cells was related to the ability of B cells to express CD86 in response to in vitro stimulation. We measured surface PD-1 expression on T cells and B cells in our cohort; there was no correlation between frequencies of PD-1-expressing CD4+ T cells and viremia (Figure 2-5A; r=0.33, p=0.17), but higher frequencies of PD-1-expressing CD8+ T cells were associated with increasing levels of viremia (Figure 2-5B; r=0.56, p=0.01). While the frequency of PD-1+ CD4+ T cells (p=0.06) and B cells (Figure 2-5C; p=0.04) was higher in HIV+ compared to HIV- individuals, PD-1 expression on these cell subsets did not correlate with viral load. PD-1

expression on CD19+ B cells was significantly lower than that of CD8+ T cells and CD4+ T cells for all individuals studied (Figure 2-5D; $p<0.0001$).

To determine whether lymphocyte PD-1 expression affects B cell stimulation, we evaluated the effect of PD-1 blockade on in vitro B cell activation in response to HIV antigen. We observed that increased CD86 expression on B cells in response to inactivated HIV-1 MN was enhanced by in vitro PD-1 blockade in HIV-infected individuals (Figure 2-6; $p=0.003$). However, while statistically significant, the median increase in CD86 expression in the presence of anti-PD-1 was modest (median absolute increase of 1.4%; median relative increase of 36%). We found no correlations between the effect of PD-1 blockade and viral load, baseline expression of CD86 on B cells, or baseline expression of PD-1 on CD4+ T cells or B cells. In summary, these data suggest that the elevated PD-1 expression on B cells or CD4+ T cells in chronic HIV infection may contribute to impaired B cell responsiveness.

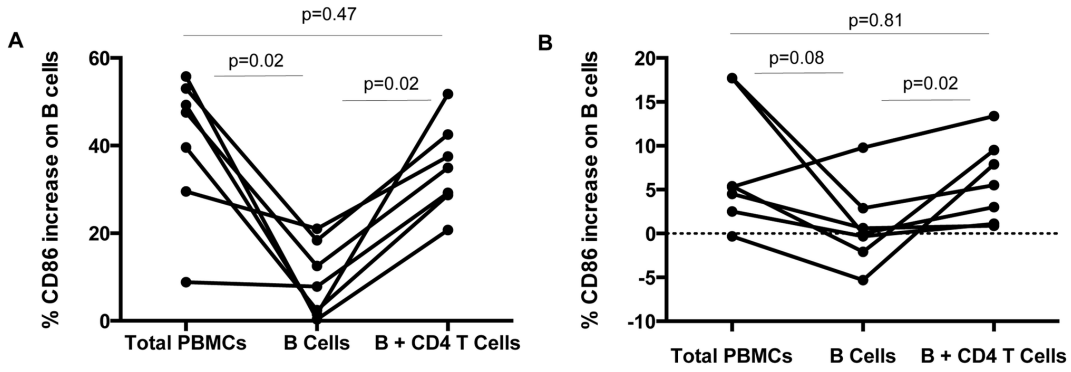


Figure 2-4: Purified B cell responses to p24 are enhanced by addition of autologous CD4+ T cells. Change in the frequency of CD86+ B cells in response to SEB (A) or HIV p24 antigen (B) was evaluated in total PBMC culture, purified B cell culture, or purified B cells co-cultured with autologous purified CD4+ T cells.

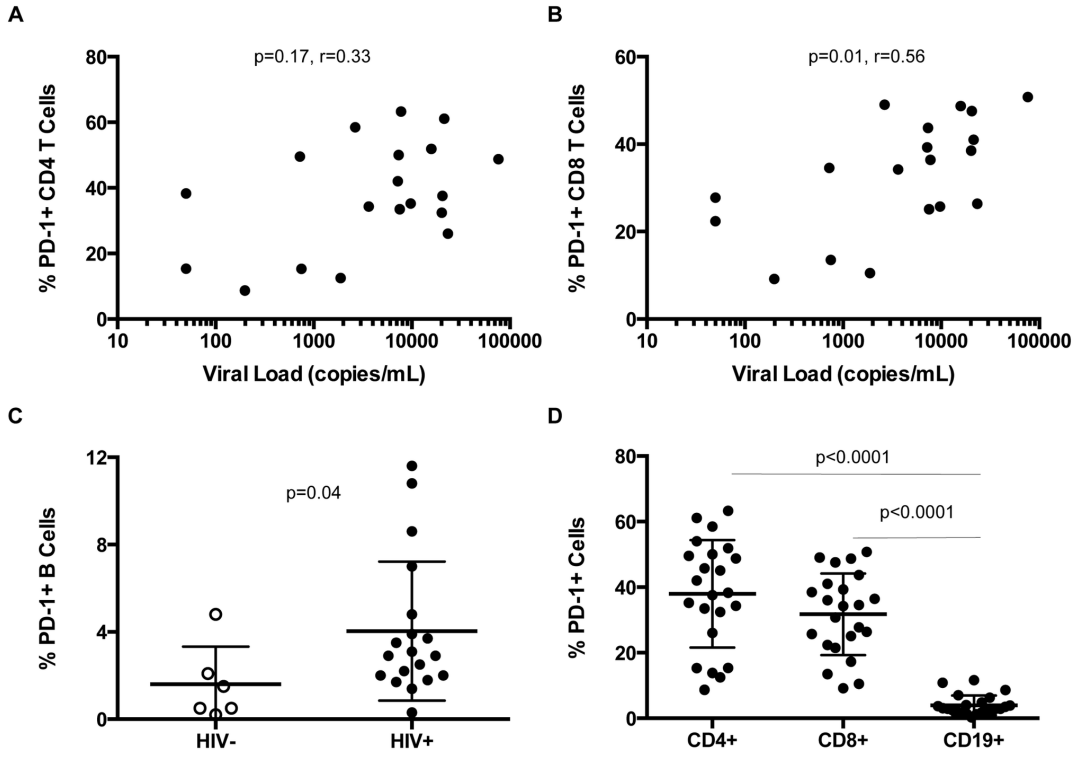


Figure 2-5: Frequency of PD-1 surface expression on lymphocytes is elevated during HIV infection. Expression of PD-1 on B cells, CD4+ T cells, and CD8+ cells was measured directly *ex vivo*. (A) In HIV-infected individuals, PD-1 expression on CD4+ is not correlated with viral load (r=0.33; p=0.17). (B) In HIV-infected individuals PD-1 expression on CD8+ T cells correlated positively with viral load (r=0.56; p=0.01). (C) PD-1 expression is higher on B cells from HIV+ (closed circles) compared to HIV- (open circles) subjects (p=0.04). (D) The frequency of PD-1 surface expression is significantly lower on B cells compared to CD4 (p<0.0001) and CD8 T cells (p<0.0001) in HIV infection.

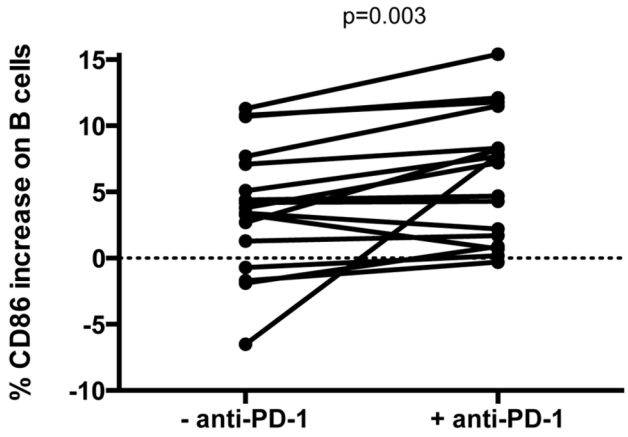


Figure 2-6: PD-1 blockade improves B cell responses to stimulation with inactivated HIV-1. PBMCs were cultured overnight with or without anti-PD-1 and stimulated with inactivated HIV-1 MN protein. Change in response to MN stimulation was calculated by subtracting stimulation with MN control protein from stimulation with HIV-1 MN protein (p=0.003).

Discussion

HIV replication causes B cell hyperactivation, susceptibility to apoptosis, and poor proliferative capacity. This dysfunction in HIV-infected individuals is characterized by hypergammaglobulinemia and deficient antibody responses to both neo and recall antigens (Klatt et al., 2011; H. C. Lane et al., 1983; Lange et al., 2003; L. Morris et al., 1998; R. Pahwa, R. A. Good, & S. Pahwa, 1987; A. Shirai, M. Cosentino, S. F. Leitman-Klinman, & D. M. Klinman, 1992; Steinhoff et al., 1991; Valdez et al., 2000). Here we confirm prior studies that the degree of B cell activation, as measured by the frequency of circulating CD86-expressing B cells, is not only higher in HIV+ individuals (A. Malaspina et al., 2003; Moir, Malaspina, Pickeral, Donoghue, Vasquez, Miller, Krishnan, Planta, Turney, Justement, et al., 2004), but also correlates with the level of viremia. We also extend these studies by demonstrating that the ability of B cells to respond to HIV antigen is a strong correlate of control of viremia.

Few studies have evaluated the relationship between B cell responses to antigen and viral load. The most consistent correlates of control of viremia are the ability of CD4+ T cells (Kalams et al., 1999; Rosenberg et al., 1997) and CD8+ T cells (Migueles et al., 2008) to proliferate for several days in culture in response to HIV antigen. The frequency of HIV-specific interferon gamma-producing T cells has not been shown to correlate with control of viral replication (Betts et al., 2001), but the frequency of T cells able to secrete multiple cytokines and exert effector function is associated with proliferation following antigen exposure, and thus may be a better correlate of immune control of viremia. However, significant frequencies of polyfunctional T cells are typically observed only in subjects with viral loads <1,000 copies/mL (Akinsiku et al., 2011; Betts et al., 2006). While we did not perform assays for cytokine responses to HIV stimulation in this study, we measured changes in CD25 expression on CD4+ T cells after stimulation with inactivated HIV-1, and the ability of B cells to respond to HIV antigen was a better correlate of viremia.

B cell activation has been linked to decreased B cell responsiveness to stimulation and decreased proliferative capacity (A. Malaspina et al., 2003; S. Moir et al., 2003). Moir et. al. found that after T cell stimulation with anti-CD3, the ability of B cells to express CD25 was diminished with increasing viral load, but a strict correlation was not observed (S Moir et al., 2003). We confirmed this finding in our cohort, but also found CD86 expression on B cells in response to inactivated HIV-1 to be a strong negative correlate of control of viremia and. Our study was not designed to determine which regions of the virus were targeted by these responses. AT-2 inactivated HIV-1 MN contains structurally intact viral proteins, and while we suspect these B cell responses are directed against Env, we can't rule out recognition of other structural proteins (e.g. Gag) that are exposed during the inactivation process. Nevertheless, B cell responsiveness to HIV antigen is potentially an even stronger negative correlate of control of viremia than the frequency of virus-specific T cell responses.

We next evaluated whether the lack of CD4+ T cell help contributes to decreased B cell responsiveness. Initially, we performed stimulations after depleting CD4+ T cells by negative selection, but found considerable B cell responses to HIV antigen. This raised the possibility that either B cells were directly responding to antigen, or antigen presenting cells remaining in culture were providing significant help (data not shown). Repeat experiments were performed with positively selected B cells with or without the addition of autologous CD4+ T cells. B cells alone had a limited ability to respond to the T cell stimulant SEB, and this ability to respond to antigen was reconstituted with the addition of CD4+ T cells. Isolated B cells also had a diminished response to p24 stimulation, which likewise was restored by the addition of CD4+ T cells to the culture. This isolation procedure had no effect on the ability of B cells to respond directly to stimulation with anti-IgM and CD40L. We also ensured that our isolation methods were not altering the activation state of the B cells by comparing the difference in CD86 expression on B cells before and after isolation. While we cannot rule out the possibility that some of the B cell activation we observed was

due to the direct recognition of HIV antigen by B cells, the results of our B and T cell isolation experiments suggest that antigen-specific CD4+ T cell help enhances B cell activation.

To investigate a potential mechanism for poor responses of B and T cells in HIV infection, we evaluated the role of immune exhaustion through expression of PD-1 on these cell populations. In accordance with prior studies, we found that while PD-1 expression on CD8+ T cells correlated with the level of viremia, there was a weaker relationship between PD-1 expression of CD4+ T cells and the level of viremia (Day et al., 2006; C. Petrovas et al., 2006; Trautmann et al., 2006). PD-1 expression on B cells was much lower than that of CD4+ and CD8+ T cells, but higher on B cells from HIV-infected compared to HIV-uninfected individuals. Boliar et. al. recently reported a correlation between the frequency of PD-1+ B cells and viral load (Boliar et al., 2012), but this relationship was driven by individuals with CD4+ T cell counts <200/mm^3. We did not see this correlation in our study cohort, likely because all but one of our study subjects had CD4+ T cell counts >300/mm^3.

In vitro PD-1 blockade of PBMC from HIV-infected individuals has been shown to augment HIV-specific CD4+ and CD8+ T cell function (Freeman et al., 2006). We found *in vitro* PD-1 blockade to modestly increase the ability of B cells to respond to HIV antigen. These experiments do not explain whether decreased B cell responsiveness to stimulation reflects increased PD-1 expression on B cells, CD4+ T cells, or both. However, since these interactions are bi-directional it is reasonable to assume increased PD-1 expression on both subsets of cells play a role in reducing B cell activation. Furthermore, we did not find a direct relationship between the frequency of PD-1 high CD4+ T cells or B cells, and the ability of PD-1 blockade to increase the magnitude of B cell responses. We did not investigate other pathways, such as CTLA-4, Tim-3 or LAG-3 (Antonelli et al., 2010; Jones et al., 2008; Khaitan & Unutmaz, 2011) that may also contribute to immune exhaustion, but

our results demonstrate reversal of immune exhaustion may be one of many modalities that could serve as a potential immunotherapy to help restore CD4-mediated B cell help.

We simultaneously evaluated surface expression markers of activation on T and B cells, and did not evaluate specific subsets of B cells that may be preferentially impaired (Agematsu et al.). Furthermore, some studies have demonstrated decreased ability of HIV-infected individuals B cells to differentiate toward plasmablasts in long-term culture. This has been attributed to decreased ability of T follicular helper cells (CD4+CXCR5+) to secrete IL-21 (Baba et al., 2000). Our study consisted of overnight assays not designed to evaluate B cell differentiation or antibody secretion. Future studies will be designed to evaluate the specific subsets of antigen-specific T cells responsible for the activation and differentiation of B cells.

Here we show B cell responsiveness to HIV antigen is a sensitive correlate of control of viremia. We also show that B cell responsiveness to non-specific and HIV-specific stimulation can be enhanced by the presence of CD4+ T cells and blockade of the inhibitory receptor PD-1. Understanding the interactions between CD4+ T helper cells and B cells will increase our understanding of the humoral immune response over the course of HIV infection, as well as factors which contribute to its preservation or dysfunction. A successful HIV vaccine will likely need to generate both robust cellular immune responses and broadly neutralizing antibodies (B. D. Walker et al., 2011), therefore understanding the precise interactions between CD4+ T cells and B cells will be of paramount importance. Our ongoing experiments will more specifically characterize subsets of CD4+ T cells able to efficiently stimulate B cells to differentiate into antibody-producing plasmablasts, and will help evaluate vaccines designed to elicit helper responses and neutralizing antibodies.

CHAPTER III

CHRONIC HIV-1 INFECTION IMPAIRS SUPERANTIGEN-INDUCED ACTIVATION OF pT_{FH} CELLS, WITH RELATIVE PRESERVATION OF ANTIGEN-SPECIFIC RESPONSES

Introduction

T follicular helper (T_{FH}) cells are a subset of CD4+ T cells that help B cells differentiate into antibody-secreting plasmablasts (Crotty, 2011). Along with PD-1, CXCR5 is the canonical T_{FH} marker, binding to CXCL13 to properly migrate within the germinal centers (GC). There is also a subset of CD4+CXCR5+PD-1+ T cells in the periphery, which are superior to other CD4+ subsets in their ability to help B cells (Bentebibel et al., 2011; N. Chevalier et al., 2011; Morita et al., 2011). Based on functional properties, peripheral CD4+CXCR5+PD-1+ T cells are a putative circulating T_{FH} population, although their precise relation to GC T_{FH} cells is still under investigation.

Defining T_{FH}-like cells in the periphery has been challenging since the transcription factor that promotes T_{FH} differentiation, Bcl-6, is not detectable in protein form in cells outside the germinal center(Cohen, Altfeld, Alter, & Stamatatos, 2014; He et al., 2013; Locci et al., 2013; Nurieva et al., 2009). Early studies relied only on CXCR5 expression on CD4+ T cells to identify 'pT_{FH}' cells (Morita et al., 2011; Pallikkuth et al., 2012). However, subsequent studies determined that both CXCR5 and PD-1 are required to identify functional pT_{FH} memory cells (Boswell et al., 2014; He et al., 2013; Locci et al., 2013). Human GC T_{FH} cells also express ICOS, CD40L, and SAP, and secrete IL-21. These proteins are necessary for T cell – B cell co-localization, adhesion, and signaling(Crotty, 2011). Specifically, ICOS is a co-stimulatory molecule that engages ICOS ligand on B cells, resulting in T cell proliferation and production of cytokines that further support B cell differentiation (Hutloff et al., 1999). CD40L must also be expressed on the surface of T_{FH}

cells, as its interaction with CD40 on B cell surfaces is required for germinal center formation, and directly promotes B cell proliferation and isotype switching(van Kooten & Banchereau, 2000). Circulating CD4+CXCR5+PD-1+ cells express high levels of CD40L and ICOS after stimulation and thus combinations of these markers are routinely used to identify T_{FH}-like cells in the periphery (Boswell et al., 2014; Cohen et al., 2014; Havenar-Daughton et al., 2016; He et al., 2013; Locci et al., 2013; Pallikkuth et al., 2015; Simpson et al., 2010).

In HIV infection, CD4+ T cell dysfunction occurs early and precedes the absolute loss of CD4+ T cells (Connors et al., 1997; Day et al., 2006; Kaufmann et al., 2007). T helper cells from HIV-infected individuals express high levels of inhibitory receptors, resulting in a diminished ability to help B cells (Boswell et al., 2014; Crawford et al., 2014; Cubas et al., 2013; Day et al., 2006; Hong, Amancha, Rogers, Ansari, & Villinger, 2012; Klatt et al., 2011; S. Moir et al., 2003; K. J. Nicholas et al., 2013; Wherry, 2011). The role of T_{FH} cell dysfunction in the dysregulation of B cells is less clear, but GC T_{FH} cell populations have been shown to be expanded in both HIV and SIV infection (Lindqvist, 2012; C. Petrovas, et al., 2012). This is consistent with B cell dysregulation observed in HIV-1 infection, which is characterized by hypergammaglobulinemia, altered maturation patterns, and exhausted phenotypes (Moir & Fauci, 2009).

In this study I evaluated the frequency, phenotype, and responsiveness of peripheral T_{FH} cells ('pT_{FH} cells', defined as CD4+CXCR5+PD-1+ T cells) in HIV-1- and chronically infected treatment-naïve HIV-1+ individuals. In a series of *in vitro* stimulation assays I observed that pT_{FH} cells from HIV-infected individuals had decreased maximal responses to superantigen stimulation as measured by their ability to express ICOS and CD40L. These decreased maximal responses in HIV+ subjects did not correlate with clinical aspects of disease or neutralizing antibody responses. I also show for the first time that HIV-specific and tetanus-specific responses are maintained within the pT_{FH} cell population in HIV-infected individuals.

Materials and methods

Human subjects

Peripheral blood mononuclear cells (PBMCs) from 10 HIV- and 34 HIV+ individuals were separated from blood samples using a Ficoll-Paque^{TM} Plus density gradient. PBMCs were cryopreserved and stored in liquid nitrogen in media composed of 90% fetal bovine serum containing 10% DMSO. All HIV+ individuals were treatment-naïve and CD4+ T cell counts and viral loads were obtained at the time of donation (Table 3-1). The Vanderbilt University School of Medicine's Institutional Review Board approved this study, and all individuals provided written informed consent.

In vitro stimulation assays

Cyropreserved PBMCs were thawed and washed twice in phosphate-buffered saline (PBS) and either stained immediately or cultured for stimulation assays. PBMCs were cultured at 10 million cells/mL in R10 media (RPMI 1640 containing 10% heat inactivated FBS, 2 mM L-glutamine, 50 ug/mL penicillin, 50 ug/mL streptomycin, and 10 mM HEPES buffer (Gibco, Life Technologies)) and co-stimulated with anti-CD28 and anti-CD49d (1 uL/mL each, from BD). Stimulation conditions included Staphylococcal Enterotoxin B (SEB) (1 ug/mL, Sigma), HIV-1 PTE Gag peptides (1 ug/mL, NIH AIDS Reagent Program)(Kalams et al., 2013; F. Li et al., 2006), tetanus toxoid (10 ug/mL, Astarte Biologics), and AT-2 inactivated HIV-1 MN particles (0.53 ug/mL p24, generously provided by Dr. Jeff Lifson) (K. J. Nicholas et al., 2013; Rossio et al., 1998; Rutebemberwa et al., 2007). For comparison to SEB and tetanus stimulation, PBMCs were incubated in R10 media alone. As a control for HIV-1 PTE Gag peptide stimulation (suspended in 0.8% DMSO), cells were suspended in R10 media containing 0.8% DMSO. For comparison to HIV-1 MN, PBMCs were incubated with MN control particles containing AT-2 treated microvesicles prepared from matched uninfected cultures, used at a comparable total

protein concentration (K. J. Nicholas et al., 2013; Rossio et al., 1998; Rutebemberwa et al., 2007). In all stimulation assays, cells were incubated overnight at 37°C with 5% CO_2. After 16 hours cells were removed from the plate, washed twice with PBS, and stained as described below.

Table 3-1: Characteristics of HIV+ treatment-naïve subjects

PTID	Viral Load	CD4+ Count	YPI	bNAb %
		Chronic Infection		
10031	50	612	8	60%
10066	50	1326	12	45%
10071	50	881	16	5%
20016	50	735	6	0%
10070	222	810	10	0%
10002	390	682	20	60%
10055	402	599	5	25%
10040	440	704	18	30%
10024	500	800	7	40%
10067	648	930	20	50%
10060	722	688	4	25%
10068	987	754	12	20%
10003	1684	891	10	65%
20010	3740	1124	8	10%
10017	4007	418	6	25%
20001	6002	562	3	55%
10014	17159	881	3	60%
10076	17693	832	5	15%
10027	19410	547	13	20%
10042	20211	331	21	100%
10028	29345	800	13	40%
		Early Infection		
20008	137	946	1	15%
20015	147	574	1	0%
20018	1886	1374	2	5%
20023	2910	508	0.5	5%
20004	3294	666	1	5%
20013	3998	400	2	60%
20025	4856	472	0.5	5%
20005	6179	903	1.5	5%
20026	8248	300	0.5	5%
20020	18005	602	1	5%
20049	18034	680	0.1	0%
20027	30000	300	0.5	55%
10006	38799	357	2	10%

Subjects are categorized by chronic and early infection and then arranged by increasing viral load. CD4+ counts and viral load were obtained at time of blood draw. Neutralizing antibody breadth was determined as described in methods (results shown in Figure 3-6). PTID = patient identification. YPI = years post-infection. bNAb = broadly neutralizing antibody breadth. 34 subjects are listed here, but due to sample availability, not all individuals were evaluated with each *ex vivo* and *in vitro* cellular assay.

Multicolor flow cytometry

Surface markers were evaluated using combinations of fluorochrome-conjugated monoclonal antibodies that were each titrated individually for their optimal stain index. PBMCs were stained at 10 million cells/mL in 200 uL PBS. All PBMCs were incubated for 10 minutes with an amine-reactive viability dye (LIVE/DEAD Aqua, Invitrogen), washed twice, and then stained for 15 minutes at room temperature with combinations of monoclonal antibodies (Table 3-2). For *ex vivo* phenotyping, cells were stained with CD3-AF700 (UCHT1, BD), CD4-PECy5 (RPA-T4, BD), CD8-APC-AF750 (3B5, Invitrogen), CD45RO-PETR (UCHL1, Beckman Coulter), CCR7-BV421 (150503, BD), CXCR5-AF488 (RF8B2, BD), PD-1-PE (EH12.2H7, BioLegend), CD14-V500 (M5E2, BD), and CD19-V500 (HIB19, BD). *In vitro* phenotyping was performed with combinations of CD3-AF700, CD4-PECy5, CD8-APC-AF750, CD45RO-PETR, CXCR5-AF488, CD14-V500, CD19-V500, ICOS-PE (DX29, BD), CD40L-PE (TRAP1, BD) and PD-1-BV421 (EH12.2H7, BioLegend). All PBMCs were washed twice after staining, fixed with 2% paraformaldehyde, and analyzed on a BD LSR Fortessa (BD Biosciences) at the VMC Flow Cytometry Shared Resource.

Flow cytometry data was analyzed using BD Biosciences FACSDiva Software. In all experiments, forward and side scatter were used to identify lymphocytes and from that population non-viable, CD14+, CD19+, CD8+ cells were excluded from further analysis (Figure 3-1A).

Table 3-2 - Fluorescence cytometry panels to study pT_{FH} cells

Instrument characteristics			Reagent panels		
Laser emission and output power	Bandwidth transmitted to PMT	Fluorochrome	*Ex vivo*	*In vitro* - CD40L	*In vitro* - ICOS
639nm (40mW)	750 - 810	APCA750	CD8	CD8	CD8
	705-750	AF700	CD3	CD3	CD3
488nm (50mW)	505 - 550	AF488	CXCR5	CXCR5	CXCR5
561nm (150mW)	600 - 620	PETR	CD45RO	CD45RO	CD45RO
	655 - 685	PE-Cy5	CD4	CD4	CD4
	575 - 590	PE	PD-1	CD40L	ICOS
404nm (100mW)	430 - 470	BV421	CCR7	PD-1	PD-1
	505 - 550	Aqua, V500	Live/dead, CD14, CD19	Live/dead, CD14, CD19	Live/dead, CD14, CD19

Three fluorescence cytometry panels were designed to identify and characterize T_{FH} cells. Panel 1 measured the memory phenotype of pT_{FH} cells *ex vivo*. Panels 2-3 measured the activation of pT_{FH} cells by assessing CD40L and ICOS expression on pT_{FH} cells *in vitro*.

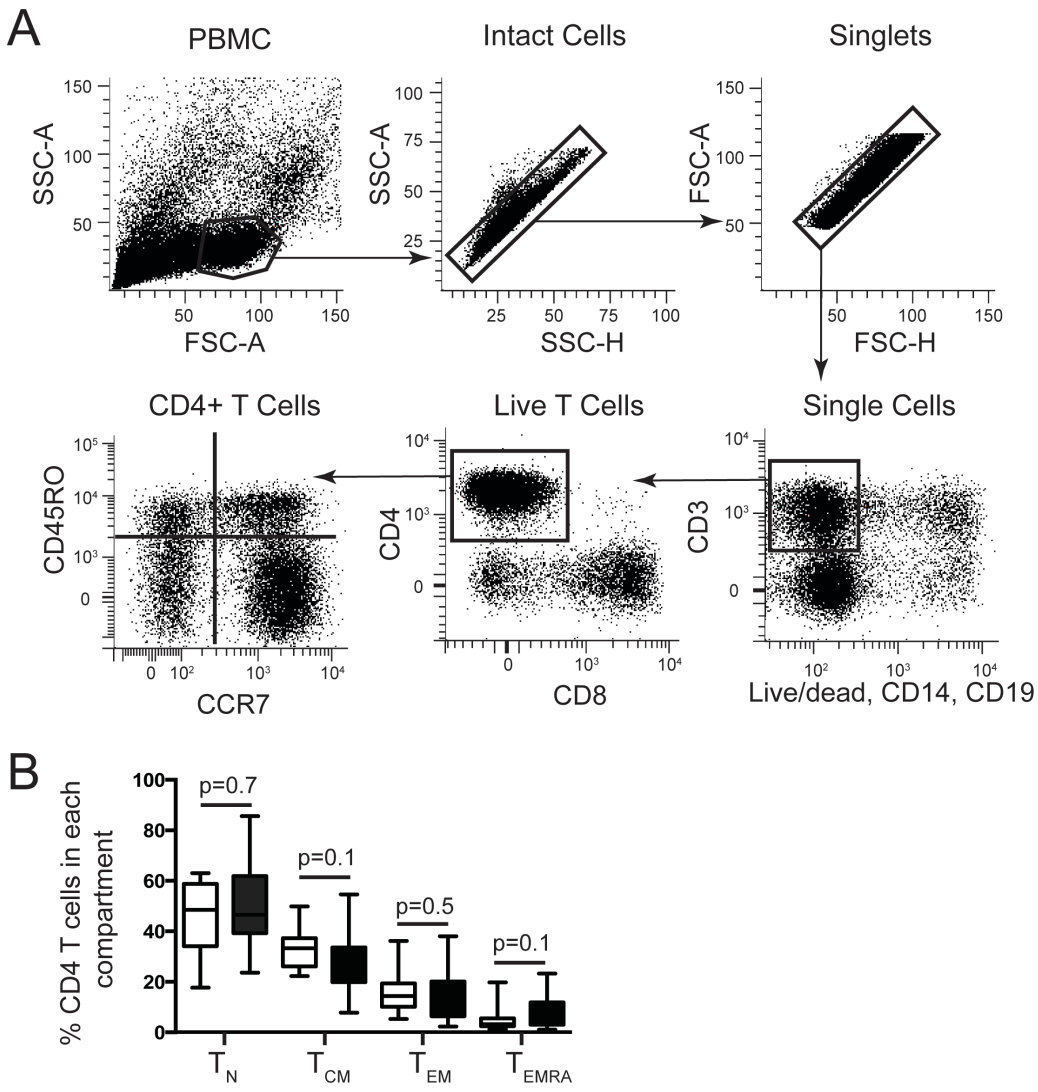


Figure 3-1: Representative gating strategy. (A) Lymphocytes were first distinguished based on the size and granularity of lymphocytes, follow by doublet exclusion. Live T cells were identified based on CD3 expression and the exclusion of CD14 and CD19 and expression and the viability dye Live/Dead Aqua. T cells were then analyzed for CD4 and CD8 expression and CD4+ T cells were then classified into the four memory subsets based on CD45RO and CCR7 expression. (B) Memory distribution of peripheral CD4+ T cells from HIV- (open bars) and HIV+ (black bars) individuals. Statistics represent p values from Mann Whitney unpaired t-tests.

Antibody neutralization assays

Neutralization assays were performed using e*nvelopes* from clades A, B, and C in the TZM-bl cell based pseudovirus assay, as previously described (Sather et al., 2009). The clade B and C *env* clones were chosen from standard *env* panels (M. Li et al., 2005; Y. Li et al., 2007), and the clade A *env* clones were isolated from Kenyan sex workers (Blish et al., 2009). The *env* clones chosen for this study represent a range of neutralization sensitivities of transmitted HIV-1 viruses. Plasma samples were titrated 2 fold from 1:20 to 1:2560 and were incubated for 90 minutes at 37°C in the presence of single-round-competent virions (pseudovirus). The neutralization values reported here are the IC50. Only 30 individuals were evaluated in the antibody neutralization assays due to sample availability.

Statistical analysis

Analysis was performed using GraphPad Prism Software (GraphPad, La Jolla, CA, USA). Paired comparisons (within a single subject) were analyzed with the Wilcoxon matched paired t test. Comparisons between healthy controls and HIV+ subjects were analyzed with the Mann-Whitney t tests. Correlation data was evaluated for statistical dependence using Spearman's rank correlation coefficient rho (ρ). All tests were 2-tailed and were considered statistically significant at $p<0.05$.

Results

Frequency of CD4+CXCR5+PD-1+ T cells in the blood of HIV+ and HIV- individuals

T follicular helper (T_{FH}) cells are more frequent in the lymph nodes during HIV and SIV infection compared to lymph nodes from uninfected controls (Hong et al., 2012; Lindqvist, 2012; Perreau et al., 2013; C. Petrovas, et al., 2012); however, there are conflicting reports comparing the frequency of circulating T_{FH}-like cells among HIV-infected

and HIV-uninfected individuals (Boswell et al., 2014; Locci et al., 2013; Pallikkuth et al., 2012). I first assessed the expression of the canonical T_{FH} markers CXCR5 and PD-1 on circulating CD4+ T cells (Figure 3-1A and Figure 3-2A). The frequency of CD4+CXCR5+PD-1+ T cells was measured in a cohort of 34 treatment-naïve HIV-infected individuals with CD4+ T cell counts > 300 cells/mm^3 and a wide range of viral loads (Table 3-1). There was no difference in the frequency of CD4+ cells with dual CXCR5 and PD-1 expression (pT_{FH} cells) between HIV- and HIV+ individuals, which constituted an average of 2% of CD4+ T cells (Figure 3-2B).

The distribution of pT_{FH} cells within memory T cell subsets was then evaluated. We distinguished CD4+ memory subsets based on CCR7 and CD45RO expression: T naive (CCR7+CD45RO-), T central memory (T_{CM}) (CCR7+CD45RO+), T effector memory (CCR7-CD45RO+), and T effector memory cells expressing CD45RA (CCR7-CD45RO-) (Figure 3-2C and Figure 3-1A) (Oswald-Richter et al., 2007; Sallusto, Lenig, Forster, Lipp, & Lanzavecchia, 1999). There were no significant differences between the distribution of CD4+ T cell memory populations between HIV+ and HIV- individuals in our cohort (Figure 3-1B), and most pT_{FH} cells had a T_{CM} phenotype (Figure 3-2D). In the absence of stimulation, few pT_{FH} cells expressed CD40L (<5%) or ICOS (<10%) (Figure 3-3A-B). After stimulation, however, significantly more pT_{FH} cells were ICOS+ (mean 47%) and CD40L+ (44%) compared to other non-pT_{FH} CD4+ populations (CXCR5+PD-1- and CXCR5- cells) ($p<0.0001$, Figure 3-3C-D).

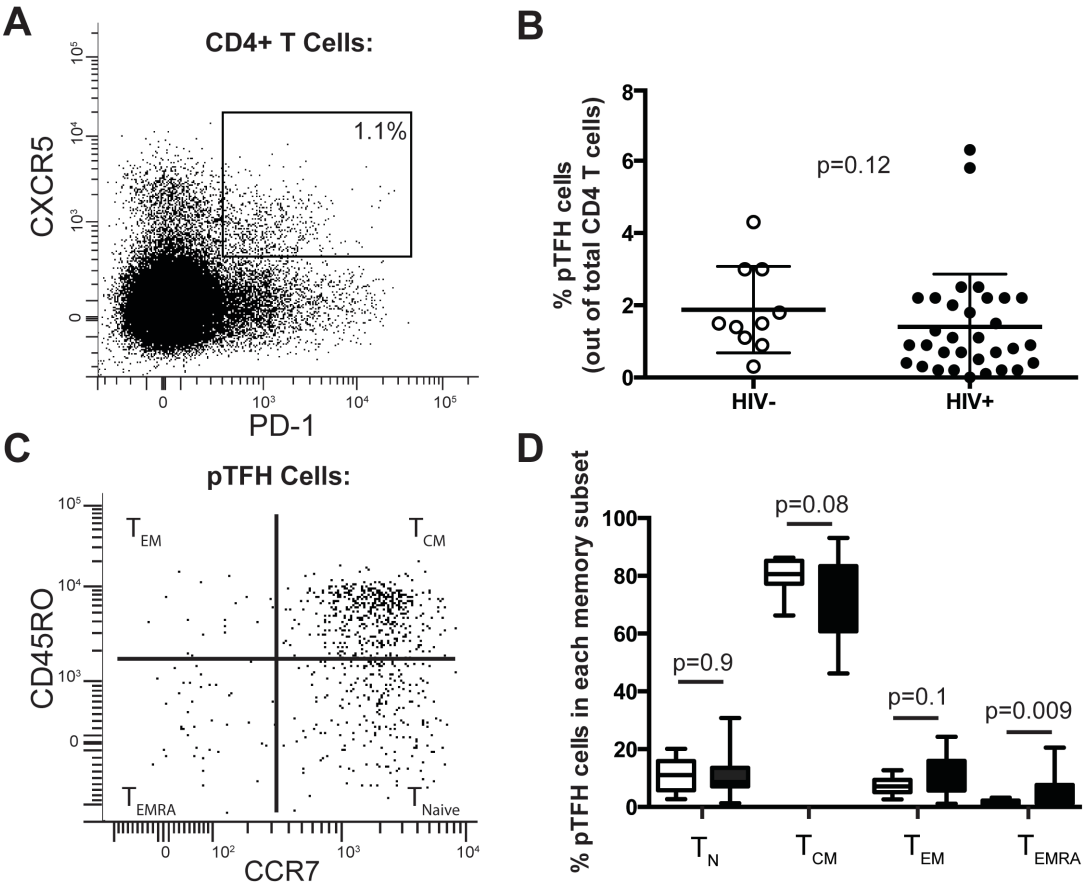


Figure 3-2: *Ex vivo* identification of peripheral CD4+ T cells expressing CXCR5 and PD-1 in HIV+ and HIV- individuals. (A) Representative plot of CXCR5 and PD-1 expression on live CD19-CD14-CD8-CD3+CD4+ cells. Gate indicates frequency of pT_{FH} cells out of total CD4+ T cells. (B) Frequency of pT_{FH} cells (CXCR5+PD-1+) out of total CD4+ T cells in HIV- (open circles) and HIV+ individuals (closed circles). (C) Representative plot of CD45RO and CCR7 expression on pT_{FH} cells. A quadrant gate was used to distinguish the four memory subsets. (D) Distribution of pT_{FH} cells among each memory compartment in HIV- (open bars) and HIV+ (closed bars) individuals. p values are from Mann-Whitney t tests.

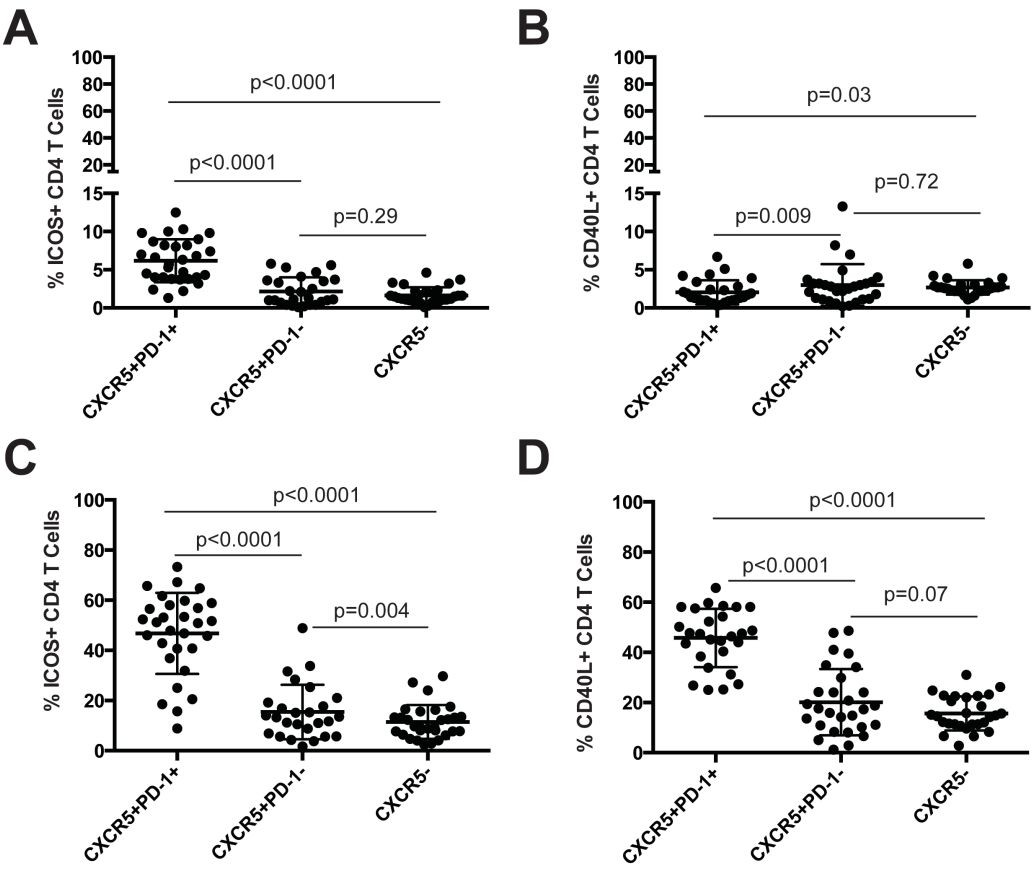


Figure 3-3: Frequency of CD40L and ICOS expression on pT_{FH} and non-pT_{FH} subsets in the presence and absence of SEB superantigen stimulation. (A-B) Frequency of ICOS (A) and CD40L (B) expression on CD4+ T cells subsets after incubation in media for 16 hours. (C-D) Frequency of ICOS (C) and CD40L (D) expression on CD4+ T cell subsets after stimulation for 16 hours with SEB. All data shown is from HIV+ individuals. The same patterns were found with statistical significance in HIV- individuals (data not shown).

pT_{FH} cells from HIV+ individuals have decreased maximal responses to in vitro stimulation compared to healthy donors

I next compared the ability of pT_{FH} cells from HIV- and HIV+ individuals to express ICOS and CD40L in response to SEB stimulation (Figure 3-4). The frequency of ICOS expression without stimulation was higher on pT_{FH} cells from HIV+ individuals compared to HIV- individuals (median 6.5% and 2.2% respectively, p=0.0005; Figure 3-4A-B). After stimulation with SEB, the frequency of ICOS-expressing pT_{FH} cells increased dramatically but was lower in HIV+ compared to HIV- subjects (median 51.1% and 63.8% respectively, p=0.03; Figure 3-4A-B). Accordingly, the change in the frequency of ICOS expression on pT_{FH} cells in response to potent stimulation was decreased in HIV+ individuals compared to HIV- individuals (p=0.003; Figure 3-4C).

The frequency of CD40L expression on pT_{FH} cells was similar in HIV- and HIV+ subjects without stimulation (median 2.5% and 1.4% respectively, p=0.24; Figure 3-4D-E). After SEB stimulation, however, the frequency of CD40L expression on pT_{FH} cells isolated from healthy individuals was higher (64.6%) compared to HIV+ individuals (47.4%; p=0.002; Figure 3-4D-E). Similarly to ICOS, the change in the frequency of CD40L+ expressing pT_{FH} cells in HIV- individuals was higher than that of HIV+ individuals after SEB stimulation (p=0.02; Figure 3-4F).

Decreased maximal pT_{FH} responses did not correlate with clinical aspects of disease

I evaluated whether expression of T_{FH} surface markers or responsiveness to *in vitro* stimulation was associated with either clinical aspects of the disease or the T cell phenotypes of our cohort. I found no correlation between the degree of response to SEB stimulation and viral load, CD4+ T cell count, or duration of infection (Figure 3-5). Although PD-1 is a marker that identifies T_{FH} cells, it is also a marker of immune exhaustion. I therefore evaluated whether the degree of PD-1 expression on CXCR5+ T

cells predicted the ability of these cells to respond to *in vitro* stimulation. No relationship existed between the mean fluorescence intensity (MFI) of PD-1 on CXCR5+ cells in the HIV+ individuals and the ability of these cells to express ICOS or CD40L responses after SEB stimulation.

No correlation between pT_{FH} responses and antibody neutralization breadth

pT_{FH} cells in the blood are capable of stimulating B cells to differentiate to immunoglobulin-secreting plasmablasts (Bentebibel et al., 2011; Boswell et al., 2014; Locci et al., 2013; Morita et al., 2011). Despite B cell dysregulation in HIV-1 infection, 20-30% of HIV+ subjects produce antibodies able to neutralize heterologous HIV-1 strains across clades A, B and C (Mikell et al., 2011; Sather et al., 2009). To assess whether the frequency of pT_{FH} cells or responsiveness to antigenic stimulation correlated with the production of broadly neutralizing antibodies, Dr. Sathers analyzed the serum from all HIV+ subjects for the ability to neutralize heterologous HIV-1 envelopes (Fig. 3-6). The subjects in our cohort ranged widely (0-100%) in their ability to neutralize 20 different HIV-1 isolates from clades A, B, and C (Fig. 3-6). I found no correlation between the degree of neutralizing antibody breadth and the frequency of pT_{FH} cells ($p=0.96$, $r=-0.01$). Additionally I found no correlation between the degree of neutralizing antibody breadth and the frequency or ability of pT_{FH} cells to express ICOS or CD40L after *in vitro* stimulation with SEB (Figure 3-5).

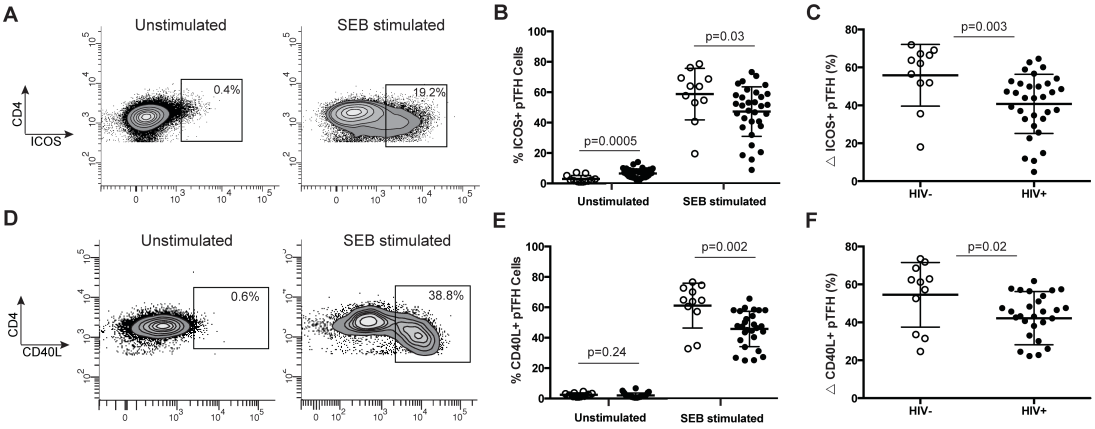


Figure 3-4: pT_{FH} cells from HIV+ individuals have decreased responsiveness to superantigen stimulation as measured by CD40L and ICOS expression. (A) Representative plots of ICOS expression on CD4+ T cells with and without SEB stimulation. (B) Frequency of ICOS on pT_{FH} cells with and without SEB stimulation in healthy controls (open circles) and HIV+ individuals (closed circles). (C) Change in frequency of ICOS expression on pT_{FH} cells in response to SEB stimulation was measured by subtracting the unstimulated from the stimulated conditions. (D) Representative plots of CD40L expression on CD4+ T cells. (E) Frequency of CD40L on pT_{FH} cells with and without SEB stimulation in healthy controls (open circles) and HIV+ individuals (closed circles). (F) Change in frequency of CD40L expression on pT_{FH} cells in response to SEB stimulation. All p values are derived from Mann-Whitney t tests.

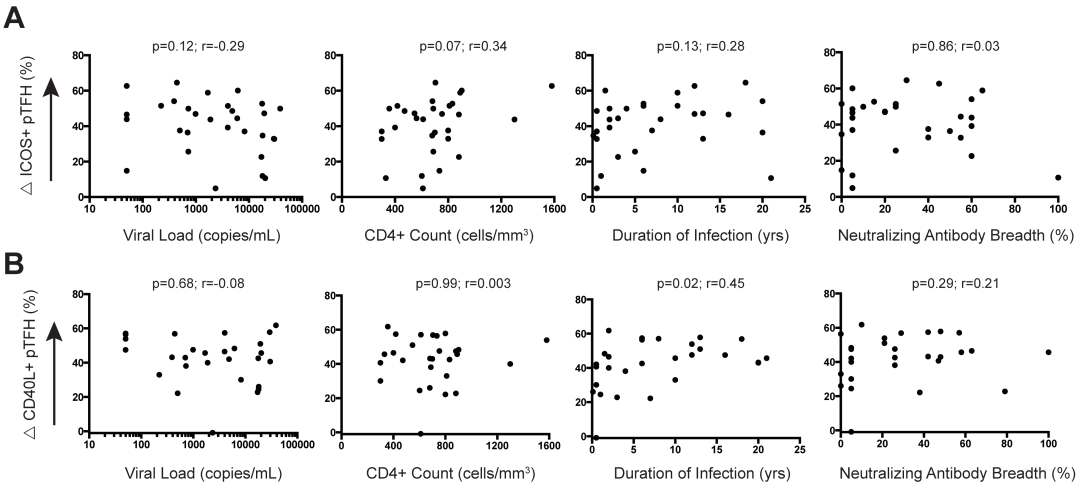
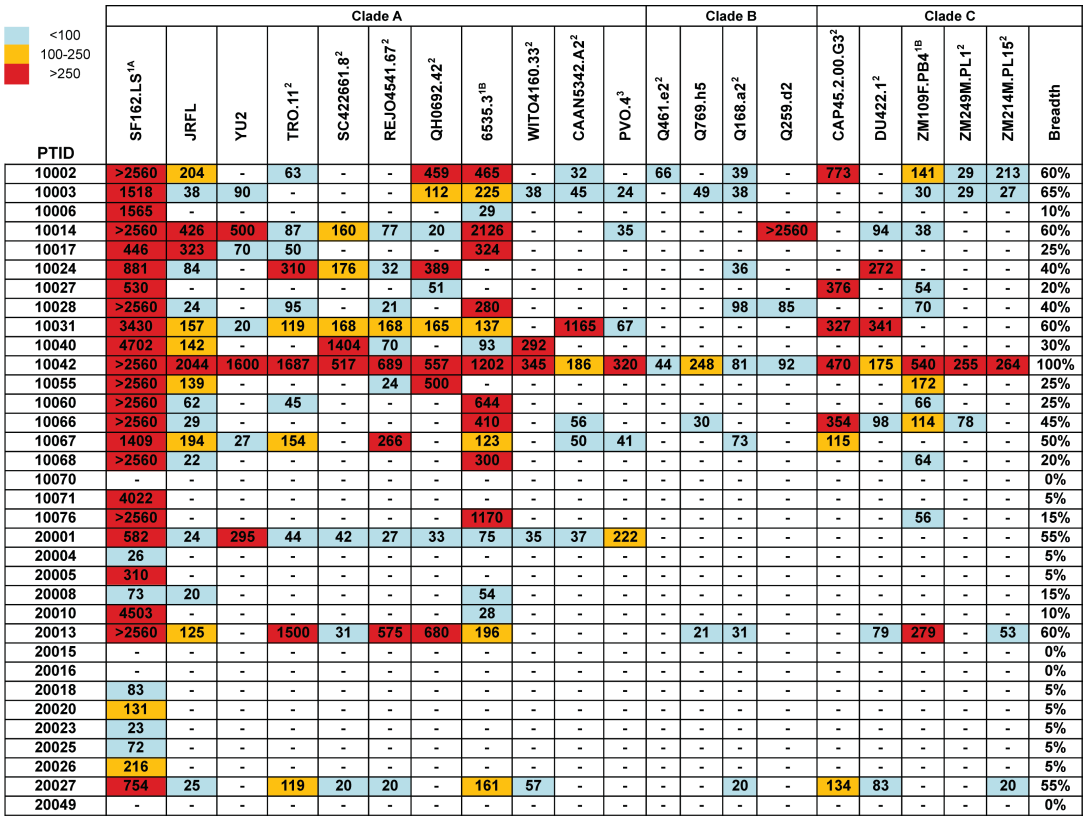


Figure 3-5: Decreased maximal pT_{FH} responses did not correlate with clinical aspects of HIV infection or neutralizing antibody breadth. Change in frequency of ICOS (A) or CD40L (B) expression on pT_{FH} cells in response to SEB stimulation did not correlate with viral load, CD4+ T cell count, duration of infection, or neutralizing antibody breadth in HIV+ individuals. There was a weak correlation between change in CD40L+ pT_{FH} cells and duration of infection. p and r values were derived from Spearman rank tests.



	Clade A											Clade B				Clade C					
PTID	SF162.LS[1A]	JRFL	YU2	TRO.11[2]	SC422661.8[2]	REJO4541.67[2]	QH0692.42[2]	6535.3[1B]	WITO4160.33[2]	CAAN5342.A2[2]	PVO.4[3]	Q461.e2[2]	Q769.h5	Q168.a2[2]	Q259.d2	CAP45.2.00.G3[2]	DU422.1[2]	ZM109F.PB4[1B]	ZM249M.PL1[2]	ZM214M.PL15[2]	Breadth
10002	>2560	204	-	63	-	-	459	465	-	32	-	66	-	39	-	773	-	141	29	213	60%
10003	1518	38	90	-	-	-	112	225	38	45	24	-	49	38	-	-	-	30	29	27	65%
10006	1565	-	-	-	-	-	-	29	-	-	-	-	-	-	-	-	-	-	-	-	10%
10014	>2560	426	500	87	160	77	20	2126	-	-	35	-	-	-	>2560	-	94	38	-	-	60%
10017	446	323	70	50	-	-	-	324	-	-	-	-	-	-	-	-	-	-	-	-	25%
10024	881	84	-	310	176	32	389	-	-	-	-	-	-	36	-	-	272	-	-	-	40%
10027	530	-	-	-	-	-	51	-	-	-	-	-	-	-	-	376	-	54	-	-	20%
10028	>2560	24	-	95	-	21	-	280	-	-	-	-	-	98	85	-	-	70	-	-	40%
10031	3430	157	20	119	168	168	165	137	-	1165	67	-	-	-	-	327	341	-	-	-	60%
10040	4702	142	-	-	1404	70	-	93	292	-	-	-	-	-	-	-	-	-	-	-	30%
10042	>2560	2044	1600	1687	517	689	557	1202	345	186	320	44	248	81	92	470	175	540	255	264	100%
10055	>2560	139	-	-	-	24	500	-	-	-	-	-	-	-	-	-	-	172	-	-	25%
10060	>2560	62	-	45	-	-	-	644	-	-	-	-	-	-	-	-	-	66	-	-	25%
10066	>2560	29	-	-	-	-	-	410	-	56	-	-	30	-	-	354	98	114	78	-	45%
10067	1409	194	27	154	-	266	-	123	-	50	41	-	-	73	-	115	-	-	-	-	50%
10068	>2560	22	-	-	-	-	-	300	-	-	-	-	-	-	-	-	-	64	-	-	20%
10070	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0%
10071	4022	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5%
10076	>2560	-	-	-	-	-	-	1170	-	-	-	-	-	-	-	-	-	56	-	-	15%
20001	582	24	295	44	42	27	33	75	35	37	222	-	-	-	-	-	-	-	-	-	55%
20004	26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5%
20005	310	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5%
20008	73	20	-	-	-	-	-	54	-	-	-	-	-	-	-	-	-	-	-	-	15%
20010	4503	-	-	-	-	-	-	28	-	-	-	-	-	-	-	-	-	-	-	-	10%
20013	>2560	125	-	1500	31	575	680	196	-	-	-	-	21	31	-	-	79	279	-	53	60%
20015	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0%
20016	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0%
20018	83	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5%
20020	131	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5%
20023	23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5%
20025	72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5%
20026	216	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5%
20027	754	25	-	119	20	20	-	161	57	-	-	-	-	20	-	134	83	-	-	20	55%
20049	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0%

Figure 3-6: Plasma neutralization breadth of HIV+ individuals. The cross-neutralizing activities of plasmas from HIV+ individuals were evaluated against the indicated clade A, B and C viruses. The values are the plasma titers at which 50% neutralization (IC50) was recorded. IC50 values are color-coded: (blue) IC50<1:100; (yellow) 1:100≤IC50≥1:250; (red) IC50>1:250. A (-) symbol indicates less than 50% neutralization was recorded. The term 'breadth' represents the percent of isolates neutralized by a plasma sample, out of the total number of isolates tested, irrespective of the potency of neutralization. With the exception of SF162.LS, which is a tier 1 virus, all viruses were tier 2.

pT_{FH} cells from HIV+ individuals maintain recall antigen-specific responses

I next investigated recall antigen-specific responses of pT_{FH} and non-pT_{FH} (defined as CD3+CD4+CXCR5-) T cells. Since the majority of naïve T cells reside in the non-pT_{FH} population, I only assessed memory (CD45RO+) CD4+ T cells in this assay. I performed stimulations with tetanus toxoid, HIV-1 Gag peptides, and inactivated HIV-1 MN to measure antigen-specific responses in HIV+ individuals (Figure 3-7, Figure 3-8, and data not shown). While the overall responses to tetanus toxoid were low, a higher fraction of pT_{FH} cells from HIV+ individuals increased ICOS and CD40L expression compared to non-pT_{FH} cells ($p=0.02$; Figure 3-7A-B). There were no differences in ICOS or CD40L expression on pT_{FH} and non-pT_{FH} cell after tetanus toxoid stimulation in HIV- individuals ($p=0.38$ (ICOS), $p=0.82$ (CD40L)). Furthermore, the responses of pT_{FH} cells to tetanus were not different between HIV- and HIV+ individuals though the responses appear to be more robust in HIV+ individuals ($p=0.9$ (ICOS), $p=0.31$ (CD40L)).

Responses to Gag peptides as measured by the change in ICOS and CD40L on CD4+ T cells was greater on pT_{FH} compared to non-pT_{FH} cells in HIV+ individuals ($p=0.002$ and $p=0.0003$; Figure 3-7C-D). There were no significant changes in ICOS or CD40L expression in HIV- individuals stimulated with Gag peptides ($p=0.34$, data not shown). Since the majority of CD4+ T cells are CXCR5- a higher absolute number of CXCR5- cells increased ICOS expression in response to antigen stimulation; however, a higher fraction of CXCR5+PD-1+ cells were antigen-specific. These data demonstrate that recall antigen-specific memory pT_{FH} cells are preserved in HIV+ individuals.

I also evaluated responses to AT-2 inactivated HIV-1 MN particles, which contain conformationally intact envelope proteins on their surface (data not shown). Compared to control particles, MN did not induce changes in ICOS on pT_{FH} in HIV- subjects ($p=0.13$). In HIV+ individuals, MN induced changes in ICOS on pT_{FH} cells compared to control particles ($p<0.0001$) and responses were greater in memory pT_{FH} cells compared to non-pT_{FH} cells ($p=0.02$). Changes in CD40L in response to MN, however, were rare (only 12 of 28

subjects in the assay) and weak and did not allow us to accurately compare non-pT_{FH} and pT_{FH} responses. No antigen-specific responses to any of the recall antigens correlated with neutralizing antibody breadth in HIV+ individuals (data not shown).

Since the core identity markers of pT_{FH} cells, PD-1 and CXCR5, can also go up with activation, I assessed the frequency of pT_{FH} cells before and after stimulation. The median increase in the frequency of pT_{FH} cells in response to SEB stimulation was 1.95%. Stimulation of PBMC with recall antigens only slightly increased the frequency of pT_{FH} cells (median increase of 0.2% with Gag stimulation; median increase of 0.5% with tetanus stimulation). To further investigate the possibility of CXCR5- cells contributing to our pT_{FH} population after overnight stimulation, we FACS sorted CD4+ T cells into four populations based on CXCR5 and PD-1 expression in 4 healthy individuals. Cells that were sorted CXCR5- (regardless of PD-1 expression) became CXCR5+ after SEB stimulation at a frequency of <2.5% and the MFI of CXCR5 after SEB stimulation never changed (Figure 3-9 and data not shown). Thus while I cannot exclude the possibility that a small number of cells we termed 'pT_{FH}' in these assays were recently activated T cells, the large majority of this 'pT_{FH}' population expressed CXCR5 and PD-1 prior to stimulation.

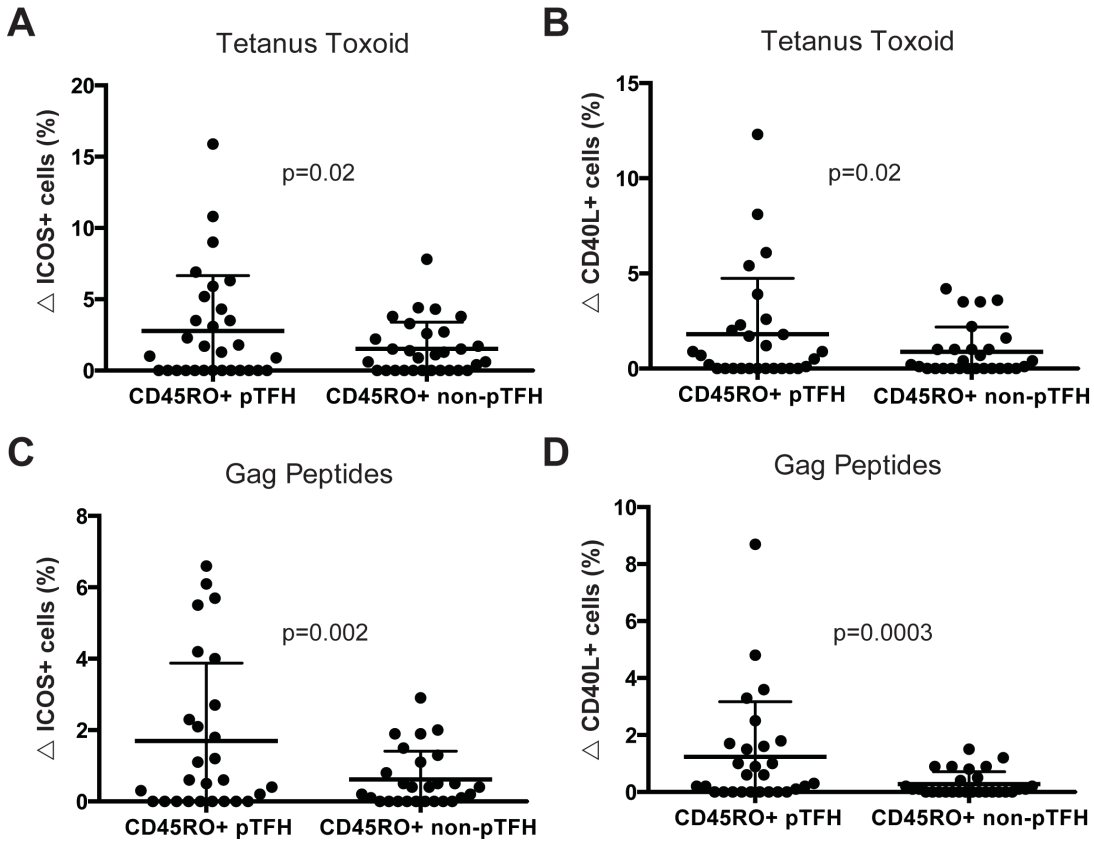


Figure 3-7: Memory pT_{FH} cells preferentially express ICOS and CD40L after recall antigen stimulation compared to memory non-pT_{FH} cells. Cells analyzed were either memory pT_{FH} cells (defined as CD45RO+CXCR5+PD-1+ CD4+ T cells) or memory non-pT_{FH} cells (defined as CD45RO+CXCR5-CD4+ T cells). (A-B) Change in the frequency of ICOS+ (A) or CD40L+ (B) memory pT_{FH} or non-pT_{FH} cells in HIV+ individuals in response to 16 hours of stimulation with tetanus toxoid. (C-D) Change in the frequency of ICOS+ (C) or CD40L+ (D) memory pT_{FH} or non-pT_{FH} cells in HIV+ individuals in response to 16 hours of stimulation with Gag peptides. All p values are derived from Wilcoxon matched-pairs t-tests.

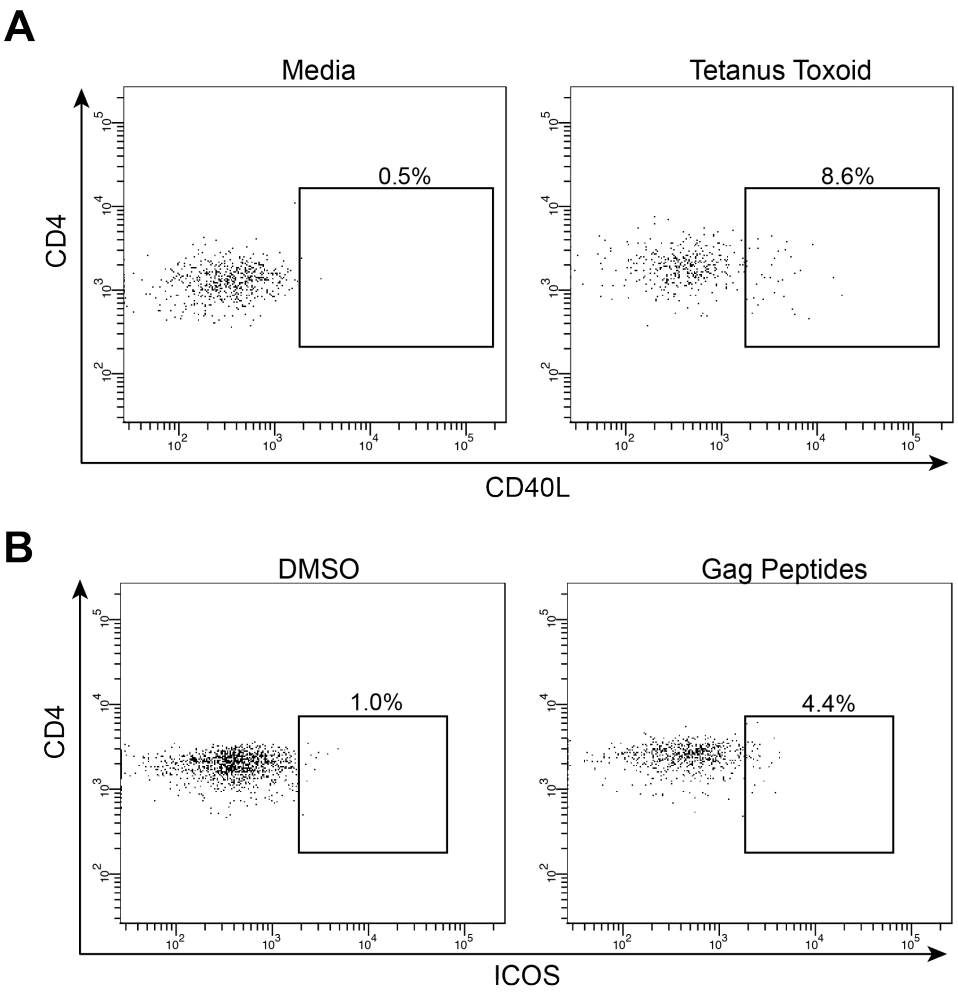


Figure 3-8. CD45RO+ pT_{FH} cell antigen-specific responses. Representative fluorescence cytometry plots measuring antigen-specific responses of CD45RO+ pT_{FH} cells. Examples from one HIV-infected individual are shown. (A) CD40L expression on CD45RO+ pT_{FH} cells after overnight incubation in media alone or tetanus toxoid. (B) ICOS expression on CD45RO+ pT_{FH} cells after overnight incubation in DMSO diluent or Gag peptides with the same concentration of DMSO.

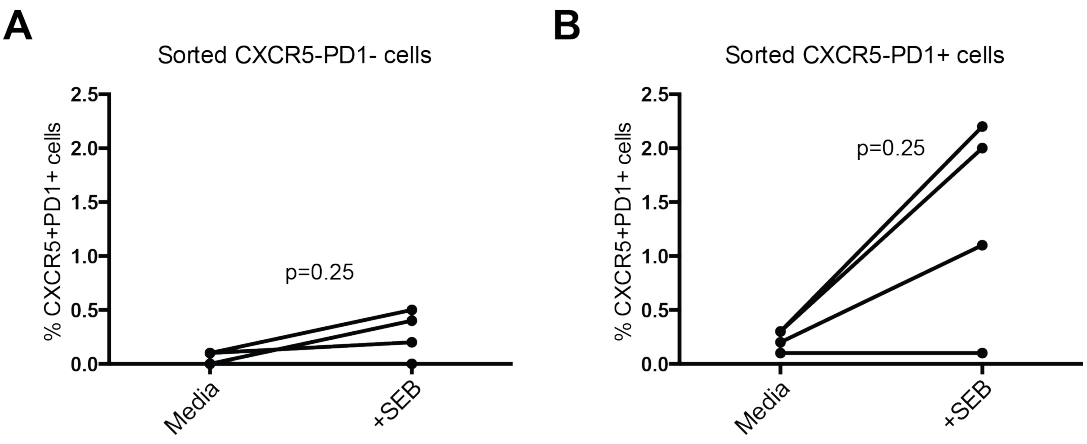


Figure 3-9. Phenotype of sorted CXCR5- cells after stimulation. CD4+ T cells were isolated from PBMC using a CD4+ T cell enrichment kit (Miltenyi). CD4+ T cells were then stained with fluorochrome-antibody conjugates and FACS sorted into 4 populations based on CXCR5 and PD-1 expression: CXCR5-PD-1-, CXCR5-PD-1+, CXCR5+PD-1-, and CXCR5+PD-1+. The sorted populations were then cultured separately overnight with or without SEB. This experiment was performed 4 times with cells from 4 different healthy individuals. (A) Frequency of sorted CXCR5-PD-1- cells that expressed CXCR5 and PD-1 after overnight SEB stimulation. (B) Frequency of sorted CXCR5-PD-1+ cells that expressed CXCR5 and PD-1 after overnight SEB stimulation. p values were derived from Wilcoxen matched-pairs t-tests.

Discussion

Multiple studies have demonstrated pT_{FH} cells are similar to GC T_{FH} cells in their ability to provide B cell help (Bentebibel et al., 2011; N. Chevalier et al., 2011; He et al., 2013; Locci et al., 2013; Morita et al., 2011; Schultz et al., 2016). I performed an *ex vivo* phenotypic analysis of pT_{FH} cells in healthy and chronically infected HIV+ individuals, and we assessed the *in vitro* responses of this cell population to superantigen and recall antigen stimulation. CXCR5+ and PD-1 are currently accepted canonical markers required to identify pT_{FH} cells (Boswell et al., 2014; Cohen et al., 2014; Havenar-Daughton et al., 2016; He et al., 2013; Locci et al., 2013; Pallikkuth et al., 2015; Simpson et al., 2010). While additional cell markers such as CXCR3 (Locci et al., 2013), chemokine receptor 6 (CCR6) (Boswell et al., 2014), and T-cell immunoreceptor with immunoglobulin (Ig) and ITIM domains (TIGIT) (Godefroy, Zhong, Pham, Friedman, & Yazdanbakhsh, 2015) have been suggested to further discriminate pT_{FH} cells, I focused on CD4+ T cells expressing the main pT_{FH} identifying canonical markers. I evaluated the ability of these cells to respond to various stimuli, and identified tetanus-specific and HIV-specific pT_{FH} populations in HIV-infected individuals. I focused on ICOS and CD40L expression of pT_{FH} cells, since these surface receptors must engage ICOS ligand and CD40 on the surface of B cells to drive B cell differentiation to antibody-secreting plasmablasts.

I observed pT_{FH} cells circulate at similar frequencies in healthy and HIV+ treatment-naïve individuals. The frequencies of circulating pT_{FH} cells I measured are in agreement with those measured in a cohort with CD4+ T cell numbers similar to ours (Pallikkuth et al., 2012). In separate studies, however, decreased pT_{FH} cell frequencies were reported in a cohort with a mean CD4+ T cell count of 320 cells/mm^3 (Boswell et al., 2014), and increased frequencies were found in a cohort of individuals that were only infected for an average of three years (CD4+ T cell values were not reported) (Locci et al., 2013). Thus, variability within these cohorts likely explains the variability in measured frequencies of pT_{FH} cells. I also show that the distribution of pT_{FH} cells within T cell

memory compartments in HIV infection is not perturbed by HIV infection, and confirm that most pT_{FH} cells reside in the T central memory compartment in both HIV- and HIV+ individuals (N. Chevalier et al., 2011; Morita et al., 2011; Pallikkuth et al., 2012).

GC T_{FH} cells and pT_{FH} cells isolated from HIV and SIV infected individuals are impaired in their ability to provide help necessary for B cells to differentiate to plasmablasts (Boswell et al., 2014; Cubas et al., 2013; Hong et al., 2012; Lindqvist, 2012; Pallikkuth et al., 2012; C. Petrovas, et al., 2012). However, the underlying changes in expression of key surface receptors on pT_{FH} cells required for proper function have not been described. Here I provide an analysis of the surface expression of ICOS and CD40L in response to stimuli in chronic HIV-1 infection.

I found that after *in vitro* superantigen stimulation, pT_{FH} cells from HIV-infected individuals expressed less ICOS and CD40L than pT_{FH} cells from HIV-uninfected individuals. This reduced maximal expression of ICOS and CD40L might explain the decreased function of these cells observed in HIV and SIV infection (Boswell et al., 2014; Cubas et al., 2013; Hong et al., 2012; Lindqvist, 2012; Pallikkuth et al., 2012; C. Petrovas, et al., 2012). I attempted to determine correlates of this decreased response. I hypothesized that the MFI of PD-1 expression on pT_{FH} cells would correlate with decreased responses in HIV-infected individuals. While PD-1 is required for proper T_{FH} cell function (Good-Jacobson et al., 2010; N. M. Haynes et al., 2007), it is also associated with functional exhaustion of T cells during chronic HIV-1 infection (Day et al., 2006; Trautmann et al., 2006). Blocking PD-1 and PD-L1 has been shown to enhance the ability of T cells to activate B cells in culture (K. J. Nicholas et al., 2013) and improves the ability of pT_{FH} cells to stimulate B cells to produce IgG in HIV+ but not HIV- individuals (Cubas et al., 2013). I found no correlation between PD-1 expression and decreased pT_{FH} cell responsiveness in HIV-infected individuals. I also did not find a correlation between the responsiveness of pT_{FH} cells with viral load, CD4 + T cell count, years post-infection, or neutralizing antibody breadth.

Recall-antigen specific pT_{FH} cells have been identified in a few prior studies: tetanus-specific pT_{FH} cells were identified using tetramers in healthy individuals (Locci et al., 2013) and in HIV-infected individuals, small populations of Gag-specific pT_{FH} cells were identified by CD40L expression and IL-21 secretion (Boswell et al., 2014). More recently, HIV-specific pT_{FH} cells (identified by IL-21 secretion after *in vitro* stimulation, and subsequently found to express transcripts for CXCR5, CD40L, and ICOS) were shown to recognize both HIV-1 Gag and Env peptides by ELISpot.(Schultz et al., 2016). In agreement with these studies, I found the majority of recall memory responses resides within the CD4+CXCR5- population, which makes up the majority of CD4+ T cells. However, the proportion of pT_{FH} cells that increase ICOS or CD40L expression is significantly higher than that of non-pT_{FH} cells in response to HIV Gag peptides and tetanus toxoid in HIV+ individuals. This was also true for ICOS responses to HIV-1 MN in HIV+ individuals, but not CD40L which I was unable to assess accurately since responses were so rare. HIV- individuals did not respond to Gag or MN stimulation as expected, but did have weak responses to tetanus toxoid stimulation. There was no difference in the frequency of these weak tetanus responses in pT_{FH} compared to non-pT_{FH} cells in HIV- individuals. It is difficult to make accurate conclusions from these findings, however, since we have no tetanus vaccination records from our study populations. However, the HIV+ individuals are followed very closely and more likely to be up-to-date on all vaccinations. My novel findings show that HIV-specific CD4+ T cells are maintained within the pT_{FH} population during chronic infection.

HIV and SIV infection have been shown to drive the expansion of GC T_{FH} cells (Cubas et al., 2013; Lindqvist, 2012), but the relationship between the frequencies of GC T_{FH} and peripheral T_{FH} cells is unclear. Recent gene expression and functional studies disagree on precisely which combination of surface receptors identifies T_{FH}-like cells in the periphery; however, there appears to be consensus that CXCR5 and PD-1 are required for identifying pT_{FH} cells (Boswell et al., 2014; He et al., 2013; Locci et al., 2013). My

observation of preferential expression of CD40L and ICOS expression on CD4+CXCR5+PD-1+ T cells in response to recall antigens further supports the identification of pT_{FH} cells as a circulating counterpart of GC T_{FH} cells. It is important to note that superantigen and recall antigen stimulation increased the frequency of CXCR5+PD-1+ cells *in vitro*. This increased frequency could reflect proliferation of pT_{FH} cells or a new population of recently activated T cells. Cell sorting experiments, however, demonstrated that few sorted CXCR5- cells became CXCR5+PD-1+ after short-term superantigen stimulation, thus the majority of this population was likely CD4+CXCR5+PD-1+ cells prior to stimulation.

Future studies should recapitulate pT_{FH} phenotypic changes in response to antigen with IL-21 measurement to establish the trends are the same. I tested many IL-21 antibody clones in intracellular cytokine assays under many stimulation conditions (PMA/ionomycin, anti-CD3/anti-CD28, and SEB) at 4, 6, 24, 48, and 72 hours without obtaining reliable results. Unfortunately, current antibodies to IL-21 protein are poor, making intracellular cytokine assays unable to address this question accurately (Porichis et al., 2014). The development of new commercially available IL-21 detection kits (of protein or transcript) will be valuable for future single-cell IL-21 research (Porichis et al., 2014).

The generation of HIV-neutralizing antibody responses through vaccination has remained an elusive goal; understanding how naturally infected individuals are able to generate broadly neutralizing antibodies will be critical to our understanding of vaccine-induced immune responses. Here we analyzed the neutralization breadth of plasma from 30 HIV+ individuals in our cohort. In agreement with prior studies I did not find a direct correlation between neutralization breadth and the frequency of pT_{FH} cells during chronic HIV infection (Cohen et al., 2014; Locci et al., 2013). I also found no correlation between neutralization breadth and the frequency of ICOS+ pT_{FH} cells. This is in contrast to findings by Locci, et al., however, subjects in that study were grouped into ‘top’ and ‘low’ neutralizers and statistical correlations were not performed (Locci et al., 2013). Despite a

lack of correlation during chronic infection, Cohen, et al., found correlations between the frequency of pT_{FH} cells and broadly neutralizing antibody responses in early infection (0.1-1 year after infection) (Cohen et al., 2014). One interpretation of these data is that during early HIV infection pT_{FH} responses may not yet be impaired and thus can facilitate the generation of broadly neutralizing antibodies, and these antibody responses can be maintained over the course of chronic infection. Future studies should investigate the responsiveness of pT_{FH} cells to HIV antigens longitudinally from early through chronic HIV infection.

In this study I describe our efforts to determine the responsiveness of pT_{FH} cells in chronic HIV-1 infection. Specifically, I investigated two surface receptors required by T_{FH} cells to provide help to B cells, ICOS and CD40L. I demonstrate that pT_{FH} cells expressed the highest levels of CD40L and ICOS in response to superantigen and recall antigen stimulation compared to CD4+CXCR5+PD-1- T cells and CD4+CXCR5- T cells. I found the pT_{FH} cells of HIV-infected individuals had impaired ability to increase expression of CD40L and ICOS in response to superantigen stimulation. Despite impaired maximal responses, however, this cell subset maintained the ability to respond to recall antigens. These results suggest that the evaluation of immune responses of pathogen-specific circulating pT_{FH} cells will be important for future studies of natural infection and immune responses to vaccines.

CHAPTER IV

MULTIPARAMETER ANALYSIS OF STIMULATED HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS: A COMPARISON OF MASS AND FLUORESCENCE CYTOMETRY

Introduction

Fluorescence cytometry has driven forward our understanding of cell biology in human immune monitoring and disease studies for decades by quantitatively characterizing single cells based on cell surface and intracellular features (Bendall, Nolan, Roederer, & Chattopadhyay, 2012; Chattopadhyay, Gierahn, Roederer, & Love, 2014; Di Palma & Bodenmiller, 2014; Irish & Doxie, 2014; Maecker, McCoy, & Nussenblatt, 2012). In Chapters II and III I describe my studies using fluorescence cytometry to study surface expression of proteins on pT_{FH} and B cells. Mass cytometry is a new quantitative single cell flow cytometry approach that employs antibodies conjugated to stable isotopes of metals and time of flight mass spectrometry as a detection technology (Bandura et al., 2009; O. Ornatsky et al., 2010). Due to the precision of mass resolution, hundreds of features can theoretically be measured on each cell simultaneously using a mass cytometer. Recently mass cytometry has emerged as a powerful tool for high dimensional single cell analysis that has been used to characterize diverse populations of immune cells (Becher et al., 2014; Bendall et al., 2014; Bendall et al., 2011; Horowitz et al., 2013; Mingueneau et al., 2014; Newell, Sigal, Bendall, Nolan, & Davis, 2012; Polikowsky, Wogsland, Diggins, Huse, & Irish, 2015; Sen et al., 2014). Future studies of pT_{FH} and B cells using mass cytometry will be greatly beneficial to the field, but I felt it was first important to carefully compare mass cytometry with fluorescence cytometry to optimize detection of various proteins.

While several studies have highlighted the potential of mass cytometry for

describing cellular subsets in great detail (Becher et al., 2014; Bendall et al., 2014; Bendall et al., 2011; Horowitz et al., 2013; Mingueneau et al., 2014; Newell et al., 2012; Sen et al., 2014), only a few of these studies have directly compared mass cytometry with traditional fluorescence cytometry for evaluating human cell populations (Behbehani, Bendall, Clutter, Fantl, & Nolan, 2012; Bendall et al., 2011; Polikowsky et al., 2015; Wang et al., 2012). Furthermore, despite its promise, mass cytometry is still a relatively new technology, and extensive optimization of panel design, protocols, and analysis workflows will be required to acquire and appropriately analyze the vast amount of data generated (Diggins, Ferrell, & Irish, 2015; Gaudilliere et al., 2015; Tricot et al., 2015). Here a direct comparison of mass cytometry and traditional fluorescence cytometry is described in detail for human subjects. A panel of 20 well-established surface markers of lymphocytes was used to assess whether mass cytometry provided equivalent per-marker and per-subset information on a one-to-one basis with traditional fluorescence cytometry. Unstimulated and stimulated human PBMC from six donors were analyzed with five established fluorescence cytometry panels in our laboratory and one newly optimized mass cytometry panel. The results of the two platforms were highly concordant, suggesting that mass and fluorescence cytometry will be complementary technologies used for characterizing the complex, dynamic cellular phenotypes that exemplify immune responses.

Materials and methods

Cell isolation and culture

Peripheral blood mononuclear cells (PBMC) from healthy donors (N = 6) were isolated using density gradient separation (Ficoll-Paque™ Plus, GE Healthcare, Piscataway, NJ, USA). PBMC were pelleted by low speed centrifugation (400 x *g*), resuspended in media composed of 90% fetal bovine serum (Atlanta Biologicals, Norcross, GA, USA) containing 10% DMSO (Sigma-Aldrich, St. Louis, MO, USA), frozen slowly in the

vapor phase of liquid nitrogen in multiple cryotubes, and stored in liquid nitrogen, as previously described (Irish, Czerwinski, Nolan, & Levy, 2006). The Vanderbilt University's Institutional Review Board approved this study, and all individuals provided written informed consent.

Individual PBMC cryotubes were thawed in 2 mL of warm phosphate buffered saline (PBS, Gibco, Life Technologies, Grand Island, NY, USA), pelleted by centrifugation (650 x *g*), divided for immediate *ex vivo* phenotyping or phenotyping following 16 hours of *in vitro* SEB (EMD Millipore, Billerica, MA) stimulation, and then pelleted again before resuspension in room temperature PBS (*ex vivo*) or R10 media (*in vitro*) at 10 x 10^6 cells/mL. R10 media contained RPMI 1640 Medium (Gibco), 2 mM L-glutamine (Gibco), 50 μg/mL penicillin (Gibco), 50 μg/mL streptomycin (Gibco), 10% FBS, and 10 mM HEPES buffer (Thermo Fisher Scientific, Waltham, MA, USA). Cells for *ex vivo* staining were further divided among flow cytometry tubes (Falcon 2052, BD-Biosciences, San Jose, CA) for fluorescence or mass cytometry staining, described below. Cells for *in vitro* culture were stimulated by addition of SEB to achieve a final concentration of 1 μg/mL in 200uL of 10 x 10^6 cells/mL in 48-well flat bottom culture plates (Costar, Corning Incorporated, Corning, NY, USA). After 16 hours of incubation at 37°C in a 5% CO_2 incubator, cells were removed from the plate, washed twice in PBS, and stained as described below.

Fluorescence cytometry

For each healthy donor, 2 x 10^6 PBMC were stained in 200μL PBS. PBMC were incubated first with a viability dye for 10 minutes (LIVE/DEAD Aqua, Life Technologies), washed once in PBS, and then stained with combinations of fluorescently-tagged antibodies (Table 4-1). For *ex vivo* phenotyping, cells were stained with Panels 1-5 from Table 1 (for antibody information see Table 4-2). For phenotyping following *in vitro* stimulation, cells were stained with Panels 3-5 from Table 4-1 at 16 hours after addition of SEB. After staining, all cells were washed twice in PBS and fixed with 2%

paraformaldehyde (PFA, Electron Microscopy Services, Fort Washington, PA, USA) and refrigerated up to 24 hours until analysis on the Special Order Research Product BD LSRFortessa (BD Biosciences, San Jose, CA) at the Vanderbilt Flow Cytometry Shared Resource.

Mass Cytometry

For each healthy donor, 2 x 10^6 PBMC were stained in 50 μL PBS. PBMC were incubated first with a viability reagent (50 μM cisplatin, Enzo Life Sciences, Farmingdale, NY, USA) in 1 mL serum-free RPMI for 3 minutes. Cisplatin was quenched by washing once with RPMI containing 10% FBS followed by two washes in PBS. A master mix containing 21 antibody-metal conjugates (Table 4-3, Table 4-2) was added to each sample (50 μL total staining volume) and incubated at room temperature for 25 minutes. Cells were then washed twice with PBS, fixed for 10 minutes with 1.6% PFA at room temperature, washed once with PBS, and then permeabilized at -20°C in 1 mL 100% cold methanol for 20 minutes. Following permeabilization, cells were washed at 800 x *g*, stained with 250 nM Iridium intercalator (O. I. Ornatsky et al., 2008) (Fluidigm/DVS Sciences, Sunnyvale, CA) for 16 hours at 4°C, washed twice in PBS, washed once with ddH_2O, and then re-suspended in 500 μL ddH_2O for mass cytometry analysis that day. Cells were filtered immediately before injection into the mass cytometry using a 35μm nylon mesh cell-strainer cap (BD Biosciences).

Samples were analyzed using a CyTOF 1.0 (Fluidigm Sciences, Sunnyvale, CA) and CyTOF software version 5.1.615 (Fluidigm) at the Vanderbilt Flow Cytometry Shared Resource. Dual count calibration (on the "data") and noise reduction (cell length 10-75, lower convolution threshold 10) were applied during acquisition.

Table 4-1: Fluorescence cytometry instrument and antibody panel information

Instrument characteristics			Reagent panels				
Laser emission and output power	Bandwidth transmitted to PMT	Fluoro-chrome	1	2	3	4	5
639nm (40mW)	750 - 810	APC-Cy7, APC-A750	HLA-DR	CD8	HLA-DR	HLA-DR	CD8
	663 - 677	APC		CD3	CD3	CD20	CD3
488nm (50mW)	505 - 550	FITC	CD57	CD45RO	CD25	CD27	
561nm (150mW)	685 - 735	PE-Cy5.5		CD127			
	600 - 620	PETR	CD14				CD45RO
	750 - 810	PE-Cy7			CD38	CD38	
	655 - 685	PE-Cy5			CD62L		CD4
	575 - 590	PE	CD16	CD27	CD69	CD86	CD40L
404nm (100mW)	430 - 470	PB, BV421, V450	CD3	CCR7	CD8		PD-1
	685 - 735	BV711	CD19			CD19	
	595 - 620	BV605		CD4	CD4		
	505 - 550	Aqua	LD	LD	LD	LD	LD

Five fluorescence cytometry panels were designed to measure PBMC populations with 21 parameters comparable to those measured in mass cytometry: 20 fluorophore-labeled antibodies and 1 viability marker. Panel 1: PBMC subsets; Panel 2: T cell memory subsets; Panel 3: T cell activation; Panel 4: B cell activation; Panel 5: T cell activation and exhaustion. LD=Live/dead

Table 4-2: Antibody-metal and antibody-fluorophore information

Target	Conjugate	Clone	Source	Unitless Dilution Used	Final Concentration (ng/mL)
CD19	142Nd	HIB19	DVS	1:100	12.5
CD40L	143Nd	24-31	BioLegend	1:100	2000
CD4	145Nd	RPA-T4	DVS	1:100	20
CD8a	146Nd	RPA-T8	DVS	1:1000	2
CD20	147Sm	2H7	DVS	1:100	30
CD38	148Nd	HIT2	BioLegend	1:100	4000
CD62L	153Eu	DREG-56	DVS	1:200	3.75
CD45	154Sm	HI30	DVS	1:2000	1.25
CD86	156Gd	IT2.2	DVS	1:200	1500
CCR7	159Tb	G043H7	DVS	1:200	25
CD14	160Gd	M5E2	DVS	1:67	30
CD69	162Dy	FN50	DVS	1:1000	2.5
HLA-DR	163Dy	L243	BioLegend	1:100	2000
CD45RO	164Dy	UCHL1	DVS	1:200	25
CD16	165Ho	3G8	DVS	1:200	3.75
CD27	167Er	L128	DVS	1:50	15
CD25	169Tm	2A3	DVS	1:400	1.5
CD3	170Er	UCHT1	DVS	1:133	5.625
CD57	172Yb	HCD57	DVS	1:400	1.875
PD-1	174Yb	EH12.2H7	BioLegend	1:100	1000
CD127	176Yb	A019D5	DVS	1:50	12
Nucleic Acid	191/193Ir	n/a	DVS	1:50	250nM
Live/dead	195Pt	n/a	Enzo Life Sciences	1:500	50uM
CD19	BV711	SJ25C1	BD	1/40	300
CD40L	PE	Trap1	BD	1/200	62.5
CD4	BV605	RPA-T4	BD	1/100	500
CD4	PECy5	RPA-T4	BD	1/200	15
CD8	APCA750	3B5	Invitrogen	1/400	1000
CD8	V450	RPA-T8	BD	1/200	250
CD20	APC	L27	BD	1/200	200
CD38	PE-Cy7	HIT2	eBioScience	1/800	250
CD62-L	PE-Cy5	DREG-56	BD	1/100	60
CD86	PE	2331 (FUN-1)	BD	1/40	250
CCR7	BV421	150503	BD	1/40	625
CD14	PETR	TüK4	Invitrogen	1/4000	25
CD69	PE	FN50	BD	1/100	30
HLA-DR	APCy7	L243	BioLegend	1/100	2000
CD45RO	FITC	UCHL1	BD	1/20	2500
CD45RO	PETR	UCHL1	Beckman Coulter	1/400	50
CD16	PE	B73.1	BD	1/2000	50
CD27	PE	M-T271	BD	1/40	625
CD27	FITC	M-T271	BD	1/400	1250
CD25	FITC	2A3	BD	1/400	25
CD3	PB	UCHT1	BD	1/100	2000
CD3	APC	UCHT1	BioLegend	1/800	600
CD57	FITC	NK-1	BD	1/40	312.5
PD-1	BV421	EH12.2H7	BioLegend	1/100	500
CD127	PE-Cy5.5	R34.34	Beckman Coulter	1/400	1000
Viability	V500	n/a	Invitrogen	1/2000	proprietary

Information for all staining reagents. Unitless dilutions were chosen based on the highest stain index (fluorescently) for each antibody determined by single antibody titrations. Final concentrations were calculated using the concentration of each reagent (specific to Lot #) provided by each source.

Table 4-3: Mass cytometry panel to identify PBMC populations

	Target	Metal	Mass	Cell type
1	CD19*	Neodymium (Nd)	142	B cells
2	**CD40L***	Neodymium (Nd)	143	Activated T cells
3	CD4	Neodymium (Nd)	145	T helper cells
4	CD8*	Neodymium (Nd)	146	Cytotoxic T cells
5	CD20*	Samarium (Sm)	147	B cells
6	**CD38**	Neodymium (Nd)	148	Activated lymphocytes
7	CD62L	Europium (Eu)	153	Activated lymphocytes
8	CD86*	Gadolinium (Gd)	156	Activated lymphocytes
9	CCR7*	Terbium (Tb)	159	T cell memory subsets
10	CD14*	Gadolinium (Gd)	160	Monocytes
11	CD69	Dysprosium (Dy)	162	Activated lymphocytes
12	**HLA-DR**	Dysprosium (Dy)	163	APCs; activated T cells
13	CD45RO	Dysprosium (Dy)	164	T cell memory subsets
14	CD16*	Holmium (Ho)	165	NK cells
15	CD27*	Erbium (Er)	167	T cell memory subsets
16	CD25	Thulium (Tm)	169	Activated lymphocytes
17	CD3	Erbium (Er)	170	T cells
18	CD57*	Ytterbium (Yb)	172	T cell memory subsets
19	**PD-1**	Ytterbium (Yb)	174	Exhausted T cells
20	CD127*	Ytterbium (Yb)	176	T cell memory subsets
21	CD45	Samarium (Sm)	154	White blood cells
22	Nuc acid --Ir	Iridium (Ir)	191/193	DNA+ Cells
23	Cisplatin	Platinum	195	Viable cells

One panel was used to measure 23 parameters using mass cytometry: 21 metal-conjugated antibodies, 1 DNA marker, and 1 viability marker (cisplatin). Bolded antibodies were custom conjugated using MaxPar Metal Conjugation Kits. “Cell type” indicates the type of cell that was identified for correlative purposes with fluorescence cytometry data. Asterisks (*) denote when different clones were used in mass and fluorescence cytometry. Bolded antibodies were custom conjugated to metals using purified antibodies from BioLegend and metal-labeling kits from Fluidigm. Markers #21-23 were not used in direct comparison to similar fluorescence parameters. Conjugates were chosen to minimize crosstalk.

Data processing and statistical analysis

All fluorescence and mass cytometry flow cytometry standard (FCS) files were evaluated using Cytobank software and established methods (Diggins et al., 2015; Kotecha, Krutzik, & Irish, 2010). Data were transformed to arcsinh scales with varying cofactors: mass cytometry cofactors ranged from 15 to 50 while fluorescence cytometry cofactors ranged from 150 to 3,000. Software compensation was applied to all fluorescence cytometry FCS files. For viSNE analysis in Figure 3, 150,000 total cells were analyzed and with equal sampling from each FCS file. 8-parameter viSNE maps were created using the 8 antibodies listed in Panel 3 of Table 4-1. GraphPad Prism (GraphPad, La Jolla, CA, USA) was used to determine statistical correlations between fluorescence and mass cytometry values (Table 4-4).

Table 4-4: Correlations of fluorescence and mass cytometry analysis of percent positive cells for proteins measured on healthy human PBMC

		Spearman rank		Pearson		Frequency detected (fluorescence)		Frequency detected (mass)	
Antibody	Gated off of	ρ (rho)	p	r	p	Mean (*ex vivo*)	Mean (+ SEB)	Mean (*ex vivo*)	Mean (+ SEB)
CD57*	Live CD16+	1.00	0.003	0.99	<0.0001	33.28	n/a	31.18	n/a
CD27	Live CD3+	1.00	0.003	1.00	<0.0001	75.05	n/a	71.25	n/a
CD45RO	Live CD3+	0.94	0.017	0.94	0.005	46.98	n/a	48.64	n/a
CD3	Singlets	0.92	<0.0001	0.92	<0.0001	62.24	39.28	62.36	36.81
CD62L	Live CD3+	0.90	0.0002	0.93	<0.0001	48.60	55.24	41.18	53.16
HLA-DR	Live CD20+	0.88	0.0003	0.87	0.0002	72.74	97.59	74.18	91.46
CD69	Live CD4+CD3+	0.86	0.0006	0.95	<0.0001	1.51	29.84	0.87	36.27
PD-1	Singlets	0.85	0.008	0.72	0.007	9.39	13.72	16.73	14.69
CCR7*	Live CD3+	0.83	0.058	0.96	0.002	59.32	n/a	59.12	n/a
CD38	Live CD8+CD3+	0.83	0.001	0.96	<0.0001	37.64	39.72	24.89	22.67
CD40L*	Singlets	0.81	0.002	0.83	0.0008	0.52	18.47	4.84	18.13
CD25	Live CD4+CD3+	0.80	0.003	0.92	<0.0001	8.58	22.72	3.22	33.93
CD20*	Singlets	0.78	0.004	0.80	0.002	7.33	7.82	6.91	7.29
CD16*	Singlets	0.77	0.100	0.67	0.14	11.22	n/a	9.86	n/a
CD86*	Live CD19+	0.76	0.006	0.97	<0.0001	3.20	77.48	13.06	68.29
CD19*	Singlets	0.73	0.009	0.78	0.0030	6.66	7.46	6.15	9.29
CD4	Live CD3+	0.72	0.01	0.74	0.006	75.31	73.92	63.10	57.25
CD8*	Live CD3+	0.72	0.01	0.79	0.002	15.35	13.55	16.73	12.85
CD27*	Live CD20+	0.69	0.016	0.50	0.09	21.67	11.73	18.17	11.44
CD127*	Live CD3+	0.60	0.240	0.75	0.08	86.05	n/a	50.81	n/a
CD14*	Singlets	0.43	0.420	0.23	0.66	10.51	n/a	11.04	n/a

Antibodies are listed in order of decreasing Spearman's rank correlation coefficient rho (ρ). Asterisks (*) denote when different clones were used in mass and fluorescent cytometry. Bolded antibodies were custom conjugated to metals using purified antibodies from BioLegend and metal-labeling kits from Fluidigm. The starting population of cells was used to determine the percent positive of cells for each protein. N indicates the number of values used in the Spearman rank and Pearson analysis: N was 6 when only *ex vivo* data was used (antibodies solely used in Panel 1 or 2, Table 1); N was 12 when an *ex vivo* and *in vitro* value were used from each subject.

Results

Fluorescence and mass cytometry panels to track T cell identity

Panel design

Five fluorescence cytometry panels currently in use in our laboratory were used to measure 20 well-established cell surface markers chosen to provide a systematic view of T cell activation after SEB stimulation (Table 4-1). Fluorochrome and antibody conjugates were chosen based upon current availability in the laboratory and their compatibility with the BD LSRFortessa at the Vanderbilt Flow Cytometry Shared Resource.

A single mass cytometry panel was developed to measure the same set of 20 surface markers captured by the five fluorescence cytometry panels (Table 4-3). While mass cytometry avoids the severity of channel overlap that affects fluorescence cytometry, 'crosstalk' between channels exists. Crosstalk leads to false signals and must be taken into consideration when designing a panel for mass cytometry and gating cellular populations. The three sources of crosstalk result from variations in abundance sensitivity, isotope purity, and oxide formation (Fluidigm.com "Maxpar Panel Designer User Guide"). These types of crosstalk can contribute to signal in the M±1 and M+16 masses from the dominant signal at mass M. To minimize crosstalk within this panel, four of the 20 antibodies were custom conjugated to metals (Table 4-3, bolded).

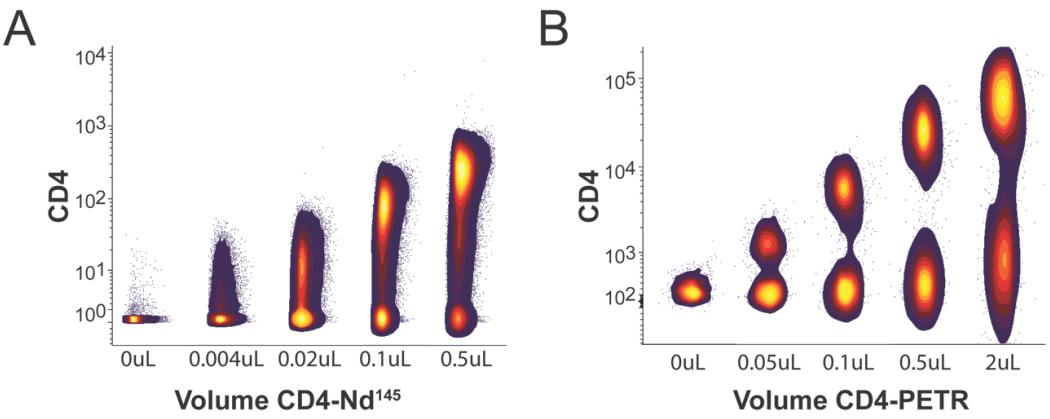
Antibody titrations

Single antibody titrations were performed for all fluorochrome-conjugated antibodies (FCAs) and metal-conjugated antibodies (MCAs) as needed. As an example, Figure 4-1 shows the titration of CD4-Nd^{145} and CD4-PETR (PE-Texas Red). CD4-Nd^{145} was titrated from 0uL to 0.5uL (recommended amount) with DNA intercalator to identify single cells for analysis (Figure 4-1A). The mean mass intensity (MMI) of the CD4-Nd^{145}+

population shifted from 6.63 to 264.48 while the MMI of the CD4-Nd^{145}- population stayed between -0.40 and -0.19 (Figure 4-1A and 4-1C). The standard deviation of the CD4-Nd^{145}- population was always below 1 (Figure 4-1A and 4-1C). With increasing antibody concentrations the frequencies of the CD4-Nd^{145}+ populations increased from 0.18% to 60.73% and stain index values increased from 11.78 to 149.16 (Figure 4-1A and 4-1C).

A single antibody titration was also performed with CD4-PETR from 0uL to 2uL and FSC and SSA properties were used to identify single cells for analysis (Figure 4-1B). The mean fluorescence intensity (MFI) of the CD4-PETR+ population increased from 715.13 to 57736.25 while the CD4-PETR- population shifted from 113.71 to 898.64 (Figure 4-1B and 4-1C). The standard deviation of the CD4-PETR- population ranged between 100-1100 (Figure 4-1B and 4-1C). The frequencies of CD4-PETR detected ranged from 0.56% to 55.42% and the stain index of CD4-PETR ranged from 2.92% up to 31.07% (Figure 4-1B and 4-1C). The highest stain index was achieved at the 0.5uL concentration (Figure 4-1B and 4-1C).

The remaining MCAs and FCAs were conjugated and titrated in a similar manner (Tables 4-1, 4-2, and 4-3). For fluorescence cytometry, single antibody titrations were performed for all FCAs and stain index determined final. For markers not typically expressed on resting cells (e.g. CD69), antibody titrations were performed on SEB-stimulated cells. To optimally titrate antibodies in the mass cytometry panel, final concentrations were chosen based on the frequency of detected populations (considering their fluorescence counterparts), stain index, and crosstalk of each MCA into their M+1, M-1, and M+16 channels. For mass cytometry, custom conjugates were titrated in groups as they were created. PBMC were then stained with the full mass cytometry panel (Table 4-3) at recommended volumes and adjustments were made as needed for each antibody. As needed for antibodies with non-bimodal distributions, additional antibodies were included to determine optimal staining volumes. For example, final adjustments of PD-1 staining volumes were made after plotting PD-1 versus CD45RO on CD4+ T cells (Figure 4-2).



C

Conjugate	Volume (µL)	% CD4+ cells	MMI/MFI (-)	MMI/MFI (+)	SD (-)	Stain index
CD4-Nd^{145}	0	0.18	-0.49	6.63	0.30	11.78
	0.004	13.41	-0.37	7.78	0.53	7.62
	0.02	49.69	-0.27	15.42	0.60	13.04
	0.1	60.46	-0.32	82.97	0.59	70.56
	0.5	60.73	-0.19	264.48	0.89	149.16
CD4-PETR	0	0.56	113.71	715.13	102.95	2.92
	0.05	44.83	128.30	1429.07	106.38	6.11
	0.1	49.78	185.01	5646.98	177.43	15.39
	0.5	51.92	357.86	24078.51	381.68	31.07
	2	55.42	898.64	57736.25	1080.30	26.31

Figure 4-1: Titration of anti-CD4 by mass and fluorescence cytometry. (A) PBMC were stained with Intercalator and different volumes of CD4-Nd^{145}. CD4 staining on single cells (as identified in Figure 4-4B) is shown as detected by mass cytometry. (B) PBMC were stained only with CD4-PETR at indicated volumes. Singlets (identified as in Figure 4-4A) were analyzed for CD4 expression using fluorescence cytometry. (C) The mean mass intensity (MMI) and mean fluorescence intensity (MFI) of cells within CD4- or CD4+ gates are reported. The standard deviation of CD4 intensity in the CD4- gate was also calculated (SD (-)). Stain index was calculated using the following equation: $\frac{MFI(+)-MFI(-)}{2\times SD}$ (or MMI where appropriate).

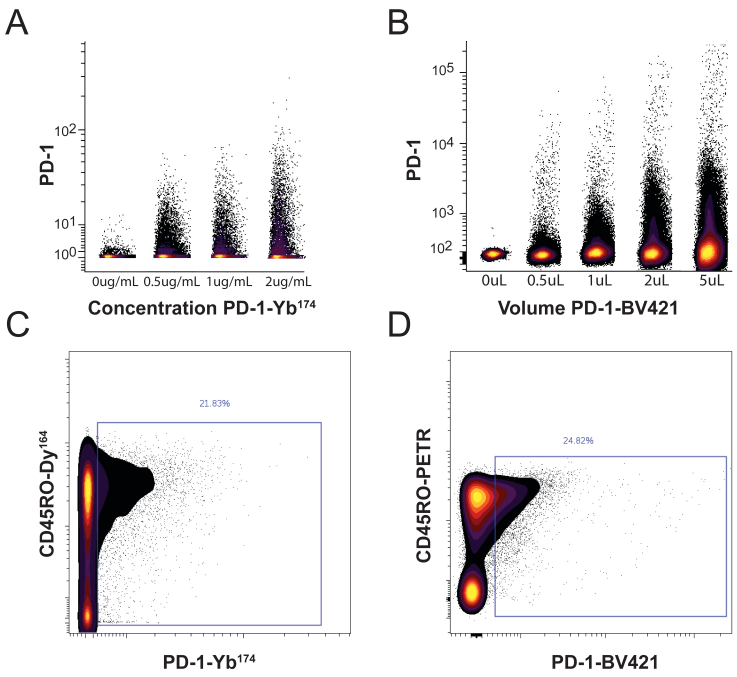


Figure 4-2: Titration of anti-PD-1 by mass and fluorescence cytometry. (A) PD-1-Yb^{174} staining on single cells (as identified in Figure 4-4A). (B) PD-1-BV421 staining on singlets (as identified in Figure 4-4B). (C-D) Since CD45RO helps to gate PD-1+ cells, PBMC were stained with CD3, CD4, CD45RO, and various volumes of PD-1. Optimal staining volumes of PD-1 were chosen based on staining patterns of PD-1 on CD4+ T cells (identified as in Figure 4-4A and 4-4B) using mass (C) and fluorescence (D) cytometry.

Values derived from fluorescence and mass cytometry were closely correlated

Considerations for setting gates in mass cytometry

Several factors were taken into consideration when setting gates on mass cytometry data to ensure that only true signal was being reported. In the absence of background signal, the gate for a particular metal could theoretically be set at 10^0. However, sources of background including nonspecific binding of antibodies and crosstalk from other channels require gates to be set at least at 10^1 since the abundance sensitivity for our instrument is 1% and the MMIs of the antibodies ranged up to 1000 (data not shown, Table 4-4). To help determine where a gate should be set, mass minus one (MMO) controls can be used to ensure that only signal from a single antibody is being detected within a gate. High-density antigens with bimodal staining patterns (MMIs between 10^2 and 10^3) did not require MMOs since the mass intensity of the signal was significantly beyond 10^1. MMOs were especially important, however, when the MCA had a non-bimodal staining pattern, was a dim antigen, and was at the M+1, M-1, or M+16 position of a MCA with an intense, abundant signal.

Figure 4-3 illustrates how gates were set for such an antibody, CD25-Tm^{169}, using a mass minus one (MMO) control. The frequency of Tm^{169}+ events when PBMC were only stained with DNA intercalator and CD4-Nd^{145} was 0.02% when the gate was set at 10^1 (dashed line gate) and 0% when the gate was set at 22 (solid line gate) which represents the actual gate used to identify CD25+ cells (Figure 4-3A). When PBMC were stained with the full panel (Table 4-3) except CD25-Tm^{169} –an MMO control—the frequency of Tm^{169}+ events when the gate was at 10^1 was 2.63% (Figure 4-3B, dashed line gate). This signal results from crosstalk into Tm^{169} by the rest of the panel: most likely from CD3-Er^{170} and CD62L-Eu^{153}. When the gate was set at 22, however, the frequency of non-specific signal was reduced to 0.19% (Figure 4-3B, solid line gate). This gate, which avoided analyzing artifact signals, was used in the full panel to detect true CD25+ events (Figure 4-3C).

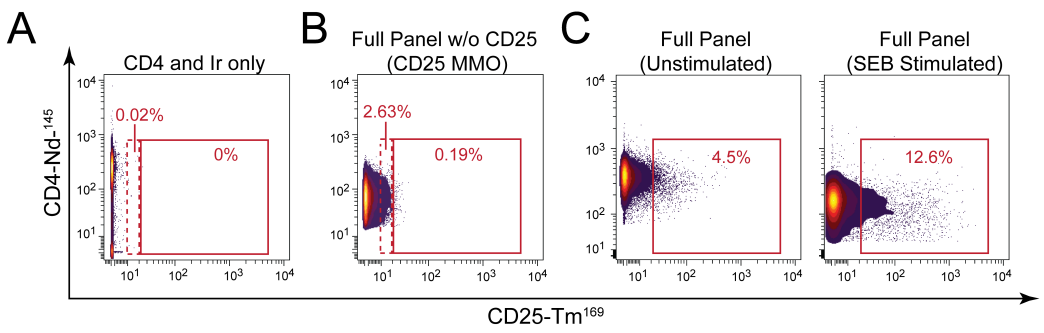


Figure 4-3: MMOs guided gating for CD25 expression on live single CD4+ T cells. (A) PBMC were stained only with DNA intercalator and CD4-Nd^{145}. Single cells were analyzed for CD25 expression. The gate with the dashed red line is set at 10, the same as in (B). The gate with the solid red line is set at 22, the same place as in (B-C) and Figure 4-4. (B) PBMC were stained with all the MCAs (Table 4-3) *except* CD25-Tm^{169} (termed a mass minus one (MMO) control). Gates are set the same as in (A), and frequencies inside each gate represent non-specific signal in the Tm^{169} channel. (C) PBMC were stained with all the MCAs (Table 4-3) with or without SEB stimulation (the same plots from Figure 4-4 are shown).

Final decisions for gating took into consideration many factors including crosstalk, fluorescence/mass counterpart staining, published staining pattern data and frequencies, and staining patterns on multiple cell populations (e.g. evaluating markers present on particular cell types and absent on others). Gates were set for antibodies that were not at the M±1 or M+16 position of another antibody between 1 and 10. Alternatively, when gating on antibodies that were subject to M+16 and/or and M±1 crosstalk gates were set at or beyond 10, preferably with an MMO to indicate its exact placement. MMOs were not always required, however, when antigens are highly-expressed and/or have bimodal staining pattern. These examples further demonstrate the need for careful panel design.

Identification of live single cells

To directly compare mass and fluorescence cytometry, an equivalent starting population of live single cells was identified in healthy human PBMC (Figure 4-4). In fluorescence cytometry (Figure 4-4A), forward and side light scatter signal properties were used to identify intact single cells and exclusion of the LIVE/DEAD Aqua dye identified live single cells. In mass cytometry (Figure 4-4B), event length and intercalator uptake were used to identify intact single cells and exclusion of cisplatin (Fienberg, Simonds, Fantl, Nolan, & Bodenmiller, 2012) identified live single cells. Although PBMC were stained with CD45-Sm^{154} it was not included in analysis since we did not have a comparable marker fluorescently and correlations improved between the two technologies when identical phenotypic gating strategies were used. After single cells were identified, live CD3+ cells and CD4+ and CD8+ T cells were gated by mass and fluorescence cytometry (Figure 4-4A-B). Both mass and fluorescence cytometry measured comparable increases in CD25 expression following SEB stimulation (Figure 4-4C-D).

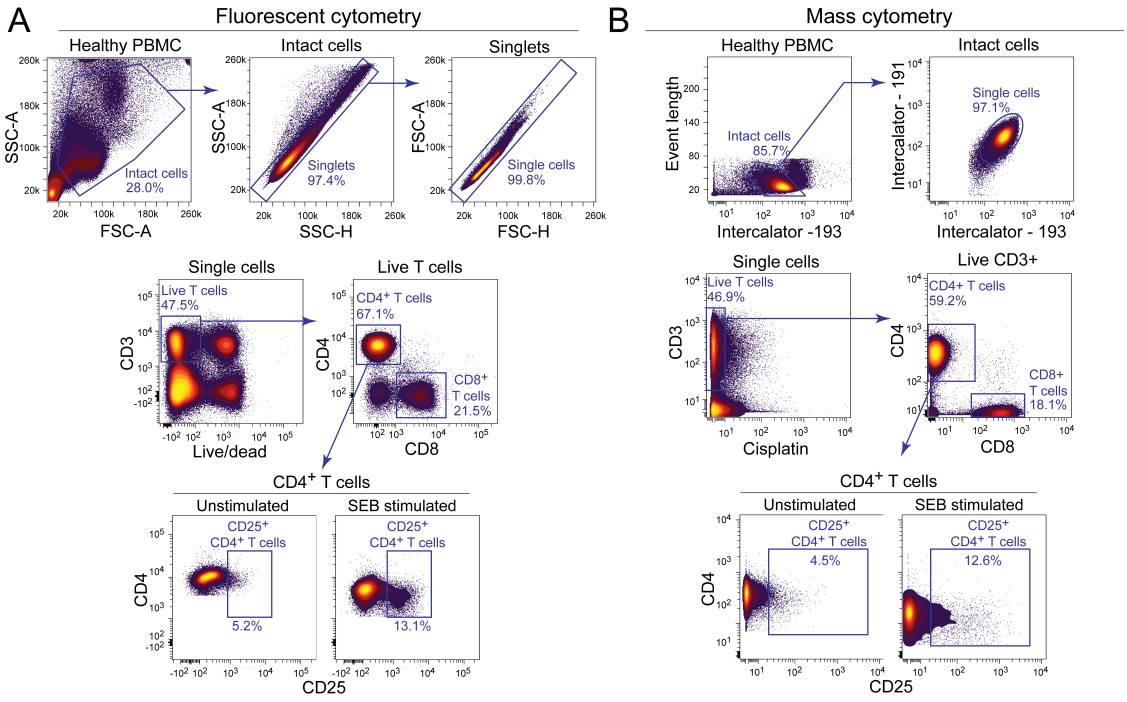


Figure 4-4: Gating schemes for fluorescence and mass cytometry. Plots show PBMC from a single healthy human donor. (A) Representative biaxial plots show the gating scheme for fluorescence flow cytometry. Intact single cells were gated using forward and side scatter area and height properties. Single cells were then assessed for viability and expression of CD3. This population was further gated as $CD8^+$ and $CD4^+$ T cells. CD25 expression on $CD4^+$ T cells was compared in PBMC from an individual healthy donor with or without SEB stimulation. (B) Representative biaxial plots show the gating scheme for mass cytometry. Single cells were identified using event length and intercalator uptake and then gated and compared as for fluorescence cytometry.

Correlation between fluorescence and mass cytometry data

Statistical correlation between fluorescence and mass cytometry was determined using Spearman's rank (Table 4-4) for all 20 measured proteins. Samples analyzed in parallel by mass and fluorescence cytometry included 12 populations of PBMC from 6 individual healthy donors under 2 conditions (unstimulated *ex vivo* and 16 hours after *in vitro* SEB stimulation). Frequencies of cellular populations identified by 20 MCAs and FCAs were directly compared using biaxial gating plots (as in Figure 4-4). The frequency of each antibody was gated from the same starting population, which is indicated in the "gated on" column (Table 4-4). The range of frequencies detected, mean frequencies of unstimulated and stimulated populations, and average intensity of each marker by fluorescence and mass cytometry is indicated (Table 4-4).

Statistically significant correlations were observed for all 9 proteins detected using the same antibody clones (custom or commercially conjugated). Eleven of the metal conjugated antibodies in the mass cytometry panel did not match the FCAs in our existing panels, (Table 4-4, asterisks) but 9 out of 11 of these antibodies still identified similar frequencies of populations by both technologies.

CD14 and CD127 were the only two antibodies that did not provide consistent values between the two technologies. We compared the two CD14 clones (Tük4-PETR and M5E2-FITC) using fluorescence cytometry and found that despite showing bimodal staining patterns they did not detect similar frequencies of CD14+ singlets (N=6, p=0.36, r=0.49). When we compared the two CD127 clones using fluorescence cytometry (R34.34-PECy5.5 and A019D5-PECy7) we found they did in fact detect similar frequencies of CD127+ T cells. Staining improved dramatically with a new lot of CD127-Yb^{176}: the MMI increased from 8 to 65 (data not shown). We then evaluated the frequency of CD127+ T cells in six new subjects and demonstrated a high concordance between the two technologies (N=6, p=0.002, r=0.96). These additional experiments suggest the two discrepancies we found in Table 4-4 were not due to differences in mass and fluorescence

cytometry, but far more likely to be due to differences in the particular antibody clone, or the particular lot of antibody-conjugate used.

viSNE identified similar frequencies of activated CD4+ T cell populations using data from each technology

Large data sets resulting from mass cytometry have resulted in the development of new computational approaches to analyzing complex single cell data. Having found a high degree of correlation between the two technologies, we next compared the ability of an unsupervised high-dimensional analysis program, viSNE (visualization of t-distributed stochastic neighbor embedding algorithm), to analyze data sets derived from both mass and fluorescence cytometry. viSNE was developed for mass cytometry data and approximates high dimensional relationships using a two dimensional scatter plot, or map, where each dot represents a single cell (Amir el et al., 2013). To read a viSNE map, one can visually identify a cluster or 'island' of cells and then determine the cellular identity based on marker expression. To determine whether viSNE could identify similar populations using mass and fluorescence cytometry data sets we chose to study frequencies of activated CD4+ T cells. The viSNE map in Figure 4-5 was generated with an equal number of cells from the FCS files of each healthy donor before and after stimulation. The viSNE analysis was restricted to eight markers of T cell activation and analyzed FCS files stained either with Panel 3 (Table 4-1) or the identical eight markers in the mass cytometry panel (Table 4-3). While cells identified by mass cytometry were tagged with greater than eight antibodies, only the same eight antibodies from the fluorescence T cell activation panel were considered when creating the viSNE maps with mass cytometry data (Figure 4-5B).

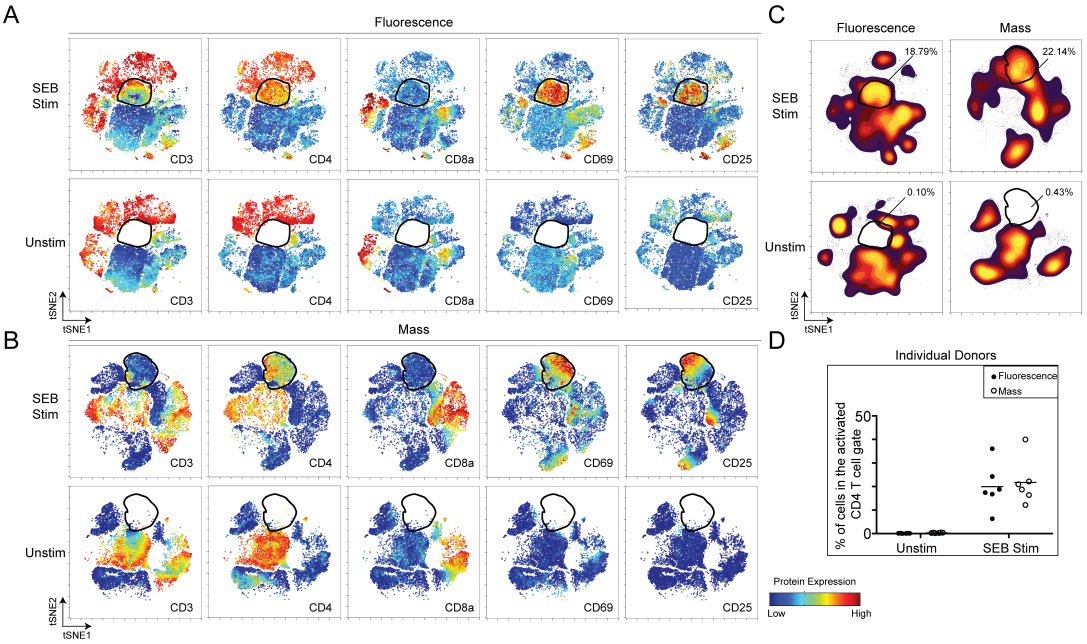


Figure 4-5: viSNE identified activated T cells in 8-dimensional analysis of fluorescence and mass cytometry. (A-B) viSNE plots show unstimulated and SEB stimulated PBMC compared according to 8 proteins (Table 4-3, Panel 3) detected by fluorescence cytometry (A) or mass (B) cytometry. For each cell, color indicates the intensity of the labeled protein on a rainbow heat scale (arcsinh scales). Activated $CD4^+$ T cells ($CD3^{lo/-}CD4^{med}CD8^-CD69^{hi}CD25^{hi}$) are outlined in black. Maps from one representative donor are shown. (C) Maps displaying density of cells from the same donor by fluorescence and mass cytometry highlight the absence of activated cells in the unstimulated condition. (D) SEB induced T cell activation in all 6 individuals, with similar percentages measured by fluorescence and mass cytometry (Wilcoxen matched pairs t-tests p=n.s). Figure created by Alison Greenplate (K. J. Nicholas et al., 2016).

When applied to fluorescence and mass cytometry data, viSNE created similar maps that demonstrated patterns of T cell activation. One fluorescence cytometry viSNE map (Figure 4-5A) and one mass cytometry viSNE map (Figure 4-5B) is each shown ten times highlighting the intensity of CD3, CD4, CD8, CD69 and CD25 on SEB stimulated (top rows) or unstimulated (bottom rows) PBMC. A gate was drawn (black outline) to highlight the population of activated CD4+ T cells. Cells within this island displayed characteristics of activated $CD4^+$ T cells that included little or no CD3, no CD8, moderate CD4, and high expression of CD69 and CD25 (Figure 4-5A-B). The activated cells are not present in the unstimulated condition (Figure 4-5A-B) and this is further highlighted both by the frequency and density of cells within the gate on the viSNE maps (Figure 4-53C). viSNE identified similar frequencies of activated CD4+ T cells analyzed by mass and fluorescence cytometry ($p=0.007$, $\rho=0.74$) and all subjects had cells that fell within this gate after stimulation (Figure 4-5D).

Discussion

The ability to combine high-dimensional single cell biology with unsupervised analysis approaches is powering a new era of systems immunology. Here, a high-dimensional mass cytometry panel was developed evaluate T cell memory and activation. The frequencies of markers detected with 20 antibodies within the mass cytometry panel were compared on a one-to-one basis with antibodies from five fluorescence cytometry panels. The resulting data indicate that mass and fluorescence cytometry data are highly comparable. We also show that unsupervised viSNE analysis provides valuable insight into single cell data, regardless of the instrumentation used to collect that data. The 20 antibody T cell panel developed and validated in this study is expected to be particularly useful for detailed characterization of human T cell populations in a variety of settings such as longitudinal immune monitoring of viral infections, immune disorders, and cancer.

Mass cytometry has the potential to greatly expand the number of observable

features on small populations of cells (Yao et al., 2014). Recent studies achieved 38- and 44-parameter single cell analysis using mass cytometry (Becher et al., 2014; Bendall et al., 2014). Alternatively, the number of measurable parameters using polychromatic fluorescence cytometry has increased to 20 and is growing still with the advent of new instruments and fluorochromes (Chattopadhyay et al., 2006). We previously used multiparameter fluorescence flow cytometry with many of the same markers used in this study to evaluate the activation status of subpopulations of virus-specific T cells in memory compartments of peripheral blood (Conrad et al., 2012b; Meyer-Olson et al., 2006; Meyer-Olson et al., 2010; Simons et al., 2008) and cerebrospinal fluid (Sadagopal et al., 2008; Sadagopal et al., 2010). Here, we used previously established panels in our laboratory that focused on T cell memory and markers of immune activation to provide a detailed comparison of mass and fluorescence cytometry.

A high dimensional mass cytometry approach provided equivalent per-marker and per-subset information when compared directly with traditional fluorescence cytometry (Figure 4-4, Table 4-4). For example, the average difference in CD3+ cells detected by both technologies was 4.8% even though healthy variation before and after stimulation spanned a range of 25-75%. The findings in this study align well with published comparisons of mass and fluorescence cytometry (Behbehani et al., 2012; Bendall et al., 2011; Wang et al., 2012). Other than expected differences due to using different antibody clones, the minor discrepancies observed between the two technologies (Table 4-4) likely resulted from differences in gating for 'live single cells'. Prior to viability gating, fluorescence cytometry employed forward and side scatter while mass cytometry employed DNA content and cell length (Figure 4-4). Overall, these results provide further support for the concordance between the two technologies.

We demonstrate that the high-dimensional visualization tool, viSNE, was still effective even in a 'low-dimensional' 8-parameter analysis. Amir and colleagues demonstrated previously that viSNE successfully identifies blood cell populations even

when using non-canonical markers (Amir el et al., 2013). This ability to detect obscure or unexpected cells is one of the most powerful attributes of new unsupervised analysis programs (Irish, 2014). In this study, viSNE identified activated CD4+ T cells based on their multidimensional phenotypes without requiring cells to express CD3. Additionally, viSNE returned comparable results with mass and fluorescence cytometry data considering 8 parameters, further strengthening the correlations of antibody detection between the two platforms. Going forward, familiarity with these tools, and learning their strengths and weaknesses, is likely to become a core skill for immunologists, especially since they apply well to any type 'event list' format data, such as single cell cytometry data from flow and imaging instruments.

One disadvantage to mass cytometry is that the samples must be destroyed for analysis, so this technology is not suitable for cell sorting. Hence, these should be viewed as complementary technologies. After identification of cell populations of interest with an extensive mass cytometry panel and high dimensional analysis with unsupervised algorithms, more focused fluorescence cytometry panels can be designed to sort cells for further analysis (Conrad et al., 2011; Meyer-Olson et al., 2006; Meyer-Olson et al., 2010; Tricot et al., 2015). For this approach to work, however, it will be necessary to have matched panels of antibodies that can reliably detect the same markers with each technology, and we demonstrate that this is feasible.

This rigorous comparison of mass and fluorescence cytometry suggests that the technologies are highly comparable. Traditional biaxial gating and an unsupervised high-dimensional analysis approach, viSNE, identified similar patterns of protein expression and frequencies of cellular populations in superantigen stimulated human blood. These results demonstrate that multidimensional analysis using either platform will be particularly useful for the comprehensive characterization of cells, including cells with dynamic or unexpected cell phenotypes in health and disease.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

HIV is the causative agent of AIDS, and has caused >34 million deaths globally since its discovery in 1983 (Barre-Sinoussi et al., 1983; Gallo et al., 1984; Popovic et al., 1984). Infection with HIV induces defects of both cellular and humoral immune responses. In this thesis I describe my work studying the frequency, phenotype, and functional response to antigen stimulation of pT_{FH} and B cells in chronically infected HIV+ individuals. Impaired pT_{FH} cell help and B cell dysfunction may partially explain the low frequency of broadly neutralizing antibodies in HIV-infected individuals.

In Chapter II I evaluated the extent of B cell dysfunction during HIV infection by assessing the level of B cell activation at baseline and after stimulation. Increased levels of viremia were associated with higher baseline expression of the activation marker CD86 on B cells and with decreased ability of B cells to increase expression of CD86 after *in vitro* stimulation with inactivated HIV-1 antigen. In a series of cell isolation experiments B cell responses to antigen were enhanced in the presence of autologous CD4+ T cells, demonstrating the importance of CD4+ T cell help for B cell function. HIV infected individuals had a higher frequency of PD-1 expression on B cells compared to HIV- subjects and PD-1 blockade improved B cell responsiveness to HIV antigen, suggesting that inhibitory molecule expression during HIV-1 infection may contribute to some of the observed B cell defects in HIV infection. Our findings demonstrate that during chronic HIV infection, B cells are activated and lose full capacity to respond to antigen, but suppression of inhibitory pressures as well as a robust CD4+ T cell helper response may help preserve B cell function.

In Chapter III I described my studies of pT_{FH} cells in HIV+ individuals. Peripheral T_{FH} cells are a putative circulating counterpart to germinal center T follicular helper (T_{FH}) cells. They show both phenotypic and functional similarities to T_{FH} cells, which provide necessary help for the differentiation of B cells to antibody-secreting plasmablasts. I evaluated the frequency, phenotypes, and responses of peripheral T_{FH}-like (pT_{FH}) cells to superantigen and recall antigen stimulation in 10 healthy and 34 chronically infected treatment-naïve HIV-1+ individuals using fluorescence cytometry. There was no difference in the frequency of pT_{FH} cells between HIV+ and HIV- individuals. Surface expression of ICOS, but not CD40L, was higher on pT_{FH} cells at baseline in HIV+ individuals, suggesting enhanced chronic activation of these cells. Compared to HIV- individuals, pT_{FH} cells from HIV+ individuals had decreased maximal expression of ICOS and CD40L in response to *in vitro* superantigen stimulation. This decreased response did not correlate with viral control, CD4+ T cell count, duration of infection, or the degree of neutralizing antibody breadth. Despite a decreased maximal response, however, pT_{FH} responses to HIV gag and tetanus toxoid recall antigens were preserved.

In Chapter IV, I describe mass cytometry, a new technology that offers the ability to explore cell phenotypes to great depth. I felt it was important to first carefully compare this new technology to the existing standard quantitative single cell flow cytometry approach, fluorescence cytometry. I directly compared mass cytometry with fluorescence cytometry by studying phenotypes of healthy human peripheral blood mononuclear cells (PBMC) in the context of superantigen stimulation. I designed and used one mass cytometry panel and five fluorescence cytometry panels to measure 20 well-established lymphocyte markers of memory and activation. Comparable frequencies of both common and rare cell subpopulations were observed with fluorescence and mass cytometry using biaxial gating. The unsupervised high-dimensional analysis tool viSNE was then used to analyze data sets generated from both mass and fluorescence cytometry. viSNE analysis effectively characterized PBMC using eight features per cell and similar frequencies of activated

CD4+ T cells were identified with both technologies. These results support combinations of unsupervised analysis programs and extended multiparameter cytometry as indispensable tools for detecting perturbations in protein expression in both health and disease.

In summary, this thesis advances our understanding of pT_{FH} and B cells during chronic HIV infection. I found that while B cells were activated at baseline and had high PD-1 expression, antigen responses were improved with CD4+ T cell help and PD-1 blockade. pT_{FH} cells, the best providers of B cell help, had decreased maximal responses to stimulation as measured by expression of CD40L and ICOS, but maintained responses to recall antigens. There are several implications from these findings. The fact that decreased maximal responses did not correlate with any clinical aspects of disease could suggest that pT_{FH} cell dysfunction may occur early in HIV infection. Then chronic persistent activation of pT_{FH} cells in turn activates B cells, leading to the observed skewing of B cell populations and hypergammaglobulinemia in chronic HIV infection. Functional pT_{FH} cell responses to recall antigen existed in HIV+ individuals but also did not correlate with clinical aspects of disease. This maintenance of HIV-specific pT_{FH} cells may simply be a result of HIV viremia driving chronic immune activation. However, the existence of recall tetanus responses suggests that vaccines that elicit protective antibodies may still be effective in HIV+ individuals. Thus, for vaccines designed to elicit antibody responses, adjuvants to optimize T_{FH} cell help may be advantageous. Additionally, pT_{FH} cells should be investigated for a potential role identifying immune correlates of vaccination.

Future Directions

Determine the exhaustion phenotype of pT_{FH} *cells in chronic HIV infection*

High PD-1 on GC T_{FH} cells in healthy individuals indicates tight regulation of T_{FH} cells. A better understanding of inhibitory receptor expression patterns on T_{FH} cells in

healthy and HIV+ individuals will help explore the regulation, and dysregulation, of T_{FH} cells. The immediate next steps I would take, based on my findings in Chapter III, would determine the exhaustion phenotype of pT_{FH} cells in chronic HIV infection using high-dimensional single-cell analysis. In Chapter III I observed that maximal responses of pT_{FH} cells to superantigen stimulation were impaired in HIV+ individuals. I hypothesize that pT_{FH} cells from chronically-infected HIV+ individuals will be more phenotypically exhausted than HIV- individuals and that this will correlate with the decreased ICOS and CD40L on pT_{FH} cells after maximal stimulation. I also observed no correlation between the frequency of pT_{FH} cells and BNAb production. However, there is evidence that the frequency of pT_{FH} cells early in HIV infection correlates with BNAbs (Cohen et al., 2014). Accordingly, I would also hypothesize that pT_{FH} cells that are less exhausted early in HIV infection correlate with BNAbs.

To address this question I identified a cohort of HIV-infected subjects with samples at two time points: within 2 years of infection (T1) and at >2 years after infection (T2) (Figure 5-1A). Plasma samples from these individuals would be used to determine the BNAbs at both time points. Additionally, these individuals can be grouped by those with and without changes in their viral load between T1 and T2 (Figure 5-1B). Thus we will be able to evaluate whether inhibitory expression differs between individuals that do and do not progress in viral load. We will also assess whether pT_{FH} frequency correlates with BNAbs, but it is important to note that BNAbs are not associated with control.

To characterize the activation and exhaustion of pT_{FH} cells in HIV+ individuals, I designed a mass cytometry panel to identify T cell subsets (including pT_{FH} cells) and their activation, exhaustion, trafficking, and memory status (Table 5-1). While the canonical markers of pT_{FH} cells are CXCR5 and PD-1, several groups have published data suggesting additional cell markers should be used such as CXCR3 (Locci et al., 2013), CCR6 (Boswell et al., 2014), and TIGIT+ (Godefroy et al., 2015). The results from this panel will offer the most robust data to-date on the phenotype of pT_{FH} cells in HIV infection.

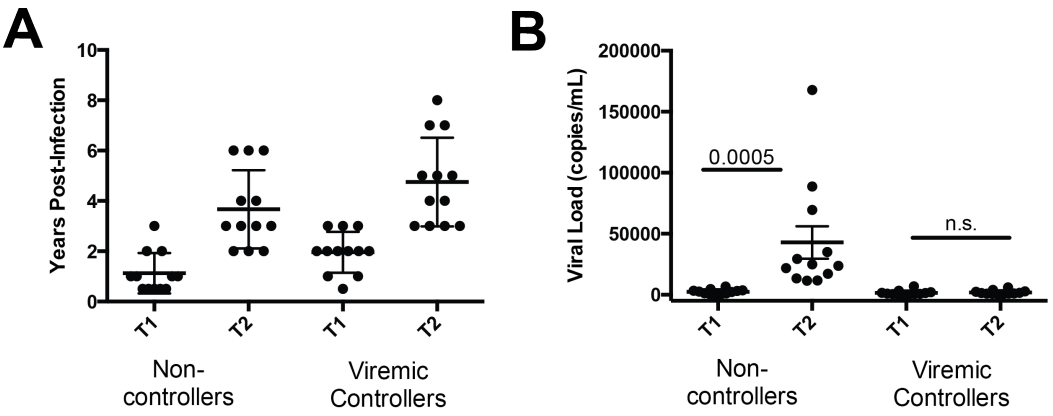


Figure 5-1: Characteristics of HIV-infected individuals for future pT_{FH} and inhibitory receptor studies. (A) Sample time points of non-controllers and viremic controllers. (B) Viral load of non-controllers and viremic controllers at the two time points. T1 = time-point 1; T2 = time-point 2.

Table 5-1: Markers to characterize T cell phenotypes		
	Antibody	**Phenotype identified**
1	**CD3**	*T cell subsets*
2	**CD4**	
3	CD8	
4	CCR7	*T cell memory status*
5	CD45RO	
6	CD127	
7	CD200	*T cell activation*
8	SLAM	
9	CD137	
10	Ki-67	
11	**CD40L**	
12	CD69	
13	CD25	
14	CD38	
15	OX40	
16	**ICOS**	
17	HLA-DR	
18	CD57	*T cell exhaustion*
19	Lag-3	
20	Tim-3	
21	**PD-1**	
22	CD160	
23	BTLA	
24	CTLA-4	
25	2B4	
26	CCR5	*T cell trafficking/CD4 subsets*
27	CCR6	
28	CXCR3	
29	CXCR4	
30	**CXCR5**	
31	CCR4	

31 cell surface proteins to identify T cell subsets, divided by memory, activation, exhaustion, and trafficking markers. Canonical markers used previously in this dissertation to identify pT_{FH} cells are shown in bold.

Table 5-2: Mass cytometry panel to evaluate pT_{FH} cells in HIV infection

	Metal Tag	Tag Isotope #	Target
1	Praseodymium (Pr)	141	CCR6
2	Neodymium (Nd)	142	CD200
3	Neodymium (Nd)	143	CTLA-4
4	Neodymium (Nd)	144	CCR5
5	Neodymium (Nd)	145	CD4
6	Neodymium (Nd)	146	CD8
7	Samarium (Sm)	147	BTLA
8	Samarium (Sm)	148	
9	Samarium (Sm)	149	CCR4
10	Neodymium (Nd)	150	CD137
11	Europium (Eu)	151	ICOS
12	Samarium (Sm)	152	
13	Europium (Eu)	153	CXCR5
14	Samarium (Sm)	154	
15	Gadolinium (Gd)	155	CD160
16	Gadolinium (Gd)	156	CXCR3
17	Gadolinium (Gd)	158	2B4
18	Terbium (Tb)	159	Tim-3
19	Gadolinium (Gd)	160	Lag-3
20	Dysprosium (Dy)	161	SLAM
21	Dysprosium (Dy)	162	CD69
22	Dysprosium (Dy)	163	CD38
23	Dysprosium (Dy)	164	CD45RO
24	Holmium (Ho)	165	
25	Erbium (Er)	166	CD40L
26	Erbium (Er)	167	CCR7
27	Erbium (Er)	168	Ki-67
28	Thulium (Tm)	169	CD25
29	Erbium (Er)	170	CD3
30	Ytterbium (Yb)	171	OX40
31	Ytterbium (Yb)	172	CD57
32	Ytterbium (Yb)	173	CXCR4
33	Ytterbium (Yb)	174	HLA-DR
34	Lutetium (Lu)	175	PD-1
35	Ytterbium (Yb)	176	CD127
36	Iridium (Ir)	191/193	DNA
37	Platinum	195	Dead cells

31 antibodies, DNA intercalator, and cisplatin will be used to determine pT_{FH} cell phenotypes. Channels 148, 152, 154, and 165 will be empty.

I strategically designed an antibody panel comprised of commercially available and custom-made conjugates to optimize CXCR5 staining (Table 5-2). The CXCR5 antibodies commercially available for use with CyTOF had been limited to clones that could detect high but not dim CXCR5 expression. CXCR5 expression is high on B cells but dim on T cells (Figure 5-2, left plot). The Fluorochrome conjugated RF8B2 clone of CXCR5 is commonly used to detect CXCR5 on pT_{FH} cells, and is now available conjugated to Europrium[153]. The measured frequency of CXCR5+CD4+ T cells is similar between mass and fluorescence cytometry (Figure 5-2, two right plots). In the proposed panel CXCR5 is strategically in the optimal range of masses for detection by the CyTOF instrument, but there are no antibodies on the metals ±1 from 153 to allow for ideal detection on the 153 channel.

Due to the nature of the CyTOF instrument, >60% of cells are destroyed before reaching the detector. Using total PBMCs would require a tremendous amount of time to collect data on ~5,000 pT_{FH} cells per sample. Magnetic bead enrichment of CD4+ cell would maximize data from this subset of interest. The cells remaining could be stained with a B cell fluorescence cytometry panel so that pT_{FH} cell and B cell phenotypes can be measured simultaneously (Table 5-3).

To best analyze the large data sets generated on this cohort I would use viSNE analysis to perform unsupervised high-dimensional analysis. As described in Chapter IV of this dissertation, viSNE was developed for mass cytometry data and approximates high-dimensional relationships using a two-dimensional scatter plot, or map, where each dot represents a single cell (Amir el et al., 2013). To read a viSNE map, one can visually identify a cluster or “island” of cells and then determine the cellular identity based on marker expression. We established in the manuscript that viSNE analysis worked well to detect populations of T cells evaluated with mass or fluorescence cytometry (Katherine J. Nicholas et al., 2015). Thus, viSNE will be a powerful tool to identify new subpopulations of pT_{FH} cells and demonstrate expression patterns of activation and exhaustion markers.

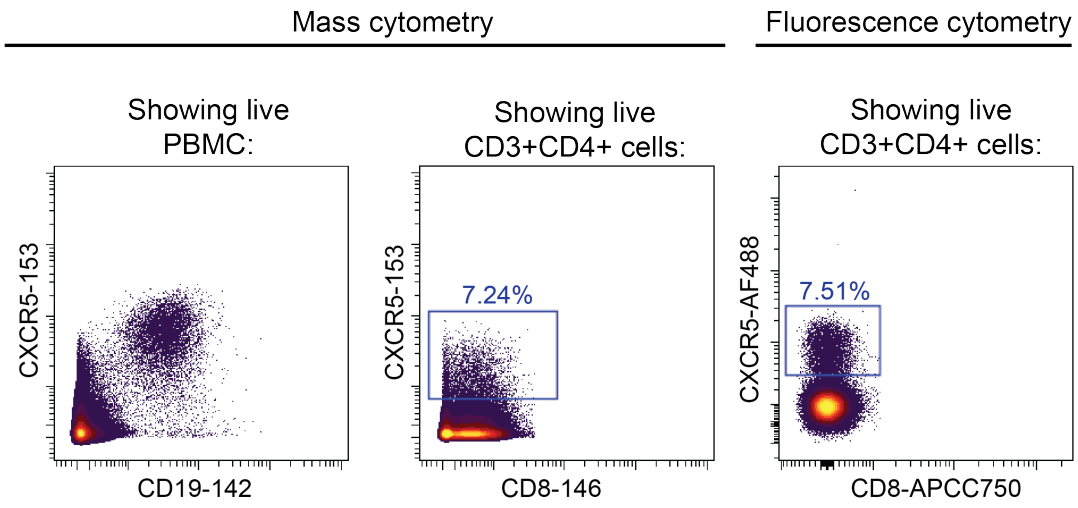


Figure 5-2: CXCR5 RF8B2 clone detects high and dim CXCR5 expression. PBMC were stained *ex vivo* with a cocktail of monoclonal antibodies conjugated to metals (mass cytometry plots) or fluorochromes (fluorescence cytometry plot). Percent indicated within gate is the frequency of CXCR5 expression on CD4+CD3+ cells.

Table 5-3: Fluorescence cytometry panel for B cells

Ex (nm)	Em (nm)	Fluor.	B cell panel
633	750 - 840	APC-Cy7	HLA-DR
	705 - 750	AF700	CD40
	656 - 684	APC	CD20
488	505 - 575	FITC	CD27
405	595 - 630	PETR	CD86
	750 - 840	PE-Cy7	CD38
	567 - 597	PE	BTLA
	685 - 760	BV711	CD19
	505 - 575	Aqua	Live/dead
	410 - 490	BV421	PD-1

Fluorescence cytometry panel designed to measure B cell popululation activation and exhaustion. Ex (excition). Em (emission). Fluor (fluorochrome).

Determine the transcriptional profile of pT_{FH} cells

I would also want to next study the transcriptional profile of pT_{FH} cells in HIV infection. The nCounter Analysis System developed by NanoString is an automated system that can robustly detect transcripts with high sensitivity. This cutting-edge technology uses 2 ~50 base pair fluorescently barcoded probes for each mRNA target and up to 800 transcripts can be detected in each reaction. Panels can be customized to detect transcripts of interest and I would select an Immune Cell Profiling panel. I hypothesize that surface protein expression determined by mass cytometry will correlate with transcript levels intracellularly, however, transcriptome analysis will provide an even more in depth analysis of the heterogeneity of pT_{FH} cell populations in HIV infection. Another benefit from transcriptome analysis is the ability to potentially measure IL-21 and Bcl-6 mRNA, of which the protein forms have been extremely difficult to measure (Cohen et al., 2014; He et al., 2013; Locci et al., 2013; Nurieva et al., 2009; Porichis et al., 2014).

Using pT_{FH} and B cell surface and transcriptional phenotypes to evaluate vaccine correlates of protection

It is well understood that T_{FH} and B cell collaboration initiates hypermutation, affinity maturation, and differentiation of B cells into long-lived plasmablasts and memory B cells, all desirable qualities for a protective immune response. Thus comprehensive studies of circulating B cells and pT_{FH} cells could be key to identifying immune correlates of vaccination. Studies of influenza vaccines demonstrate strong correlations between pT_{FH} cells and vaccine responses (Bentebibel et al., 2013; Herati et al., 2014; Pallikkuth et al., 2012). One study even suggests that simply measuring the frequency of pT_{FH} cells predicted individuals who would make antibodies to a flu vaccine (Herati et al., 2014). Thus, studies to better understand the frequencies, phenotypes, and responsiveness of circulating B cells and pT_{FH} cells in response to vaccines in healthy and HIV+ individuals are prudent.

The fluorescence and mass cytometry panels described above can be applied to samples from vaccine trials to study circulating B cells and pT_{FH} cells. viSNE analysis will be imperative to provide an unbiased analysis to identify non-canonical markers of B and pT_{FH} cell populations that would be overlooked by traditional biaxial gating schemes. We have already applied some of these techniques successfully in a submitted manuscript from Mark Pilkinton in the Kalams laboratory. Dr. Pilkinton studied the frequency and phenotype of circulating plasmablasts and pT_{FH} cells along with serological responses to standard and high doses of the 2010 inactivated influenza vaccine in adults >65 year. I applied viSNE analysis to the data and identified a unique population of pT_{FH} cells that was significantly more frequent in high dose vaccine recipients compared to standard dose recipients (Figure 5-3). This strong pT_{FH} cell activation in response to influenza vaccination predicted seroconversion. The Kalams laboratory is immediately moving forward with similar studies in response to vaccination. I recently designed a mass cytometry panel to identify the phenotype circulating B cells and pT_{FH} cells to evaluate immune responses to an HIV DNA vaccine administered by electroporation by Inovio Pharmaceuticals®.

Understanding B and pT_{FH} cell responses to vaccination will be imperative for future vaccine studies. As subsets of B and pT_{FH} cells are identified by mass cytometry and viSNE analysis, Nanostring technology (described above) can be performed for more in depth study of cell transcriptomes. Studies of these cells that are most important for GC collaboration and optimal antibody production will benefit vaccine studies in several ways: they will help identify immune correlates of protection, help predict and identify individuals less able to respond to standard vaccinations (for example: adults >65 years old or HIV+ individuals), and help direct further research towards optimizing pT_{FH} cell responses. Understanding the phenotype of pT_{FH} cells that best correlates with immune protection from vaccination will prompt studies to understand how to specifically induce that pT_{FH} cell phenotype. The conditions that optimize pT_{FH} cell interactions with B cells should then be tested as vaccine adjuvants.

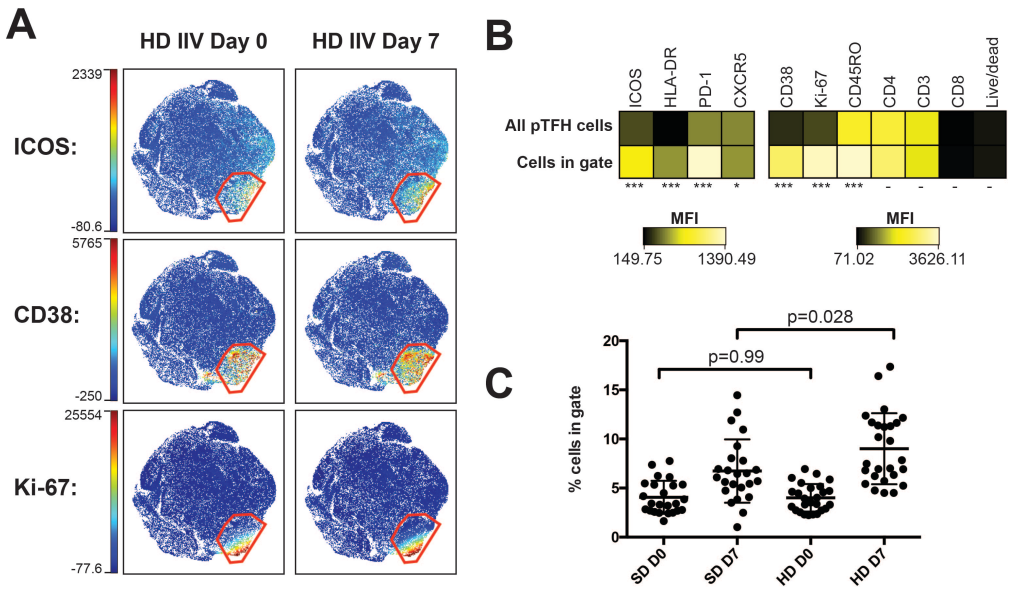


Figure 5-3: viSNE analysis reveals multi-dimensional phenotype of activated pT_{FH} cells that are significantly more frequent in high dose recipients at day 7. (A) viSNE maps displaying live pT_{FH} cells highlight expression of ICOS, CD38, and Ki-67. Intensity scales indicate the range of expression for each marker. Maps display cells from the concatenated files of groups indicated above the plots. The red gate was drawn based on dual CD38 and ICOS expression. (B) A heatmap of the mean fluorescence intensities (MFI) of all the markers for all pT_{FH} cells or cells that fell in the red gate shown in A. Heatmap is shown for high dose recipients at day 7. MFI of each marker on all pT_{FH} cells versus cells in the gate were compared for all individuals at day 7 that received high dose: - p=n.s.; * p=0.02; ***p<0.0001. (C) Statistical analysis of the frequency of cells within the gated population for all individuals. Mann Whiney unpaired t-test used to determine p values.

Determine the relationship between PD-1 expression, signaling, and T_{FH} cell dysfunction

The role of PD-1 as an exhaustion marker on T_{FH} cells in HIV infection is complicated by the fact that PD-1 is also a canonical marker of T_{FH} cells. In healthy individuals, PD-1 curbs unnecessary T cell activation by blocking PI3K activation after prolonged TCR engagement. High PD-1 expression on GC T_{FH} cells should inhibit inappropriate expansion of GC T_{FH} cells, especially in HIV infection where GC B cells express higher than normal levels of PD-L1 (Cubas et al., 2013). However, in HIV infection GC TFH populations are expanded (Cubas et al., 2013; Lindqvist, 2012). T_{FH} cells are usually the limiting factor for B cell maturation in the germinal center, and indeed the expansion of T_{FH} cells in HIV infection is accompanied by an increase in plasma cells and hypergammaglobulinemia (Lindqvist, 2012).

High levels of PD-1 expression on GC T_{FH} cells that fails to inhibit excess T_{FH} expansion despite high levels of PD-L1 on GC B cells, and poor collaboration with B cells, suggest that PD-1 signaling is dysregulated in HIV infection. I propose the next series of experiments should determine how chronic HIV infection affects PD-1 function on T_{FH} cells. Addressing this question will lend valuable insight into the specific role of PD-1 on T_{FH} cells in health and disease.

To study the effects of chronic HIV-1 on PD-1 function on T_{FH} cells, I would sort PD-1^{high} T_{FH} cells and assess T_{FH} cell signaling, proliferation and function. Since GC T_{FH} cells express higher levels of PD-1 compared to pT_{FH} cells, I propose these studies be done with GC T_{FH} cells isolated from tonsil biopsies of healthy controls and untreated HIV+ individuals with a range of viral loads. FACS could be used to sort PD-1^{high} T_{FH} cells from healthy and HIV+ donors, which could then be functionally compared by measuring proliferation (by measuring absolute numbers and dye dilution), expression of activation markers (by fluorescence cytometry), signaling (by phospho-flow), and IL-21 secretion (by ELISA). Specifically, I would measure T_{FH} function in response to stimulation through (1) the TCR (using anti-CD3/anti-CD28), (2) cytokine pathways (using IL-2) and (3) PD-1

(using PD-L1 coated beads). I would also use FACS to sort autologous GC B cells and co-culture them with PD-1^{high} and PD-1^{intermediate} GC T_{FH} cells and measure absolute B cell number and IgG secretion under the same three stimulation conditions. Conditions of decreasing and increasing PD-1 engagement (by PD-L1 blockade or PD-L1 coated beads, respectively) should be used to manipulate the system to best understand the role of PD-1 on GC T_{FH} cells.

I hypothesize that PD-1^{high} T_{FH} cells will respond equally to cytokine and TCR stimulation in both healthy and HIV+ individuals. However, I hypothesize that PD-1 stimulation by PD-L1 beads will fail to block PI3K activation in HIV+ individuals but not healthy controls. Furthermore, I believe that when PD-1^{high} T_{FH} cells are subjected to TCR or cytokine stimulation in the presence (beads) or absence (blockade) of PD-L1, responses will be drastically different between HIV+ and HIV- individuals. Specifically, I expect T_{FH} cells from HIV- controls will respond robustly when PD-L1 is blocked and poorly in the presence of excess PD-L1. This will be in contrast to T_{FH} cells from HIV+ individuals that will display small or no differences in response to stimulation regardless of PD-L1 availability. Additionally, I expect susceptibility to variations in PD-L1/PD-1 engagement to inversely correlate with viral load in HIV+ individuals.

Should there be no difference in T_{FH} responses to PD1-PDL1 engagement in HIV+ individuals, PD-L2 should be investigated. There is recent evidence that PD-L2 can outcompete PD-L1 to engage PD-1 and PD-L2 binding to PD-1 led to an increase in ICOS expression on CD4+ T cells (Karunarathne et al., 2016). As an alternative hypothesis to those listed above, PD-L2 could be overriding the suppressive activity of PD-L1/PD-1 engagement and facilitating expansion of GC T_{FH} cells.

Understanding the mechanisms behind poor quality T_{FH} responses could lead to strategies to improve T_{FH} function (Ma, 2016; Ma & Deenick, 2014; Pillai, 2013; Pissani & Streeck, 2014; Pratama & Vinuesa, 2014; Tangye, Ma, Brink, & Deenick, 2013; Yamamoto et al., 2015). The studies proposed in this section will advance our understanding of the

role of PD-1 expression and function in the regulation of T_{FH} responses and germinal center activity. These results will be of broad significance to studies of chronic viral infections, autoimmune diseases, and cancer.

LIST OF PUBLISHED MANUSCRIPTS

Nicholas KJ, Flaherty DK, Smith RM, Sather DN, Kalams SA. Chronic HIV-1 infection impairs superantigen-induced activation of peripheral CD4+CXCR5+PD-1+ cells, with relative preservation of recall antigen-specific responses. Journal of Acquired Immune Deficiency Syndrome. **2016**. Epub ahead of print Aug 5 2016.

Nicholas KJ, Greenplate AR, Flaherty DK, Matlock BK, Juan JS, Smith RM, Irish JM, Kalams SA. Multiparameter analysis of stimulated human peripheral blood mononuclear cells: A comparison of mass and fluorescence cytometry. Cytometry Part A. **2016,** 89(3): 271–280.

Chukwuma VU, Hicar MD, Chen X, **Nicholas KJ**, Joyner A, Kalams SA, Landucci G, Forthal DN, Spearman PW, Crowe JE Jr. Association of VH4-59 Antibody Variable Gene Usage with Recognition of an Immunodominant Epitope on the HIV-1 Gag Protein. PLoS One. **2015**, 10(7): e0133509.

Nicholas KJ, Zern EK, Barnett L, Smith RM, Lorey SL, Copeland CA, Sadagopal S, Kalams SA. B cell responses to HIV antigen are a potent correlate of viremia in HIV-1 infection and improve with PD-1 blockade. PLoS One. **2013**, 8(12): e84185.

BIBLIOGRAPHY

Agematsu, K., Nagumo, H., Shinozaki, K., Hokibara, S., Yasui, K., Terada, K., . . . Komiyama, A. (1998). Absence of IgD-CD27(+) memory B cell population in X-linked hyper-IgM syndrome. *J Clin Invest, 102*(4), 853-860. doi:10.1172/JCI3409

Akinsiku, O. T., Bansal, A., Sabbaj, S., Heath, S. L., & Goepfert, P. A. (2011). Interleukin-2 production by polyfunctional HIV-1-specific CD8 T cells is associated with enhanced viral suppression. *J Acquir Immune Defic Syndr, 58*(2), 132-140. doi:10.1097/QAI.0b013e318224d2e9

Albert, J., Abrahamsson, B., Nagy, K., Aurelius, E., Gaines, H., Nystrom, G., & Fenyo, E. M. (1990). Rapid development of isolate-specific neutralizing antibodies after primary HIV-1 infection and consequent emergence of virus variants which resist neutralization by autologous sera. *Aids, 4*(2), 107-112.

Allen, C. D., Okada, T., & Cyster, J. G. (2007). Germinal-center organization and cellular dynamics. *Immunity, 27*(2), 190-202. doi:10.1016/j.immuni.2007.07.009

Amir el, A. D., Davis, K. L., Tadmor, M. D., Simonds, E. F., Levine, J. H., Bendall, S. C., . . . Pe'er, D. (2013). viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. *Nat Biotechnol, 31*(6), 545-552. doi:10.1038/nbt.2594

Ansari, M. J., Salama, A. D., Chitnis, T., Smith, R. N., Yagita, H., Akiba, H., . . . Sayegh, M. H. (2003). The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. *J Exp Med, 198*(1), 63-69. doi:10.1084/jem.20022125

Ansel, K. M., McHeyzer-Williams, L. J., Ngo, V. N., McHeyzer-Williams, M. G., & Cyster, J. G. (1999). In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. *J Exp Med, 190*(8), 1123-1134.

Antonelli, L. R., Mahnke, Y., Hodge, J. N., Porter, B. O., Barber, D. L., DerSimonian, R., . . . Sereti, I. (2010). Elevated frequencies of highly activated CD4+ T cells in HIV+ patients developing immune reconstitution inflammatory syndrome. *Blood, 116*(19), 3818-3827. doi:10.1182/blood-2010-05-285080

Aubert, R. D., Kamphorst, A. O., Sarkar, S., Vezys, V., Ha, S. J., Barber, D. L., . . . Ahmed, R. (2011). Antigen-specific CD4 T-cell help rescues exhausted CD8 T cells during chronic viral infection. *Proc Natl Acad Sci U S A, 108*(52), 21182-21187.

Baba, T. W., Liska, V., Hofmann-Lehmann, R., Vlasak, J., Xu, W., Ayehunie, S., . . . Ruprecht, R. M. (2000). Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. *Nat Med, 6*(2), 200-206. doi:10.1038/72309

Bandura, D. R., Baranov, V. I., Ornatsky, O. I., Antonov, A., Kinach, R., Lou, X., . . . Tanner, S. D. (2009). Mass cytometry: technique for real time single cell multitarget immunoassay based on inductively coupled plasma time-of-flight mass spectrometry. *Anal Chem, 81*(16), 6813-6822. doi:10.1021/ac901049w

Barber, D. L., Wherry, E. J., & Ahmed, R. (2003). Cutting edge: rapid in vivo killing by memory CD8 T cells. *J Immunol, 171*(1), 27-31.

Barber, D. L., Wherry, E. J., Masopust, D., Zhu, B., Allison, J. P., Sharpe, A. H., . . . Ahmed, R. (2006). Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature, 439*(7077), 682-687.

Barre-Sinoussi, F., Chermann, J. C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., . . . Montagnier, L. (1983). Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science, 220*(4599), 868-871.

Becher, B., Schlitzer, A., Chen, J., Mair, F., Sumatoh, H. R., Teng, K. W., . . . Poidinger, M. (2014). High-dimensional analysis of the murine myeloid cell system. *15*(12), 1181-1189. doi:10.1038/ni.3006

Behbehani, G. K., Bendall, S. C., Clutter, M. R., Fantl, W. J., & Nolan, G. P. (2012). Single-cell mass cytometry adapted to measurements of the cell cycle. *Cytometry A, 81*(7), 552-566. doi:10.1002/cyto.a.22075

Bendall, S. C., Davis, K. L., Amir el, A. D., Tadmor, M. D., Simonds, E. F., Chen, T. J., . . . Pe'er, D. (2014). Single-cell trajectory detection uncovers progression and regulatory coordination in human B cell development. *Cell, 157*(3), 714-725. doi:10.1016/j.cell.2014.04.005

Bendall, S. C., Nolan, G. P., Roederer, M., & Chattopadhyay, P. K. (2012). A deep profiler's guide to cytometry. *Trends Immunol, 33*(7), 323-332. doi:10.1016/j.it.2012.02.010

Bendall, S. C., Simonds, E. F., Qiu, P., Amir el, A. D., Krutzik, P. O., Finck, R., . . . Nolan, G. P. (2011). Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science, 332*(6030), 687-696. doi:10.1126/science.1198704

Bennett, F., Luxenberg, D., Ling, V., Wang, I. M., Marquette, K., Lowe, D., . . . Carreno, B. M. (2003). Program death-1 engagement upon TCR activation has distinct effects on costimulation and cytokine-driven proliferation: attenuation of ICOS, IL-4, and IL-21, but not CD28, IL-7, and IL-15 responses. *J Immunol, 170*(2), 711-718.

Bentebibel, S. E., Lopez, S., Obermoser, G., Schmitt, N., Mueller, C., Harrod, C., . . . Ueno, H. (2013). Induction of ICOS+CXCR3+CXCR5+ TH cells correlates with antibody responses to influenza vaccination. *Sci Transl Med, 5*(176), 176ra132. doi:10.1126/scitranslmed.3005191

Bentebibel, S. E., Schmitt, N., Banchereau, J., & Ueno, H. (2011). Human tonsil B-cell lymphoma 6 (BCL6)-expressing CD4+ T-cell subset specialized for B-cell help outside germinal centers. *Proc Natl Acad Sci U S A, 108*(33), E488-497.

Betts, M. R., Ambrozak, D. R., Douek, D. C., Bonhoeffer, S., Brenchley, J. M., Casazza, J. P., . . . Picker, L. J. (2001). Analysis of total human immunodeficiency virus (HIV)-specific CD4(+) and CD8(+) T-cell responses: relationship to viral load in untreated HIV infection. *J Virol, 75*(24), 11983-11991. doi:10.1128/JVI.75.24.11983-11991.2001

Betts, M. R., Nason, M. C., West, S. M., De Rosa, S. C., Migueles, S. A., Abraham, J., . . . Koup, R. A. (2006). HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood, 107*(12), 4781-4789. doi:10.1182/blood-2005-12-4818

Blackburn, S. D., Shin, H., Haining, W. N., Zou, T., Workman, C. J., Polley, A., . . . Wherry, E. J. (2009). Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol, 10*(1), 29-37. doi:10.1038/ni.1679

Blankson, J. N. (2011). The study of elite controllers: a pure academic exercise or a potential pathway to an HIV-1 vaccine? *Curr Opin HIV AIDS, 6*(3), 147-150. doi:10.1097/COH.0b013e3283457868

Blish, C. A., Jalalian-Lechak, Z., Rainwater, S., Nguyen, M. A., Dogan, O. C., & Overbaugh, J. (2009). Cross-subtype neutralization sensitivity despite monoclonal antibody resistance among early subtype A, C, and D envelope variants of human immunodeficiency virus type 1. *J Virol, 83*(15), 7783-7788. doi:10.1128/JVI.00673-09

Boliar, S., Murphy, M. K., Tran, T. C., Carnathan, D. G., Armstrong, W. S., Silvestri, G., & Derdeyn, C. A. (2012). B-lymphocyte dysfunction in chronic HIV-1 infection does not prevent cross-clade neutralization breadth. *J Virol, 86*(15), 8031-8040. doi:10.1128/JVI.00771-12

Boswell, K. L., Paris, R., Boritz, E., Ambrozak, D., Yamamoto, T., Darko, S., . . . Koup, R. A. (2014). Loss of circulating CD4 T cells with B cell helper function during chronic HIV infection. *PLoS Pathog, 10*(1), e1003853. doi:10.1371/journal.ppat.1003853

Breitfeld, D., Ohl, L., Kremmer, E., Ellwart, J., Sallusto, F., Lipp, M., & Forster, R. (2000). Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med, 192*(11), 1545-1552.

Brenchley, J. M., Schacker, T. W., Ruff, L. E., Price, D. A., Taylor, J. H., Beilman, G. J., . . . Douek, D. C. (2004). CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med, 200*(6), 749-759. doi:10.1084/jem.20040874

Burton, D. R., Ahmed, R., Barouch, D. H., Butera, S. T., Crotty, S., Godzik, A., . . . Wyatt, R. (2012). A Blueprint for HIV Vaccine Discovery. *Cell Host Microbe, 12*(4), 396-407. doi:10.1016/j.chom.2012.09.008

Burton, D. R., Stanfield, R. L., & Wilson, I. A. (2005). Antibody vs. HIV in a clash of evolutionary titans. *Proc Natl Acad Sci U S A, 102*(42), 14943-14948.

Bussmann, B. M., Reiche, S., Bieniek, B., Krznaric, I., Ackermann, F., & Jassoy, C. (2010). Loss of HIV-specific memory B-cells as a potential mechanism for the dysfunction of the humoral immune response against HIV. *Virology, 397*(1), 7-13. doi:S0042-6822(09)00698-9 [pii]

10.1016/j.virol.2009.11.003

Cao, Y., Qin, L., Zhang, L., Safrit, J., & Ho, D. D. (1995). Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. *N Engl J Med, 332*(4), 201-208. doi:10.1056/NEJM199501263320401

CDC. (1993). 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. *MMWR, 41*(No. RR-17).

CDC. (2015, September 2015). HIV in the United States: At A Glance. Retrieved from http://www.cdc.gov/hiv/statistics/overview/ataglance.html

Chattopadhyay, P. K., Gierahn, T. M., Roederer, M., & Love, J. C. (2014). Single-cell technologies for monitoring immune systems. *Nat Immunol, 15*(2), 128-135. doi:10.1038/ni.2796

Chattopadhyay, P. K., Price, D. A., Harper, T. F., Betts, M. R., Yu, J., Gostick, E., . . . Roederer, M. (2006). Quantum dot semiconductor nanocrystals for immunophenotyping by polychromatic flow cytometry. *Nat Med, 12*(8), 972-977. doi:10.1038/nm1371

Chevalier, M. F., Julg, B., Pyo, A., Flanders, M., Ranasinghe, S., Soghoian, D. Z., . . . Streeck, H. (2011). HIV-1-specific interleukin-21+ CD4+ T cell responses contribute to durable viral control through the modulation of HIV-specific CD8+ T cell function. *J Virol, 85*(2), 733-741.

Chevalier, N., Jarrossay, D., Ho, E., Avery, D. T., Ma, C. S., Yu, D., . . . Mackay, C. R. (2011). CXCR5 expressing human central memory CD4 T cells and their relevance for humoral immune responses. *J Immunol, 186*(10), 5556-5568.

Cohen, K., Altfeld, M., Alter, G., & Stamatatos, L. (2014). Early preservation of CXCR5+ PD-1+ helper T cells and B cell activation predict the breadth of neutralizing antibody responses in chronic HIV-1 infection. *J Virol, 88*(22), 13310-13321. doi:10.1128/JVI.02186-14

Connors, M., Kovacs, J. A., Krevat, S., Gea-Banacloche, J. C., Sneller, M. C., Flanigan, M., . . . Lane, H. C. (1997). HIV infection induces changes in CD4+ T-cell phenotype and depletions within the CD4+ T-cell repertoire that are not immediately restored by antiviral or immune-based therapies. *Nat Med, 3*(5), 533-540.

Conrad, J. A., Ramalingam, R. K., Duncan, C. B., Smith, R. M., Wei, J., Barnett, L., . . . Kalams, S. A. (2012a). Anti-retroviral therapy reduces the magnitude and T cell receptor repertoire diversity of HIV-specific T cell responses without changing T cell clonotype dominance. *J Virol In Press*. doi:10.1128/JVI.06000-11

Conrad, J. A., Ramalingam, R. K., Duncan, C. B., Smith, R. M., Wei, J., Barnett, L., . . . Kalams, S. A. (2012b). Antiretroviral therapy reduces the magnitude and T cell receptor repertoire diversity of HIV-specific T cell responses without changing T cell clonotype dominance. *J Virol, 86*(8), 4213-4221. doi:10.1128/JVI.06000-11

Conrad, J. A., Ramalingam, R. K., Smith, R. M., Barnett, L., Lorey, S. L., Wei, J., . . . Kalams, S. A. (2011). Dominant clonotypes within HIV-specific T cell responses are programmed death-1high and CD127low and display reduced variant cross-reactivity. *J Immunol, 186*(12), 6871-6885. doi:10.4049/jimmunol.1004234

Crawford, A., Angelosanto, J. M., Kao, C., Doering, T. A., Odorizzi, P. M., Barnett, B. E., & Wherry, E. J. (2014). Molecular and Transcriptional Basis of CD4(+) T Cell Dysfunction during Chronic Infection. *Immunity, 40*(2), 289-302. doi:10.1016/j.immuni.2014.01.005

Crotty, S. (2011). Follicular helper CD4 T cells (TFH). *Annu Rev Immunol, 29*, 621-663.

Cubas, R. A., Mudd, J. C., Savoye, A. L., Perreau, M., van Grevenynghe, J., Metcalf, T., . . . Haddad, E. K. (2013). Inadequate T follicular cell help impairs B cell immunity during HIV infection. *Nat Med*. doi:10.1038/nm.3109

Day, C. L., Kaufmann, D. E., Kiepiela, P., Brown, J. A., Moodley, E. S., Reddy, S., . . . Walker, B. D. (2006). PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature, 443*(7109), 350-354. doi:10.1038/nature05115

Deeks, S. G., Schweighardt, B., Wrin, T., Galovich, J., Hoh, R., Sinclair, E., . . . Hecht, F. M. (2006). Neutralizing antibody responses against autologous and heterologous viruses in acute versus chronic human immunodeficiency virus (HIV) infection: evidence for a constraint on the ability of HIV to completely evade neutralizing antibody responses. *J Virol, 80*(12), 6155-6164. doi:10.1128/JVI.00093-06

Deeks, S. G., & Walker, B. D. (2007). Human immunodeficiency virus controllers: mechanisms of durable virus control in the absence of antiretroviral therapy. *Immunity, 27*(3), 406-416. doi:10.1016/j.immuni.2007.08.010

Di Palma, S., & Bodenmiller, B. (2014). Unraveling cell populations in tumors by single-cell mass cytometry. *Curr Opin Biotechnol*. doi:10.1016/j.copbio.2014.07.004

Diggins, K. E., Ferrell, P. B., & Irish, J. M. (2015). Methods for discovery and characterization of cell subsets in high dimensional mass cytometry data. *Methods*. doi:10.1016/j.ymeth.2015.05.008

Doitsh, G., Cavrois, M., Lassen, K. G., Zepeda, O., Yang, Z., Santiago, M. L., . . . Greene, W. C. (2010). Abortive HIV infection mediates CD4 T cell depletion and inflammation in human lymphoid tissue. *Cell, 143*(5), 789-801. doi:10.1016/j.cell.2010.11.001

Dorfman, D. M., Brown, J. A., Shahsafaei, A., & Freeman, G. J. (2006). Programmed death-1 (PD-1) is a marker of germinal center-associated T cells and angioimmunoblastic T-cell lymphoma. *Am J Surg Pathol, 30*(7), 802-810. doi:10.1097/01.pas.0000209855.28282.ce

Doria-Rose, N. A., Klein, R. M., Daniels, M. G., O'Dell, S., Nason, M., Lapedes, A., . . . Connors, M. (2010). Breadth of human immunodeficiency virus-specific neutralizing activity in sera: clustering analysis and association with clinical variables. *J Virol, 84*(3), 1631-1636. doi:10.1128/JVI.01482-09

Easterbrook, P. J. (1994). Non-progression in HIV infection. *Aids, 8*(8), 1179-1182.

Elsaesser, H., Sauer, K., & Brooks, D. G. (2009). IL-21 is required to control chronic viral infection. *Science, 324*(5934), 1569-1572.

Emu, B., Sinclair, E., Favre, D., Moretto, W. J., Hsue, P., Hoh, R., . . . Deeks, S. G. (2005). Phenotypic, functional, and kinetic parameters associated with apparent T-cell control of human immunodeficiency virus replication in individuals with and without antiretroviral treatment. *J Virol, 79*(22), 14169-14178. doi:10.1128/JVI.79.22.14169-14178.2005

Eto, D., Lao, C., DiToro, D., Barnett, B., Escobar, T. C., Kageyama, R., . . . Crotty, S. (2011). IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. *PLoS One, 6*(3), e17739. doi:10.1371/journal.pone.0017739

Fauci, A. S., & Desrosiers, R. C. (1997). Pathogenesis of HIV and SIV. In J. M. Coffin, S. H. Hughes, & H. E. Varmus (Eds.), *Retroviruses*. Cold Spring Harbor (NY).

Ferrando-Martinez, S., Casazza, J. P., Leal, M., Machmach, K., Munoz-Fernandez, M. A., Viciana, P., . . . Ruiz-Mateos, E. (2012). Differential Gag-Specific Polyfunctional T Cell Maturation Patterns in HIV-1 Elite Controllers. *J Virol, 86*(7), 3667-3674.

Ferre, A. L., Hunt, P. W., McConnell, D. H., Morris, M. M., Garcia, J. C., Pollard, R. B., . . . Shacklett, B. L. (2010). HIV controllers with HLA-DRB1*13 and HLA-DQB1*06 alleles have strong, polyfunctional mucosal CD4+ T-cell responses. *J Virol, 84*(21), 11020-11029. doi:10.1128/JVI.00980-10

Fienberg, H. G., Simonds, E. F., Fantl, W. J., Nolan, G. P., & Bodenmiller, B. (2012). A platinum-based covalent viability reagent for single-cell mass cytometry. *Cytometry A, 81*(6), 467-475. doi:10.1002/cyto.a.22067

Finzi, D., Blankson, J., Siliciano, J. D., Margolick, J. B., Chadwick, K., Pierson, T., . . . Siliciano, R. F. (1999). Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. *Nat Med, 5*(5), 512-517. doi:10.1038/8394

Forster, R., Mattis, A. E., Kremmer, E., Wolf, E., Brem, G., & Lipp, M. (1996). A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell, 87*(6), 1037-1047.

Forster, R., Schweigard, G., Johann, S., Emrich, T., Kremmer, E., Nerl, C., & Lipp, M. (1997). Abnormal expression of the B-cell homing chemokine receptor BLR1 during the progression of acquired immunodeficiency syndrome. *Blood, 90*(2), 520.

Freeman, G. J., Long, A. J., Iwai, Y., Bourque, K., Chernova, T., Nishimura, H., . . . Honjo, T. (2000). Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med, 192*(7), 1027-1034.

Freeman, G. J., & Sharpe, A. H. (2012). A new therapeutic strategy for malaria: targeting T cell exhaustion. *Nat Immunol, 13*(2), 113-115. doi:10.1038/ni.2211

Freeman, G. J., Wherry, E. J., Ahmed, R., & Sharpe, A. H. (2006). Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade. *J Exp Med, 203*(10), 2223-2227. doi:10.1084/jem.20061800

Frost, S. D., Wrin, T., Smith, D. M., Kosakovsky Pond, S. L., Liu, Y., Paxinos, E., . . . Richman, D. D. (2005). Neutralizing antibody responses drive the evolution of human immunodeficiency virus type 1 envelope during recent HIV infection. *Proc Natl Acad Sci U S A, 102*(51), 18514-18519. doi:10.1073/pnas.0504658102

Gallo, R. C., Salahuddin, S. Z., Popovic, M., Shearer, G. M., Kaplan, M., Haynes, B. F., . . . et al. (1984). Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science, 224*(4648), 500-503.

Gaudilliere, B., Ganio, E. A., Tingle, M., Lancero, H. L., Fragiadakis, G. K., Baca, Q. J., . . . Angst, M. S. (2015). Implementing Mass Cytometry at the Bedside to Study the Immunological Basis of Human Diseases: Distinctive Immune Features in Patients with a History of Term or Preterm Birth. *Cytometry A*. doi:10.1002/cyto.a.22720

Godefroy, E., Zhong, H., Pham, P., Friedman, D., & Yazdanbakhsh, K. (2015). TIGIT-positive circulating follicular helper T cells display robust B-cell help functions: potential role in sickle cell alloimmunization. *Haematologica, 100*(11), 1415-1425. doi:10.3324/haematol.2015.132738

Golden-Mason, L., Palmer, B. E., Kassam, N., Townshend-Bulson, L., Livingston, S., McMahon, B. J., . . . Rosen, H. R. (2009). Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. *J Virol, 83*(18), 9122-9130. doi:10.1128/JVI.00639-09

Good-Jacobson, K. L., Szumilas, C. G., Chen, L., Sharpe, A. H., Tomayko, M. M., & Shlomchik, M. J. (2010). PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat Immunol, 11*(6), 535-542. doi:10.1038/ni.1877

Grabar, S., Selinger-Leneman, H., Abgrall, S., Pialoux, G., Weiss, L., & Costagliola, D. (2009). Prevalence and comparative characteristics of long-term nonprogressors and HIV controller patients in the French Hospital Database on HIV. *Aids, 23*(9), 1163-1169. doi:10.1097/QAD.0b013e32832b44c8

Gray, E. S., Madiga, M. C., Hermanus, T., Moore, P. L., Wibmer, C. K., Tumba, N. L., . . . Team, C. S. (2011). The neutralization breadth of HIV-1 develops incrementally over four years and is associated with CD4+ T cell decline and high viral load during acute infection. *J Virol, 85*(10), 4828-4840. doi:10.1128/JVI.00198-11

Gray, E. S., Taylor, N., Wycuff, D., Moore, P. L., Tomaras, G. D., Wibmer, C. K., . . . Morris, L. (2009). Antibody specificities associated with neutralization breadth in plasma from human immunodeficiency virus type 1 subtype C-infected blood donors. *J Virol, 83*(17), 8925-8937. doi:10.1128/JVI.00758-09

Guadalupe, M., Reay, E., Sankaran, S., Prindiville, T., Flamm, J., McNeil, A., & Dandekar, S. (2003). Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. *J Virol, 77*(21), 11708-11717.

Ha, S. J., Mueller, S. N., Wherry, E. J., Barber, D. L., Aubert, R. D., Sharpe, A. H., . . . Ahmed, R. (2008). Enhancing therapeutic vaccination by blocking PD-1-mediated

inhibitory signals during chronic infection. *J Exp Med, 205*(3), 543-555. doi:jem.20071949 [pii]

10.1084/jem.20071949

Hamid, O., Robert, C., Daud, A., Hodi, F. S., Hwu, W. J., Kefford, R., . . . Ribas, A. (2013). Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *N Engl J Med, 369*(2), 134-144. doi:10.1056/NEJMoa1305133

Hams, E., McCarron, M. J., Amu, S., Yagita, H., Azuma, M., Chen, L., & Fallon, P. G. (2011). Blockade of B7-H1 (programmed death ligand 1) enhances humoral immunity by positively regulating the generation of T follicular helper cells. *J Immunol, 186*(10), 5648-5655.

Harari, A., Petitpierre, S., Vallelian, F., & Pantaleo, G. (2004). Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1-infected subjects with progressive disease: changes after antiretroviral therapy. *Blood, 103*(3), 966-972. doi:10.1182/blood-2003-04-1203

Hardy, A. M. (1991). Characterization of long-term survivors of acquired immunodeficiency syndrome. The Long-term Survivor Collaborative Study Group. *J Acquir Immune Defic Syndr, 4*(4), 386-391.

Havenar-Daughton, C., Lindqvist, M., Heit, A., Wu, J. E., Reiss, S. M., Kendric, K., . . . Crotty, S. (2016). CXCL13 is a plasma biomarker of germinal center activity. *Proc Natl Acad Sci U S A*. doi:10.1073/pnas.1520112113

Haynes, B. F., Kelsoe, G., Harrison, S. C., & Kepler, T. B. (2012). B-cell-lineage immunogen design in vaccine development with HIV-1 as a case study. *Nat Biotechnol, 30*(5), 423-433. doi:10.1038/nbt.2197

Haynes, N. M., Allen, C. D., Lesley, R., Ansel, K. M., Killeen, N., & Cyster, J. G. (2007). Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. *J Immunol, 179*(8), 5099-5108.

He, J., Tsai, L. M., Leong, Y. A., Hu, X., Ma, C. S., Chevalier, N., . . . Yu, D. (2013). Circulating precursor CCR7(lo)PD-1(hi) CXCR5(+) CD4(+) T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. *Immunity, 39*(4), 770-781. doi:10.1016/j.immuni.2013.09.007

Hellerstein, M., Hanley, M. B., Cesar, D., Siler, S., Papageorgopoulos, C., Wieder, E., . . . McCune, J. M. (1999). Directly measured kinetics of circulating T lymphocytes in normal and HIV-1-infected humans. *Nat Med, 5*(1), 83-89. doi:10.1038/4772

Herati, R. S., Reuter, M. A., Dolfi, D. V., Mansfield, K. D., Aung, H., Badwan, O. Z., . . . Wherry, E. J. (2014). Circulating CXCR5+PD-1+ response predicts influenza vaccine antibody responses in young adults but not elderly adults. *J Immunol, 193*(7), 3528-3537. doi:10.4049/jimmunol.1302503

Hessell, A. J., Poignard, P., Hunter, M., Hangartner, L., Tehrani, D. M., Bleeker, W. K., . . . Burton, D. R. (2009). Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques. *Nat Med, 15*(8), 951-954. doi:10.1038/nm.1974

Hessell, A. J., Rakasz, E. G., Poignard, P., Hangartner, L., Landucci, G., Forthal, D. N., . . . Burton, D. R. (2009). Broadly neutralizing human anti-HIV antibody 2G12 is effective in protection against mucosal SHIV challenge even at low serum neutralizing titers. *PLoS Pathog, 5*(5), e1000433. doi:10.1371/journal.ppat.1000433

Hirano, F., Kaneko, K., Tamura, H., Dong, H., Wang, S., Ichikawa, M., . . . Chen, L. (2005). Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Cancer Res, 65*(3), 1089-1096.

Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, W., Leonard, J. M., & Markowitz, M. (1995). Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature, 373*(6510), 123-126. doi:10.1038/373123a0

Hokey, D. A., Johnson, F. B., Smith, J., Weber, J. L., Yan, J., Hirao, L., . . . Weiner, D. B. (2008). Activation drives PD-1 expression during vaccine-specific proliferation and following lentiviral infection in macaques. *Eur J Immunol, 38*(5), 1435-1445. doi:10.1002/eji.200737857

Hong, J. J., Amancha, P. K., Rogers, K., Ansari, A. A., & Villinger, F. (2012). Spatial alterations between CD4(+) T follicular helper, B, and CD8(+) T cells during simian immunodeficiency virus infection: T/B cell homeostasis, activation, and potential mechanism for viral escape. *J Immunol, 188*(7), 3247-3256. doi:10.4049/jimmunol.1103138

Horowitz, A., Strauss-Albee, D. M., Leipold, M., Kubo, J., Nemat-Gorgani, N., Dogan, O. C., . . . Blish, C. A. (2013). Genetic and environmental determinants of human NK cell diversity revealed by mass cytometry. *Sci Transl Med, 5*(208), 208ra145. doi:10.1126/scitranslmed.3006702

Hubert, J. B., Burgard, M., Dussaix, E., Tamalet, C., Deveau, C., Le Chenadec, J., . . . Rouzioux. (2000). Natural history of serum HIV-1 RNA levels in 330 patients with a known date of infection. The SEROCO Study Group. *Aids, 14*(2), 123-131.

Hunt, P. W., Landay, A. L., Sinclair, E., Martinson, J. A., Hatano, H., Emu, B., . . . Deeks, S. G. (2011). A low T regulatory cell response may contribute to both viral control and generalized immune activation in HIV controllers. *PLoS One, 6*(1), e15924. doi:10.1371/journal.pone.0015924

Hutloff, A., Dittrich, A. M., Beier, K. C., Eljaschewitsch, B., Kraft, R., Anagnostopoulos, I., & Kroczek, R. A. (1999). ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature, 397*(6716), 263-266. doi:10.1038/16717

Irish, J. M. (2014). Beyond the age of cellular discovery. *Nat Immunol, 15*(12), 1095-1097. doi:10.1038/ni.3034

Irish, J. M., Czerwinski, D. K., Nolan, G. P., & Levy, R. (2006). Kinetics of B cell receptor signaling in human B cell subsets mapped by phosphospecific flow cytometry. *J Immunol, 177*(3), 1581-1589.

Irish, J. M., & Doxie, D. B. (2014). High-dimensional single-cell cancer biology. *Curr Top Microbiol Immunol, 377*, 1-21. doi:10.1007/82_2014_367

Johnston, R. J., Poholek, A. C., DiToro, D., Yusuf, I., Eto, D., Barnett, B., . . . Crotty, S. (2009). Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science, 325*(5943), 1006-1010. doi:10.1126/science.1175870

Jones, R. B., Ndhlovu, L. C., Barbour, J. D., Sheth, P. M., Jha, A. R., Long, B. R., . . . Ostrowski, M. A. (2008). Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *J Exp Med, 205*(12), 2763-2779. doi:10.1084/jem.20081398

Kalams, S. A., Buchbinder, S. P., Rosenberg, E. S., Billingsley, J. M., Colbert, D. S., Jones, N. G., . . . Walker, B. D. (1999). Association between virus-specific cytotoxic T-lymphocyte and helper responses in human immunodeficiency virus type 1 infection. *J Virol, 73*(8), 6715-6720.

Kalams, S. A., Parker, S. D., Elizaga, M., Metch, B., Edupuganti, S., Hural, J., . . . Network, N. H. V. T. (2013). Safety and comparative immunogenicity of an HIV-1 DNA vaccine in combination with plasmid interleukin 12 and impact of intramuscular electroporation for delivery. *J Infect Dis, 208*(5), 818-829. doi:10.1093/infdis/jit236

Kardava, L., Moir, S., Wang, W., Ho, J., Buckner, C. M., Posada, J. G., . . . Fauci, A. S. (2011). Attenuation of HIV-associated human B cell exhaustion by siRNA downregulation of inhibitory receptors. *J Clin Invest, 121*(7), 2614-2624.

Karunarathne, D. S., Horne-Debets, J. M., Huang, J. X., Faleiro, R., Leow, C. Y., Amante, F., . . . Wykes, M. N. (2016). Programmed Death-1 Ligand 2-Mediated Regulation of the PD-L1 to PD-1 Axis Is Essential for Establishing CD4(+) T Cell Immunity. *Immunity, 45*(2), 333-345. doi:10.1016/j.immuni.2016.07.017

Kassu, A., Marcus, R. A., D'Souza, M. B., Kelly-McKnight, E. A., Golden-Mason, L., Akkina, R., . . . Palmer, B. E. (2010). Regulation of virus-specific CD4+ T cell function by multiple costimulatory receptors during chronic HIV infection. *J Immunol, 185*(5), 3007-3018. doi:10.4049/jimmunol.1000156

Katzenstein, T. L., Pedersen, C., Nielsen, C., Lundgren, J. D., Jakobsen, P. H., & Gerstoft, J. (1996). Longitudinal serum HIV RNA quantification: correlation to viral phenotype at seroconversion and clinical outcome. *Aids, 10*(2), 167-173.

Kaufmann, D. E., Bailey, P. M., Sidney, J., Wagner, B., Norris, P. J., Johnston, M. N., . . . Altfeld, M. (2004). Comprehensive analysis of human immunodeficiency virus type 1-specific CD4 responses reveals marked immunodominance of gag and nef and the presence of broadly recognized peptides. *J Virol, 78*(9), 4463.

Kaufmann, D. E., Kavanagh, D. G., Pereyra, F., Zaunders, J. J., Mackey, E. W., Miura, T., . . . Walker, B. D. (2007). Upregulation of CTLA-4 by HIV-specific CD4+ T cells correlates with disease progression and defines a reversible immune dysfunction. *Nat Immunol, 8*(11), 1246-1254. doi:10.1038/ni1515

Khaitan, A., & Unutmaz, D. (2011). Revisiting immune exhaustion during HIV infection. *Curr HIV/AIDS Rep, 8*(1), 4-11.

Kiepiela, P., Ngumbela, K., Thobakgale, C., Ramduth, D., Honeyborne, I., Moodley, E., . . . Goulder, P. (2007). CD8+ T-cell responses to different HIV proteins have

discordant associations with viral load. *Nat Med, 13*(1), 46-53. doi:10.1038/nm1520

Kim, C. H., Rott, L. S., Clark-Lewis, I., Campbell, D. J., Wu, L., & Butcher, E. C. (2001). Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. *J Exp Med, 193*(12), 1373-1381.

Klatt, N. R., Vinton, C. L., Lynch, R. M., Canary, L. A., Ho, J., Darrah, P. A., . . . Brenchley, J. M. (2011). SIV infection of rhesus macaques results in dysfunctional T- and B-cell responses to neo and recall Leishmania major vaccination. *Blood, 118*(22), 5803-5812. doi:10.1182/blood-2011-07-365874

Klein, F., Diskin, R., Scheid, J. F., Gaebler, C., Mouquet, H., Georgiev, I. S., . . . Nussenzweig, M. C. (2013). Somatic mutations of the immunoglobulin framework are generally required for broad and potent HIV-1 neutralization. *Cell, 153*(1), 126-138. doi:10.1016/j.cell.2013.03.018

Kotecha, N., Krutzik, P. O., & Irish, J. M. (2010). Web-based analysis and publication of flow cytometry experiments. *Curr Protoc Cytom, Chapter 10*, Unit10 17. doi:10.1002/0471142956.cy1017s53

Kwong, P. D., & Mascola, J. R. (2012). Human antibodies that neutralize HIV-1: identification, structures, and B cell ontogenies. *Immunity, 37*(3), 412-425. doi:10.1016/j.immuni.2012.08.012

Kwong, P. D., Mascola, J. R., & Nabel, G. J. (2013). Broadly neutralizing antibodies and the search for an HIV-1 vaccine: the end of the beginning. *Nat Rev Immunol, 13*(9), 693-701. doi:10.1038/nri3516

Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J., & Hendrickson, W. A. (1998). Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature, 393*(6686), 648-659. doi:10.1038/31405

Lambotte, O., Boufassa, F., Madec, Y., Nguyen, A., Goujard, C., Meyer, L., . . . Delfraissy, J. F. (2005). HIV controllers: a homogeneous group of HIV-1-infected patients with spontaneous control of viral replication. *Clin Infect Dis, 41*(7), 1053-1056.

Lane, H., Masur, H., Edgar, L., Whalen, G., Rook, A., & Fauci, A. (1983). Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. *New England Journal of Medicine, 309*(8), 453-458.

Lane, H. C., Masur, H., Edgar, L. C., Whalen, G., Rook, A. H., & Fauci, A. S. (1983). Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. *N Engl J Med, 309*(8), 453-458. doi:10.1056/NEJM198308253090803

Lange, C. G., Lederman, M. M., Medvik, K., Asaad, R., Wild, M., Kalayjian, R., & Valdez, H. (2003). Nadir CD4+ T-cell count and numbers of CD28+ CD4+ T-cells predict functional responses to immunizations in chronic HIV-1 infection. *Aids, 17*(14), 2015-2023. doi:10.1097/01.aids.0000076312.76477.b5

Lefrere, J. J., Morand-Joubert, L., Mariotti, M., Bludau, H., Burghoffer, B., Petit, J. C., & Roudot-Thoraval, F. (1997). Even individuals considered as long-term nonprogressors show biological signs of progression after 10 years of human immunodeficiency virus infection. *Blood, 90*(3), 1133-1140.

Lefrere, J. J., Roudot-Thoraval, F., Mariotti, M., Thauvin, M., Lerable, J., Salpetrier, J., & Morand-Joubert, L. (1998). The risk of disease progression is determined during the first year of human immunodeficiency virus type 1 infection. *J Infect Dis, 177*(6), 1541-1548.

Levy, J. A. (1993). HIV pathogenesis and long-term survival. *Aids, 7*(11), 1401-1410.

Li, F., Malhotra, U., Gilbert, P. B., Hawkins, N. R., Duerr, A. C., McElrath, J. M., . . . Self, S. G. (2006). Peptide selection for human immunodeficiency virus type 1 CTL-based vaccine evaluation. *Vaccine, 24*(47-48), 6893-6904. doi:10.1016/j.vaccine.2006.06.009

Li, M., Gao, F., Mascola, J. R., Stamatatos, L., Polonis, V. R., Koutsoukos, M., . . . Montefiori, D. C. (2005). Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. *J Virol, 79*(16), 10108-10125. doi:10.1128/JVI.79.16.10108-10125.2005

Li, Q., Duan, L., Estes, J. D., Ma, Z. M., Rourke, T., Wang, Y., . . . Haase, A. T. (2005). Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells. *Nature, 434*(7037), 1148-1152. doi:10.1038/nature03513

Li, Y., Migueles, S. A., Welcher, B., Svehla, K., Phogat, A., Louder, M. K., . . . Mascola, J. R. (2007). Broad HIV-1 neutralization mediated by CD4-binding site antibodies. *Nat Med, 13*(9), 1032-1034. doi:10.1038/nm1624

Lifson, A. R., Buchbinder, S. P., Sheppard, H. W., Mawle, A. C., Wilber, J. C., Stanley, M., . . . Holmberg, S. D. (1991). Long-term human immunodeficiency virus infection in asymptomatic homosexual and bisexual men with normal CD4+ lymphocyte counts: immunologic and virologic characteristics. *J Infect Dis, 163*(5), 959-965.

Lindqvist, M., et al. (2012). Expansion of HIV-specific T follicular helper cells in chronic HIV infection. *The Journal of Clinical Investigation, 122*(9), 3271-3280.

Linterman, M. A., Beaton, L., Yu, D., Ramiscal, R. R., Srivastava, M., Hogan, J. J., . . . Vinuesa, C. G. (2010). IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *J Exp Med, 207*(2), 353-363. doi:10.1084/jem.20091738

Locci, M., Havenar-Daughton, C., Landais, E., Wu, J., Kroenke, M. A., Arlehamn, C. L., . . . Crotty, S. (2013). Human Circulating PD-1CXCR3CXCR5 Memory Tfh Cells Are Highly Functional and Correlate with Broadly Neutralizing HIV Antibody Responses. *Immunity*. doi:10.1016/j.immuni.2013.08.031

Long, E. O. (1999). Regulation of immune responses through inhibitory receptors. *Annu Rev Immunol, 17*, 875-904. doi:10.1146/annurev.immunol.17.1.875

Ma, C. S. (2016). Human T Follicular Helper Cells in Primary Immunodeficiency: Quality Just as Important as Quantity. *J Clin Immunol, 36 Suppl 1*, 40-47. doi:10.1007/s10875-016-0257-6

Ma, C. S., & Deenick, E. K. (2014). Human T follicular helper (Tfh) cells and disease. *Immunol Cell Biol, 92*(1), 64-71. doi:10.1038/icb.2013.55

MacLeod, M. K., David, A., McKee, A. S., Crawford, F., Kappler, J. W., & Marrack, P. (2011). Memory CD4 T cells that express CXCR5 provide accelerated help to B cells. *J Immunol, 186*(5), 2889-2896.

Maecker, H. T., McCoy, J. P., & Nussenblatt, R. (2012). Standardizing immunophenotyping for the Human Immunology Project. *Nat Rev Immunol, 12*(3), 191-200. doi:10.1038/nri3158

Mahalanabis, M., Jayaraman, P., Miura, T., Pereyra, F., Chester, E. M., Richardson, B., . . . Haigwood, N. L. (2009). Continuous viral escape and selection by autologous neutralizing antibodies in drug-naive human immunodeficiency virus controllers. *J Virol, 83*(2), 662-672. doi:10.1128/JVI.01328-08

Malaspina, A., Moir, S., Kottilil, S., Hallahan, C., Ehler, L., Liu, S., . . . Fauci, A. (2003). Deleterious effect of HIV-1 plasma viremia on B cell costimulatory function. *The Journal of Immunology, 170*(12), 5965.

Malaspina, A., Moir, S., Kottilil, S., Hallahan, C. W., Ehler, L. A., Liu, S., . . . Fauci, A. S. (2003). Deleterious effect of HIV-1 plasma viremia on B cell costimulatory function. *J Immunol, 170*(12), 5965-5972.

Martinez-Maza, O., Crabb, E., Mitsuyasu, R., Fahey, J., & Giorgi, J. (1987). Infection with the human immunodeficiency virus (HIV) is associated with an in vivo increase in B lymphocyte activation and immaturity. *The Journal of Immunology, 138*(11), 3720.

Mascola, J. R., Stiegler, G., VanCott, T. C., Katinger, H., Carpenter, C. B., Hanson, C. E., . . . Lewis, M. G. (2000). Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat Med, 6*(2), 207-210. doi:10.1038/72318

Mattapallil, J. J., Douek, D. C., Hill, B., Nishimura, Y., Martin, M., & Roederer, M. (2005). Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature, 434*(7037), 1093-1097. doi:10.1038/nature03501

McMahan, R. H., Golden-Mason, L., Nishimura, M. I., McMahon, B. J., Kemper, M., Allen, T. M., . . . Rosen, H. R. (2010). Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence, and its blockade restores hepatocyte-directed in vitro cytotoxicity. *J Clin Invest, 120*(12), 4546-4557. doi:10.1172/JCI43127

Mehandru, S., Poles, M. A., Tenner-Racz, K., Horowitz, A., Hurley, A., Hogan, C., . . . Markowitz, M. (2004). Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. *J Exp Med, 200*(6), 761-770. doi:10.1084/jem.20041196

Mellors, J. W., Kingsley, L. A., Rinaldo, C. R., Jr., Todd, J. A., Hoo, B. S., Kokka, R. P., & Gupta, P. (1995). Quantitation of HIV-1 RNA in plasma predicts outcome after seroconversion. *Ann Intern Med, 122*(8), 573-579.

Meyer-Olson, D., Brady, K. W., Bartman, M. T., O'Sullivan, K. M., Simons, B. C., Conrad, J. A., . . . Kalams, S. A. (2006). Fluctuations of functionally distinct CD8+ T-cell clonotypes demonstrate flexibility of the HIV-specific TCR repertoire. *Blood, 107*(6), 2373-2383. doi:10.1182/blood-2005-04-1636

Meyer-Olson, D., Simons, B. C., Conrad, J. A., Smith, R. M., Barnett, L., Lorey, S. L., . . . Kalams, S. A. (2010). Clonal expansion and TCR-independent differentiation shape the HIV-specific CD8+ effector-memory T-cell repertoire in vivo. *Blood, 116*(3), 396-405. doi:10.1182/blood-2009-11-254136

Migueles, S. A., Osborne, C. M., Royce, C., Compton, A. A., Joshi, R. P., Weeks, K. A., . . . Connors, M. (2008). Lytic granule loading of CD8+ T cells is required for HIV-infected cell elimination associated with immune control. *Immunity, 29*(6), 1009-1021. doi:10.1016/j.immuni.2008.10.010

Migueles, S. A., Sabbaghian, M. S., Shupert, W. L., Bettinotti, M. P., Marincola, F. M., Martino, L., . . . Connors, M. (2000). HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proc Natl Acad Sci U S A, 97*(6), 2709-2714. doi:10.1073/pnas.050567397

Mikell, I., Sather, D. N., Kalams, S. A., Altfeld, M., Alter, G., & Stamatatos, L. (2011). Characteristics of the earliest cross-neutralizing antibody response to HIV-1. *PLoS Pathog, 7*(1), e1001251.

Mingueneau, M., Krishnaswamy, S., Spitzer, M. H., Bendall, S. C., Stone, E. L., Hedrick, S. M., . . . Benoist, C. (2014). Single-cell mass cytometry of TCR signaling: Amplification of small initial differences results in low ERK activation in NOD mice. *Proc Natl Acad Sci U S A, 111*(46), 16466-16471. doi:10.1073/pnas.1419337111

Moir, S., & Fauci, A. S. (2009). B cells in HIV infection and disease. *Nat Rev Immunol, 9*(4), 235-245.

Moir, S., Ho, J., Malaspina, A., Wang, W., DiPoto, A., O'Shea, M., . . . Proschan, M. (2008). Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. *The Journal of experimental medicine, 205*(8), 1797.

Moir, S., Malaspina, A., Ogwaro, K., Donoghue, E., Hallahan, C., Ehler, L., . . . Hwu, P. (2001). HIV-1 induces phenotypic and functional perturbations of B cells in chronically infected individuals. *Proceedings of the National Academy of Sciences of the United States of America, 98*(18), 10362.

Moir, S., Malaspina, A., Pickeral, O. K., Donoghue, E. T., Vasquez, J., Miller, N. J., . . . Justement, J. S. (2004). Decreased survival of B cells of HIV-viremic patients mediated by altered expression of receptors of the TNF superfamily. *The Journal of experimental medicine, 200*(5), 587.

Moir, S., Malaspina, A., Pickeral, O. K., Donoghue, E. T., Vasquez, J., Miller, N. J., . . . Fauci, A. S. (2004). Decreased survival of B cells of HIV-viremic patients mediated by altered expression of receptors of the TNF superfamily. *J Exp Med, 200*(5), 587-599. doi:10.1084/jem.20032236

Moir, S., Ogwaro, K., Malaspina, A., Vasquez, J., Donoghue, E., Hallahan, C., . . . Kottilil, S. (2003). Perturbations in B cell responsiveness to CD4+ T cell help in HIV-infected individuals. *Proceedings of the National Academy of Sciences of the United States of America, 100*(10), 6057.

Moir, S., Ogwaro, K. M., Malaspina, A., Vasquez, J., Donoghue, E. T., Hallahan, C. W., . . . Fauci, A. S. (2003). Perturbations in B cell responsiveness to CD4+ T cell help in HIV-infected individuals. *Proc Natl Acad Sci U S A, 100*(10), 6057-6062. doi:10.1073/pnas.0730819100

Moldt, B., Rakasz, E. G., Schultz, N., Chan-Hui, P. Y., Swiderek, K., Weisgrau, K. L., . . . Burton, D. R. (2012). Highly potent HIV-specific antibody neutralization in vitro translates into effective protection against mucosal SHIV challenge in vivo. *Proc Natl Acad Sci U S A, 109*(46), 18921-18925. doi:10.1073/pnas.1214785109

Moog, C., Fleury, H. J., Pellegrin, I., Kirn, A., & Aubertin, A. M. (1997). Autologous and heterologous neutralizing antibody responses following initial seroconversion in human immunodeficiency virus type 1-infected individuals. *J Virol, 71*(5), 3734-3741.

Morita, R., Schmitt, N., Bentebibel, S. E., Ranganathan, R., Bourdery, L., Zurawski, G., . . . Ueno, H. (2011). Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity, 34*(1), 108-121.

Morris, L., Binley, J., Clas, B., Bonhoeffer, S., Astill, T., Kost, R., . . . Ho, D. (1998). HIV-1 antigen–specific and–nonspecific B cell responses are sensitive to combination antiretroviral therapy. *The Journal of experimental medicine, 188*(2), 233.

Morris, L., Binley, J. M., Clas, B. A., Bonhoeffer, S., Astill, T. P., Kost, R., . . . Moore, J. P. (1998). HIV-1 antigen-specific and -nonspecific B cell responses are sensitive to combination antiretroviral therapy. *J Exp Med, 188*(2), 233-245.

Nakamoto, N., Cho, H., Shaked, A., Olthoff, K., Valiga, M. E., Kaminski, M., . . . Chang, K. M. (2009). Synergistic reversal of intrahepatic HCV-specific CD8 T cell exhaustion by combined PD-1/CTLA-4 blockade. *PLoS Pathog, 5*(2), e1000313. doi:10.1371/journal.ppat.1000313

Nakanishi, Y., Lu, B., Gerard, C., & Iwasaki, A. (2009). CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help. *Nature, 462*(7272), 510-513. doi:10.1038/nature08511

Newell, E. W., Sigal, N., Bendall, S. C., Nolan, G. P., & Davis, M. M. (2012). Cytometry by time-of-flight shows combinatorial cytokine expression and virus-specific cell niches within a continuum of CD8+ T cell phenotypes. *Immunity, 36*(1), 142-152. doi:10.1016/j.immuni.2012.01.002

Nicholas, K. J., Greenplate, A. R., Flaherty, D. K., Matlock, B. K., Juan, J. S., Smith, R. M., . . . Kalams, S. A. (2015). Multiparameter analysis of stimulated human peripheral blood mononuclear cells: A comparison of mass and fluorescence cytometry. *Cytometry Part A*, n/a-n/a. doi:10.1002/cyto.a.22799

Nicholas, K. J., Greenplate, A. R., Flaherty, D. K., Matlock, B. K., Juan, J. S., Smith, R. M., . . . Kalams, S. A. (2016). Multiparameter analysis of stimulated human peripheral blood mononuclear cells: A comparison of mass and fluorescence cytometry. *Cytometry A, 89*(3), 271-280. doi:10.1002/cyto.a.22799

Nicholas, K. J., Zern, E. K., Barnett, L., Smith, R. M., Lorey, S. L., Copeland, C. A., . . . Kalams, S. A. (2013). B cell responses to HIV antigen are a potent correlate of viremia in HIV-1 infection and improve with PD-1 blockade. *PLoS One, 8*(12), e84185. doi:10.1371/journal.pone.0084185

Nielsen, C., Hansen, D., Husby, S., Jacobsen, B. B., & Lillevang, S. T. (2003). Association of a putative regulatory polymorphism in the PD-1 gene with susceptibility to type 1 diabetes. *Tissue Antigens, 62*(6), 492-497.

Nixon, D. F., Townsend, A. R., Elvin, J. G., Rizza, C. R., Gallwey, J., & McMichael, A. J. (1988). HIV-1 gag-specific cytotoxic T lymphocytes defined with recombinant vaccinia virus and synthetic peptides. *Nature, 336*(6198), 484-487. doi:10.1038/336484a0

Nurieva, R. I., Chung, Y., Hwang, D., Yang, X. O., Kang, H. S., Ma, L., . . . Dong, C. (2008). Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity, 29*(1), 138-149. doi:10.1016/j.immuni.2008.05.009

Nurieva, R. I., Chung, Y., Martinez, G. J., Yang, X. O., Tanaka, S., Matskevitch, T. D., . . . Dong, C. (2009). Bcl6 mediates the development of T follicular helper cells. *Science, 325*(5943), 1001-1005. doi:10.1126/science.1176676

Nutt, S. L., & Tarlinton, D. M. (2011). Germinal center B and follicular helper T cells: siblings, cousins or just good friends? *Nat Immunol, 12*(6), 472-477.

Okulicz, J. F., & Lambotte, O. (2011). Epidemiology and clinical characteristics of elite controllers. *Curr Opin HIV AIDS, 6*(3), 163-168. doi:10.1097/COH.0b013e328344f35e

Okulicz, J. F., Marconi, V. C., Landrum, M. L., Wegner, S., Weintrob, A., Ganesan, A., . . . Infectious Disease Clinical Research Program, H. I. V. W. G. (2009). Clinical outcomes of elite controllers, viremic controllers, and long-term nonprogressors in the US Department of Defense HIV natural history study. *J Infect Dis, 200*(11), 1714-1723. doi:10.1086/646609

Ornatsky, O., Bandura, D., Baranov, V., Nitz, M., Winnik, M. A., & Tanner, S. (2010). Highly multiparametric analysis by mass cytometry. *J Immunol Methods, 361*(1-2), 1-20. doi:10.1016/j.jim.2010.07.002

Ornatsky, O. I., Lou, X., Nitz, M., Schafer, S., Sheldrick, W. S., Baranov, V. I., . . . Tanner, S. D. (2008). Study of cell antigens and intracellular DNA by identification of element-containing labels and metallointercalators using inductively coupled

plasma mass spectrometry. *Anal Chem, 80*(7), 2539-2547. doi:10.1021/ac702128m

Oswald-Richter, K., Grill, S. M., Leelawong, M., Tseng, M., Kalams, S. A., Hulgan, T., . . . Unutmaz, D. (2007). Identification of a CCR5-expressing T cell subset that is resistant to R5-tropic HIV infection. *PLoS Pathog, 3*(4), e58.

Overbaugh, J., & Morris, L. (2012). The Antibody Response against HIV-1. *Cold Spring Harb Perspect Med, 2*(1), a007039. doi:10.1101/cshperspect.a007039

Pahwa, R., Good, R., & Pahwa, S. (1987). Prematurity, hypogammaglobulinemia, and neuropathology with human immunodeficiency virus (HIV) infection. *Proceedings of the National Academy of Sciences of the United States of America, 84*(11), 3826.

Pahwa, R., Good, R. A., & Pahwa, S. (1987). Prematurity, hypogammaglobulinemia, and neuropathology with human immunodeficiency virus (HIV) infection. *Proc Natl Acad Sci U S A, 84*(11), 3826-3830.

Pallikkuth, S., Parmigiani, A., Silva, S. Y., George, V. K., Fischl, M., Pahwa, R., & Pahwa, S. (2012). Impaired peripheral blood T-follicular helper cell function in HIV-infected nonresponders to the 2009 H1N1/09 vaccine. *Blood, 120*(5), 985-993. doi:10.1182/blood-2011-12-396648

Pallikkuth, S., Sharkey, M., Babic, D. Z., Gupta, S., Stone, G. W., Fischl, M. A., . . . Pahwa, S. (2015). Peripheral T Follicular Helper Cells Are the Major HIV Reservoir within Central Memory CD4 T Cells in Peripheral Blood from Chronically HIV-Infected Individuals on Combination Antiretroviral Therapy. *J Virol, 90*(6), 2718-2728. doi:10.1128/JVI.02883-15

Pantaleo, G., Demarest, J. F., Schacker, T., Vaccarezza, M., Cohen, O. J., Daucher, M., . . . Fauci, A. S. (1997). The qualitative nature of the primary immune response to HIV infection is a prognosticator of disease progression independent of the initial level of plasma viremia. *Proc Natl Acad Sci U S A, 94*(1), 254-258.

Pantaleo, G., Menzo, S., Vaccarezza, M., Graziosi, C., Cohen, O. J., Demarest, J. F., . . . et al. (1995). Studies in subjects with long-term nonprogressive human immunodeficiency virus infection. *N Engl J Med, 332*(4), 209-216. doi:10.1056/NEJM199501263320402

Pantophlet, R., & Burton, D. R. (2006). GP120: target for neutralizing HIV-1 antibodies. *Annu Rev Immunol, 24*, 739-769. doi:10.1146/annurev.immunol.24.021605.090557

Pardoll, D. M. (2012). The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer, 12*(4), 252-264. doi:10.1038/nrc3239

Parry, R. V., Chemnitz, J. M., Frauwirth, K. A., Lanfranco, A. R., Braunstein, I., Kobayashi, S. V., . . . Riley, J. L. (2005). CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol Cell Biol, 25*(21), 9543-9553. doi:10.1128/MCB.25.21.9543-9553.2005

Pedersen, C., Katzenstein, T., Nielsen, C., Lundgren, J. D., & Gerstoft, J. (1997). Prognostic value of serum HIV-RNA levels at virologic steady state after seroconversion: relation to CD4 cell count and clinical course of primary infection. *J Acquir Immune Defic Syndr Hum Retrovirol, 16*(2), 93-99.

Perelson, A. S., Essunger, P., Cao, Y., Vesanen, M., Hurley, A., Saksela, K., . . . Ho, D. D. (1997). Decay characteristics of HIV-1-infected compartments during combination therapy. *Nature, 387*(6629), 188-191. doi:10.1038/387188a0

Pereyra, F., Addo, M. M., Kaufmann, D. E., Liu, Y., Miura, T., Rathod, A., . . . Walker, B. D. (2008). Genetic and immunologic heterogeneity among persons who control HIV infection in the absence of therapy. *J Infect Dis, 197*(4), 563-571. doi:10.1086/526786

Perreau, M., Savoye, A. L., De Crignis, E., Corpataux, J. M., Cubas, R., Haddad, E. K., . . . Pantaleo, G. (2013). Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production. *J Exp Med, 210*(1), 143-156. doi:10.1084/jem.20121932

Petrovas, C., Casazza, J. P., Brenchley, J. M., Price, D. A., Gostick, E., Adams, W. C., . . . Koup, R. A. (2006). PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. *J Exp Med, 203*(10), 2281-2292. doi:jem.20061496 [pii]

10.1084/jem.20061496

Petrovas, C., et al. (2012). CD4 T follicular helper cell dynamics during SIV infection. *The Journal of Clinical Investigation, 122*(9), 3281-3294.

Picker, L. J., Hansen, S. G., & Lifson, J. D. (2012). New paradigms for HIV/AIDS vaccine development. *Annu Rev Med, 63*, 95-111.

Pillai, S. (2013). Love the one you're with: the HIV, B cell and TFH cell triangle. *Nat Med, 19*(4), 401-402. doi:10.1038/nm.3141

Pissani, F., & Streeck, H. (2014). Emerging concepts on T follicular helper cell dynamics in HIV infection. *Trends Immunol, 35*(6), 278-286. doi:10.1016/j.it.2014.02.010

Pitcher, C. J., Quittner, C., Peterson, D. M., Connors, M., Koup, R. A., Maino, V. C., & Picker, L. J. (1999). HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. *Nat Med, 5*(5), 518-525. doi:10.1038/8400

Plata, F., Autran, B., Martins, L. P., Wain-Hobson, S., Raphael, M., Mayaud, C., . . . Debre, P. (1987). AIDS virus-specific cytotoxic T lymphocytes in lung disorders. *Nature, 328*(6128), 348-351. doi:10.1038/328348a0

Polikowsky, H. G., Wogsland, C. E., Diggins, K. E., Huse, K., & Irish, J. M. (2015). Cutting Edge: Redox Signaling Hypersensitivity Distinguishes Human Germinal Center B Cells. *J Immunol*. doi:10.4049/jimmunol.1500904

Popovic, M., Sarngadharan, M. G., Read, E., & Gallo, R. C. (1984). Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science, 224*(4648), 497-500.

Porichis, F., Hart, M. G., Griesbeck, M., Everett, H. L., Hassan, M., Baxter, A. E., . . . Kaufmann, D. E. (2014). High-throughput detection of miRNAs and gene-specific mRNA at the single-cell level by flow cytometry. *Nat Commun, 5*, 5641. doi:10.1038/ncomms6641

Porichis, F., Kwon, D. S., Zupkosky, J., Tighe, D. P., McMullen, A., Brockman, M. A., . . . Kaufmann, D. E. (2011). Responsiveness of HIV-specific CD4 T cells to PD-1 blockade. *Blood, 118*(4), 965-974.

Pratama, A., & Vinuesa, C. G. (2014). Control of TFH cell numbers: why and how? *Immunol Cell Biol, 92*(1), 40-48. doi:10.1038/icb.2013.69

Qi, H., Cannons, J. L., Klauschen, F., Schwartzberg, P. L., & Germain, R. N. (2008). SAP-controlled T-B cell interactions underlie germinal centre formation. *Nature, 455*(7214), 764-769. doi:10.1038/nature07345

Ravetch, J. V., & Lanier, L. L. (2000). Immune inhibitory receptors. *Science, 290*(5489), 84-89.

Ribeiro, R. M., Qin, L., Chavez, L. L., Li, D., Self, S. G., & Perelson, A. S. (2010). Estimation of the initial viral growth rate and basic reproductive number during acute HIV-1 infection. *J Virol, 84*(12), 6096-6102. doi:10.1128/JVI.00127-10

Richman, D. D., Wrin, T., Little, S. J., & Petropoulos, C. J. (2003). Rapid evolution of the neutralizing antibody response to HIV type 1 infection. *Proc Natl Acad Sci U S A, 100*(7), 4144-4149. doi:10.1073/pnas.0630530100

Riley, J. L. (2009). PD 1 signaling in primary T cells. *Immunological Reviews, 229*(1), 114-125.

Riley, J. L. (2009). PD-1 Signaling in primary T cells. *Immunological Reviews, 229*, 114-125.

Rosenberg, E. S., Billingsley, J. M., Caliendo, A. M., Boswell, S. L., Sax, P. E., Kalams, S. A., & Walker, B. D. (1997). Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science, 278*(5342), 1447-1450.

Rossio, J. L., Esser, M. T., Suryanarayana, K., Schneider, D. K., Bess, J. W., Jr., Vasquez, G. M., . . . Lifson, J. D. (1998). Inactivation of human immunodeficiency virus type 1 infectivity with preservation of conformational and functional integrity of virion surface proteins. *J Virol, 72*(10), 7992-8001.

Rutebemberwa, A., Bess, J. W., Jr., Brown, B., Arroyo, M., Eller, M., Slike, B., . . . Cox, J. H. (2007). Evaluation of aldrithiol-2-inactivated preparations of HIV type 1 subtypes A, B, and D as reagents to monitor T cell responses. *AIDS Res Hum Retroviruses, 23*(4), 532-542. doi:10.1089/aid.2006.0136

Saag, M., & Deeks, S. G. (2010). How do HIV elite controllers do what they do? *Clin Infect Dis, 51*(2), 239-241. doi:10.1086/653678

Sadagopal, S., Lorey, S. L., Barnett, L., Basham, R., Lebo, L., Erdem, H., . . . Kalams, S. A. (2008). Enhancement of human immunodeficiency virus (HIV)-specific CD8+ T cells in cerebrospinal fluid compared to those in blood among antiretroviral

therapy-naive HIV-positive subjects. *J Virol, 82*(21), 10418-10428. doi:10.1128/JVI.01190-08

Sadagopal, S., Lorey, S. L., Barnett, L., Sutherland, D., Basham, R., Erdem, H., . . . Haas, D. W. (2010). Enhanced PD-1 expression by T cells in cerebrospinal fluid does not reflect functional exhaustion during chronic human immunodeficiency virus type 1 infection. *J Virol, 84*(1), 131-140. doi:10.1128/JVI.01181-09

Saez-Cirion, A., Pancino, G., Sinet, M., Venet, A., Lambotte, O., & group, A. E. H. C. s. (2007). HIV controllers: how do they tame the virus? *Trends Immunol, 28*(12), 532-540. doi:10.1016/j.it.2007.09.002

Saez-Cirion, A., Sinet, M., Shin, S. Y., Urrutia, A., Versmisse, P., Lacabaratz, C., . . . Group, A. E. H. C. S. (2009). Heterogeneity in HIV suppression by CD8 T cells from HIV controllers: association with Gag-specific CD8 T cell responses. *J Immunol, 182*(12), 7828-7837. doi:10.4049/jimmunol.0803928

Sallusto, F., Lenig, D., Forster, R., Lipp, M., & Lanzavecchia, A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature, 401*(6754), 708-712. doi:10.1038/44385

Sather, D. N., Armann, J., Ching, L. K., Mavrantoni, A., Sellhorn, G., Caldwell, Z., . . . Stamatatos, L. (2009). Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection. *J Virol, 83*(2), 757-769. doi:10.1128/JVI.02036-08

Schacker, T. W., Hughes, J. P., Shea, T., Coombs, R. W., & Corey, L. (1998). Biological and virologic characteristics of primary HIV infection. *Ann Intern Med, 128*(8), 613-620.

Schaerli, P., Willimann, K., Lang, A. B., Lipp, M., Loetscher, P., & Moser, B. (2000). CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med, 192*(11), 1553-1562.

Scheid, J. F., Mouquet, H., Feldhahn, N., Seaman, M. S., Velinzon, K., Pietzsch, J., . . . Nussenzweig, M. C. (2009). Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals. *Nature, 458*(7238), 636-640. doi:10.1038/nature07930

Schultz, B. T., Teigler, J. E., Pissani, F., Oster, A. F., Kranias, G., Alter, G., . . . Streeck, H. (2016). Circulating HIV-Specific Interleukin-21(+)CD4(+) T Cells Represent Peripheral Tfh Cells with Antigen-Dependent Helper Functions. *Immunity, 44*(1), 167-178. doi:10.1016/j.immuni.2015.12.011

Schulze Zur Wiesch, J., Thomssen, A., Hartjen, P., Toth, I., Lehmann, C., Meyer-Olson, D., . . . van Lunzen, J. (2011). Comprehensive analysis of frequency and phenotype of T regulatory cells in HIV infection: CD39 expression of FoxP3+ T regulatory cells correlates with progressive disease. *J Virol, 85*(3), 1287-1297. doi:10.1128/JVI.01758-10

Sen, N., Mukherjee, G., Sen, A., Bendall, S. C., Sung, P., Nolan, G. P., & Arvin, A. M. (2014). Single-cell mass cytometry analysis of human tonsil T cell remodeling by varicella zoster virus. *Cell Rep, 8*(2), 633-645. doi:10.1016/j.celrep.2014.06.024

Sharpe, A. H., Wherry, E. J., Ahmed, R., & Freeman, G. J. (2007). The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat Immunol, 8*(3), 239-245.

Shaw, G. M., & Hunter, E. (2012). HIV Transmission. In F. D. Bushman, G. J. Nabel, & R. Swanstrom (Eds.), *HIV: From Biology to Prevention and Treatment*. Cold Spring Harbor, New York: Cold Sping Harbor Laboratory Press.

Shaw, J. M., Hunt, P. W., Critchfield, J. W., McConnell, D. H., Garcia, J. C., Pollard, R. B., . . . Shacklett, B. L. (2011). Increased frequency of regulatory T cells accompanies increased immune activation in rectal mucosae of HIV-positive noncontrollers. *J Virol, 85*(21), 11422-11434. doi:10.1128/JVI.05608-11

Sheppard, H. W., Lang, W., Ascher, M. S., Vittinghoff, E., & Winkelstein, W. (1993). The characterization of non-progressors: long-term HIV-1 infection with stable CD4+ T-cell levels. *Aids, 7*(9), 1159-1166.

Sheppard, K. A., Fitz, L. J., Lee, J. M., Benander, C., George, J. A., Wooters, J., . . . Chaudhary, D. (2004). PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. *FEBS Lett, 574*(1-3), 37-41. doi:10.1016/j.febslet.2004.07.083

Shetty, R. D., et al. (2012). PD-1 blockade during chronic SIV infection reduces hyperimmune activation and microbial translocation in rhesus macaques. *J Clin Invest, 122*(5), 1712-1716.

Shibata, R., Igarashi, T., Haigwood, N., Buckler-White, A., Ogert, R., Ross, W., . . . Martin, M. A. (1999). Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. *Nat Med, 5*(2), 204-210. doi:10.1038/5568

Shirai, A., Cosentino, M., Leitman-Klinman, S., & Klinman, D. (1992). Human immunodeficiency virus infection induces both polyclonal and virus-specific B cell activation. *Journal of Clinical Investigation, 89*(2), 561.

Shirai, A., Cosentino, M., Leitman-Klinman, S. F., & Klinman, D. M. (1992). Human immunodeficiency virus infection induces both polyclonal and virus-specific B cell activation. *J Clin Invest, 89*(2), 561-566. doi:10.1172/JCI115621

Siliciano, J. D., & Siliciano, R. F. (2006). The latent reservoir for HIV-1 in resting CD4+ T cells: a barrier to cure. *Curr Opin HIV AIDS, 1*(2), 121-128. doi:10.1097/01.COH.0000209582.82328.b8

Siliciano, R. F., & Greene, W. C. (2012). HIV Latency *HIV: From Biology to Prevention and Treatment*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Simons, B. C., Vancompernolle, S. E., Smith, R. M., Wei, J., Barnett, L., Lorey, S. L., . . . Kalams, S. A. (2008). Despite biased TRBV gene usage against a dominant HLA B57-restricted epitope, TCR diversity can provide recognition of circulating epitope variants. *J Immunol, 181*(7), 5137-5146.

Simpson, N., Gatenby, P. A., Wilson, A., Malik, S., Fulcher, D. A., Tangye, S. G., . . . Cook, M. C. (2010). Expansion of circulating T cells resembling follicular helper T cells is

a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis Rheum, 62*(1), 234-244. doi:10.1002/art.25032

Stamatatos, L., Morris, L., Burton, D. R., & Mascola, J. R. (2009). Neutralizing antibodies generated during natural HIV-1 infection: good news for an HIV-1 vaccine? *Nat Med, 15*(8), 866-870. doi:10.1038/nm.1949

Steinhoff, M. C., Auerbach, B. S., Nelson, K. E., Vlahov, D., Becker, R. L., Graham, N. M., . . . Chaisson, R. E. (1991). Antibody responses to Haemophilus influenzae type B vaccines in men with human immunodeficiency virus infection. *N Engl J Med, 325*(26), 1837-1842. doi:10.1056/NEJM199112263252603

Streeck, H., Brumme, Z. L., Anastario, M., Cohen, K. W., Jolin, J. S., Meier, A., . . . Altfeld, M. (2008). Antigen load and viral sequence diversification determine the functional profile of HIV-1-specific CD8+ T cells. *PLoS Med, 5*(5), e100. doi:10.1371/journal.pmed.0050100

Streeck, H., D'Souza, M. P., Littman, D. R., & Crotty, S. (2013). Harnessing CD4(+) T cell responses in HIV vaccine development. *Nat Med, 19*(2), 143-149. doi:10.1038/nm.3054

Tangye, S. G., Ma, C. S., Brink, R., & Deenick, E. K. (2013). The good, the bad and the ugly - TFH cells in human health and disease. *Nat Rev Immunol, 13*(6), 412-426. doi:10.1038/nri3447

Thaventhiran T, S. S., Yeang HXA, Al-Huseini L, Hamdam J, and Sathish J. (2012). T Cell Co-inhibitory Receptors: Functions and Signalling Mechanisms. *Journal of Clinical and Cellular Immunology, S12*(004). doi:10.4172/2155-9899.S12-004

Trautmann, L., Janbazian, L., Chomont, N., Said, E. A., Gimmig, S., Bessette, B., . . . Sekaly, R. P. (2006). Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat Med, 12*(10), 1198-1202. doi:nm1482 [pii]

10.1038/nm1482

Tricot, S., Meyrand, M., Sammicheli, C., Elhmouzi-Younes, J., Corneau, A., Bertholet, S., . . . Cosma, A. (2015). Evaluating the efficiency of isotope transmission for improved panel design and a comparison of the detection sensitivities of mass cytometer instruments. *Cytometry A, 87*(4), 357-368. doi:10.1002/cyto.a.22648

Ueda, H., Howson, J. M., Esposito, L., Heward, J., Snook, H., Chamberlain, G., . . . Gough, S. C. (2003). Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature, 423*(6939), 506-511. doi:10.1038/nature01621

Valdez, H., Smith, K. Y., Landay, A., Connick, E., Kuritzkes, D. R., Kessler, H., . . . Lederman, M. M. (2000). Response to immunization with recall and neoantigens after prolonged administration of an HIV-1 protease inhibitor-containing regimen. ACTG 375 team. AIDS Clinical Trials Group. *Aids, 14*(1), 11-21.

van Kooten, C., & Banchereau, J. (2000). CD40-CD40 ligand. *J Leukoc Biol, 67*(1), 2-17.

Veazey, R. S., DeMaria, M., Chalifoux, L. V., Shvetz, D. E., Pauley, D. R., Knight, H. L., . . . Lackner, A. A. (1998). Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science, 280*(5362), 427-431.

Velu, V., Kannanganat, S., Ibegbu, C., Chennareddi, L., Villinger, F., Freeman, G. J., . . . Amara, R. R. (2007). Elevated expression levels of inhibitory receptor programmed death 1 on simian immunodeficiency virus-specific CD8 T cells during chronic infection but not after vaccination. *J Virol, 81*(11), 5819-5828. doi:JVI.00024-07 [pii]

10.1128/JVI.00024-07

Velu, V., Titanji, K., Zhu, B., Husain, S., Pladevega, A., Lai, L., . . . Freeman, G. (2008). Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature, 458*(7235), 206-210.

Velu, V., Titanji, K., Zhu, B., Husain, S., Pladevega, A., Lai, L., . . . Amara, R. R. (2009). Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature, 458*(7235), 206-210.

Vinuesa, C. G., Tangye, S. G., Moser, B., & Mackay, C. R. (2005). Follicular B helper T cells in antibody responses and autoimmunity. *Nat Rev Immunol, 5*(11), 853-865.

Vogelzang, A., McGuire, H. M., Yu, D., Sprent, J., Mackay, C. R., & King, C. (2008). A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity, 29*(1), 127-137. doi:10.1016/j.immuni.2008.06.001

Walker, B. D. (2007). Elite control of HIV Infection: implications for vaccines and treatment. *Top HIV Med, 15*(4), 134-136.

Walker, B. D., Ahmed, R., & Plotkin, S. (2011). Moving ahead an HIV vaccine: use both arms to beat HIV. *Nat Med, 17*(10), 1194-1195. doi:10.1038/nm.2529

Walker, B. D., Chakrabarti, S., Moss, B., Paradis, T. J., Flynn, T., Durno, A. G., . . . Schooley, R. T. (1987). HIV-specific cytotoxic T lymphocytes in seropositive individuals. *Nature, 328*(6128), 345-348. doi:10.1038/328345a0

Walker, L. M., Huber, M., Doores, K. J., Falkowska, E., Pejchal, R., Julien, J. P., . . . Poignard, P. (2011). Broad neutralization coverage of HIV by multiple highly potent antibodies. *Nature, 477*(7365), 466-470. doi:10.1038/nature10373

Walker, L. M., Simek, M. D., Priddy, F., Gach, J. S., Wagner, D., Zwick, M. B., . . . Burton, D. R. (2010). A limited number of antibody specificities mediate broad and potent serum neutralization in selected HIV-1 infected individuals. *PLoS Pathog, 6*(8), e1001028. doi:10.1371/journal.ppat.1001028

Wang, L., Abbasi, F., Ornatsky, O., Cole, K. D., Misakian, M., Gaigalas, A. K., . . . Stebbings, R. (2012). Human CD4+ lymphocytes for antigen quantification: characterization using conventional flow cytometry and mass cytometry. *Cytometry A, 81*(7), 567-575. doi:10.1002/cyto.a.22060

Wei, X., Decker, J. M., Wang, S., Hui, H., Kappes, J. C., Wu, X., . . . Shaw, G. M. (2003). Antibody neutralization and escape by HIV-1. *Nature, 422*(6929), 307-312. doi:10.1038/nature01470

Weiss, R. A., Clapham, P. R., Cheingsong-Popov, R., Dalgleish, A. G., Carne, C. A., Weller, I. V., & Tedder, R. S. (1985). Neutralization of human T-lymphotropic virus type III by sera of AIDS and AIDS-risk patients. *Nature, 316*(6023), 69-72.

Wherry, E. J. (2011). T cell exhaustion. *Nat Immunol, 12*(6), 492-499.

Wherry, E. J., Ha, S. J., Kaech, S. M., Haining, W. N., Sarkar, S., Kalia, V., . . . Ahmed, R. (2007). Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity, 27*(4), 670-684.

WHO. (2015, Nov 2015). HIV/AIDS Fact Sheet no. 360. Retrieved from http://www.who.int/mediacentre/factsheets/fs360/en/

Williams, L. D., Bansal, A., Sabbaj, S., Heath, S. L., Song, W., Tang, J., . . . Goepfert, P. A. (2011). Interleukin-21-producing HIV-1-specific CD8 T cells are preferentially seen in elite controllers. *J Virol, 85*(5), 2316-2324.

Wu, X., Yang, Z. Y., Li, Y., Hogerkorp, C. M., Schief, W. R., Seaman, M. S., . . . Mascola, J. R. (2010). Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1. *Science, 329*(5993), 856-861. doi:10.1126/science.1187659

Wu, X., Zhou, T., Zhu, J., Zhang, B., Georgiev, I., Wang, C., . . . Mascola, J. R. (2011). Focused evolution of HIV-1 neutralizing antibodies revealed by structures and deep sequencing. *Science, 333*(6049), 1593-1602. doi:10.1126/science.1207532

Yamamoto, T., Lynch, R. M., Gautam, R., Matus-Nicodemos, R., Schmidt, S. D., Boswell, K. L., . . . Koup, R. A. (2015). Quality and quantity of TFH cells are critical for broad antibody development in SHIVAD8 infection. *Sci Transl Med, 7*(298), 298ra120. doi:10.1126/scitranslmed.aab3964

Yamamoto, T., Price, D. A., Casazza, J. P., Ferrari, G., Nason, M., Chattopadhyay, P. K., . . . Koup, R. A. (2011). Surface expression patterns of negative regulatory molecules identify determinants of virus-specific CD8+ T-cell exhaustion in HIV infection. *Blood, 117*(18), 4805-4815. doi:10.1182/blood-2010-11-317297

Yao, Y., Liu, R., Shin, M. S., Trentalange, M., Allore, H., Nassar, A., . . . Montgomery, R. R. (2014). CyTOF supports efficient detection of immune cell subsets from small samples. *J Immunol Methods, 415*, 1-5. doi:10.1016/j.jim.2014.10.010

Yi, J. S., Du, M., & Zajac, A. J. (2009). A vital role for interleukin-21 in the control of a chronic viral infection. *Science, 324*(5934), 1572-1576.

Yu, D., Rao, S., Tsai, L. M., Lee, S. K., He, Y., Sutcliffe, E. L., . . . Vinuesa, C. G. (2009). The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity, 31*(3), 457-468. doi:10.1016/j.immuni.2009.07.002

Yue, F. Y., Lo, C., Sakhdari, A., Lee, E. Y., Kovacs, C. M., Benko, E., . . . Ostrowski, M. A. (2010). HIV-specific IL-21 producing CD4+ T cells are induced in acute and chronic progressive HIV infection and are associated with relative viral control. *J Immunol, 185*(1), 498-506.