Immune Correlates of Cross-Reactive Neutralizing Antibodies in HIV-1 Infection

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Abstract

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Global Health

In spite of more than three decades of rigorous investigation, there remains no licensed vaccine against HIV-1. A major challenge in developing an effective vaccine is the extensive diversity in HIV-1, varying by as much as 35% amino acid sequence in the HIV-1 envelope protein (Env), which is the sole target of neutralizing antibodies. Therefore, an effective HIV-1 vaccine will likely require elicitation of potent neutralizing antibodies able to neutralize diverse heterologous viral isolates. Such cross-reactive neutralizing antibodies (CRNA) develop in a minority of HIV-1 infected subjects, with broad and potent monoclonal antibodies having been isolated from some. In this thesis, I have concentrated on identifying immunological correlates associated with the development of these responses during HIV-1 infection and the potential mechanisms involved. I examined immunological factors in a cohort of HIV-1 infected subjects

who have been monitored regularly during their infection; starting soon after infection and followed for up to several years and for whom the development of serum CRNA was previously documented in detail (Mikell et al., 2011b). In my first aim, I evaluated the phenotypes and frequencies of peripheral T follicular helper-like (pTFH) cells and Env-specific B cells, plasma cytokines, and B cell transcriptional profiles during early infection and at ~2.5 years post-infection when CRNA becomes detectable in some subjects. Consequently, I demonstrated significant correlations between the frequency of pTFH cells, plasma levels of CXCL13, and development of CRNA, independent of plasma viral load. Early in infection the subjects who later developed CRNA had a higher frequency of pTFH cells, reflecting levels found in HIV-1 uninfected subjects, suggesting that there may be underlying mechanisms critical for maintaining pTFH cells in those individuals in early infection. In addition, we show that B cells from these subjects expressed more activation-induced cytidine deaminase (AID) in the first year post-infection, and that AID transcript levels correlated with the frequency of pTFH cells. The pTFH cells from these individuals were also more effective at inducing class-switching in autologous B cells *in vitro.* Therefore, our results directly link the development of CRNA against HIV-1 with pTFH cell frequency and function. Furthermore, as elevated levels of the chemokine, CXCL13, were observed in the plasma of subjects with CRNA, in my second aim, I sought to determine potential pathways which may induce CXCL13 during HIV-1 infection. To this end, I identified two potential mechanisms of HIV-1 induced CXCL13 secretion; one due to direct TLR7/8 activation in monocytes by ssHIV-1 RNA and the second due to TLR7 induction of type I interferon (IFN) by pDCs and subsequent IFN stimulation of monocytes. Taken together, there may be differences in aspects of the innate immune responses during acute/early HIV-1 infection that predispose or contribute to some individuals developing CRNA later in infection. Furthermore these studies identified pathways potentially contributing to the development of CRNA as well as a possibly predictive ‘signature’ of CRNA development following HIV-1 infection and vaccination.

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Abbreviations

AA= Amino acid
AID= Activation-induced cytidine deaminase
AIDS= Acquired immunodeficiency syndrome
APC= Antigen presenting cell
ART= Antiretroviral therapy
BCR= B cell receptor
BnAbs= Broadly neutralizing antibodies
CD= Cluster of differentiation
CTL= Cytotoxic T lymphocyte
CRF= Circulating recombinant form
CRNA= Cross-Reactive Neutralizing Antibodies
DC= Dendritic cells
Env= HIV-1 envelope protein
HIV= Human immunodeficiency virus
HLA= Human leukocyte antigen
IFN= Interferon
IL= Interleukin
MHC= Major histocompatibility complex
NHP= Non-human primate
pDC= Plasmacytoid dendritic cells
PMBC= Peripheral blood mononuclear cells
SIV= Simian immunodeficiency virus
TCR= T cell receptor
TFH= T follicular helper
TLR=Toll-like receptor
VL= Viral load
YPI= Years post-infection

Introduction

Introduction

Although the etiology of the Acquired Immune Deficiency Syndrome (AIDS) was identified thirty years ago (Barre-Sinoussi et al., 1983; Chermann et al., 1983; Gallo et al., 1984; Gallo et al., 1983), it continues to be the cause of sustained morbidity and mortality with an estimated 35 million people world-wide currently living with the disease (WHO 2012). HIV/AIDS is a terminal degenerate immunological condition caused by infection with the Human Immunodeficiency Virus-1 (HIV-1). About two thirds of infected individuals do not have access to anti-retroviral therapy. Many of those receiving treatment do not have access to optimal regimens or clinical monitoring (Hall et al., 2013). While substantial progress has been made in understanding the virus, pathogenesis, and the immunology of the host response, little progress has been made in the development of an effective vaccine.

Natural History and HIV-1 Pathogenesis

The Origin of HIV

HIV originated from cross-species transmissions of the Simian Immunodeficiency Virus (SIV) from non-human primates into humans (Hahn et al., 2000). There are two distinct subtypes of HIV: HIV-1 and HIV-2, which differ in genome organization, evolutionary relationships, pathogenicity, and global burden and distribution. In contrast to HIV-1, people infected with HIV-2 have lower viral loads and slower disease progression (Gao et al., 1992). HIV-2 is primarily limited to West Africa and originated in humans as a result of zoonotic transmission of SIVsm from sooty mangabeys (*Cercocebus atys*) (Gao et al., 1992; Hirsch et al., 1989). The greatest disease burden and pathogenicity clearly lies within the HIV-1 viruses. HIV-1 originated from zoonotic transmissions of a strain of simian immunodeficiency virus (SIVcpzPtt), which infects chimpanzees (*Pan troglodytes troglodytes*), early in the 20th century (Hahn et al., 2000). HIV-1-like viruses have been isolated from captive and wild chimpanzees (Huet et al.,

1990; Keele et al., 2006). Three independent cross-species transmissions of SIVcpzPtt from *P. t. troglodytes* to humans led to three independent clusters of HIV-1 viruses grouped as major (M), outlier (O), and non-major and non-outlier (N) (Sharp et al., 2001). Group N originated in southern Cameroon and has only been detected in a restricted group of subjects from that area (Keele et al., 2006). Recently, a fourth group of HIV-1 has been identified, group P which is believed to have originated from the zoonotic transmission of a SIV (SIVgor) from gorillas (*Gorilla gorilla gorilla*) (Plantier et al., 2009; Vallari et al., 2011).

Group M, responsible for the global pandemic, was originally transmitted to humans in Cameroon and diversified into ten distinct clades (A-K) before it spread globally in the latter half of the 20th century (Gao et al., 1999; Keele et al., 2006; Korber et al., 2000). In addition, group M currently includes at least fifty-five circulating recombinant forms (CRF) of HIV-1 (Taylor et al., 2008). Clade B accounts for approximately 11% of infections globally, but because of its high prevalence in North America and Europe it has been the most thoroughly investigated (Hemelaar et al., 2011). Clade C is the single most prevalent clade of HIV-1, accounting for approximately 48% of global infections and is found primarily in sub-Saharan Africa and India (Hemelaar et al., 2011). The genetic diversity represented by these clades is substantial; for example, the HIV-1 Envelope protein can vary by 35% amino acid sequence between clades (Gaschen et al., 2002). This level of diversity creates significant challenges in the development of a vaccine. The mechanisms responsible for generating such diversity are intrinsic to the life cycle of HIV-1.

HIV-1 Virology

HIV-1 belongs to the *Retroviridea* family in the *Lentivirus* genus and has a single-stranded positive sense RNA genome encoding nine genes: Gag, Pol, Env, Nef, Vpr, Vpu, ViF Tat, and Rev. The HIV Envelope protein (Env) is the only virally-encoded surface protein and facilitates

entry into host cells. Env sequentially binds the CD4 receptor on the target cell (Klatzmann et al., 1984b), it undergoes conformational changes and then binds a co-receptor, predominantly the chemokine receptors, CCR5 or CXCR4, resulting in additional conformational changes which facilitate fusion of the viral and host membranes. The expression of these receptors dictate the susceptibility of cells to become infected, primarily CD4 T lymphocytes (Klatzmann et al., 1984a), but also myeloid cells (monocytes and macrophages) (Crowe et al., 1987), and microglial cells of the brain (He et al., 1997). Transmitted/founder viruses are almost always CCR5-tropic (Keele et al., 2008; Salazar-Gonzalez et al., 2009; Zhu et al., 1993), whereas CXCR4 and dual-tropic viruses emerge later in chronic infection (Hu et al., 2000; Nelson et al., 2000; Shankarappa et al., 1999). CCR5 is primarily expressed on activated or memory T cells (Bleul et al., 1997), which reside in large numbers in the gut-associated lymphoid tissue and are immediately depleted in acute infection (Mehandru et al., 2004). In contrast, CXCR4 is expressed on resting T cells (Bleul et al., 1997), and when the virus gains CXCR4 tropism, an additional target population becomes susceptible to infection (Blaak et al., 2000). Dendritic cells (DCs) also express CD4 and while not infected by HIV-1, they are thought to facilitate infection because they capture and transport the virus into contact with activated CD4 T cells (Altfeld et al., 2011).

After entry into the cell, the reverse transcriptase converts the RNA genome into proviral DNA. The reverse transcriptase is error-prone and does not have proofreading capabilities resulting in a high frequency of mutation, approximately 1.4 mutations per 10,000 base-pairs, which can amount to as much as 10% variation in the nucleotide sequence of the *Env* gene within a single individual (Abram et al., 2010; Korber et al., 2001; Preston et al., 1988). After reverse transcription, the proviral DNA is imported into the nucleus where the viral integrase protein incorporates the DNA into the host genome, where it is replicated by the host replication machinery. The viral genome will remain in that cell's DNA for the life of that cell and will be

passed on to progeny cells during replication and cell division (Stroud et al., 2009). The elongation of transcription is primarily moderated by the trans-activator protein (TAT) and the host transcription factor, nuclear factor kappa B (NFκB). NFκB is a transcription factor activated by multiple cytokine receptor signaling pathways. TAT and NFκB bind respective response elements encoded in the 5' long terminal repeat (LTR) of the HIV genome and cooperatively promote transcription of viral proteins (West and Karn, 1999). By this mechanism, activation of an infected cell can directly induce viral production. The initial transcripts are retained in the nucleus and undergo complete spicing, yielding short transcripts able to be exported by standard mRNA nuclear export mechanisms. Among these early transcripts is the RNA encoding Rev. Rev proteins then enter back into the nucleus and bind the Rev Response Element on the unspliced transcripts and facilitate their nuclear export (Suhasini and Reddy, 2009). The unspliced transcripts not only provide the template for the additional HIV-1 proteins, like Env, but can also serve as the genome for new virions (Suhasini and Reddy, 2009). The Gag polyproteins, which make up the viral capsid, assemble proximal to the host membrane along with other viral and host factors (Lee et al., 2012). Two copies of the genome are packaged in the protein capsid along with the viral enzymes: reverse transcriptase, protease, and integrase. As the virions bud off from the cell, they acquire a plasma membrane envelope from the host with the Env protein as the only exposed viral protein, and then undergo proteolytic processing to form mature virions (Bieniasz, 2009). Approximately 10 billion viral particles can be produced in an untreated infected individual per day (Simon and Ho, 2003).

HIV-1 Pathogenesis

Although HIV is a blood borne pathogen, the dominant form of transmission is sexually via the genital or rectal mucosa. During sexual transmission, multiple viruses may cross the mucosal barrier but usually only a single transmitted virus will seed the systemic infection (Abrahams et al., 2009; Estes, 2013; Keele et al., 2008; Salazar-Gonzalez et al., 2009). From studies in SIV-

infected rhesus macaques, it has been shown that this founder virus replicates at the site of transmission before dissemination to local lymph nodes, probably by dendritic cells, and by one week post-infection is systemic, in the plasma and distant lymphatic tissues (Fiebig et al., 2005). During acute infection, there is an exponential increase in plasma viral load followed by sequential waves of cytokines, a "cytokine storm," detected in the plasma (Stacey et al., 2009) (Fig 1.1). This can manifest as Flu-like symptoms. At the same time there is a transient drop in peripheral CD4 cell counts and a sustained depletion of CD4 cells in the gut-associated lymphoid tissue (GALT) (Mehandru et al., 2004; Veazey et al., 1998).

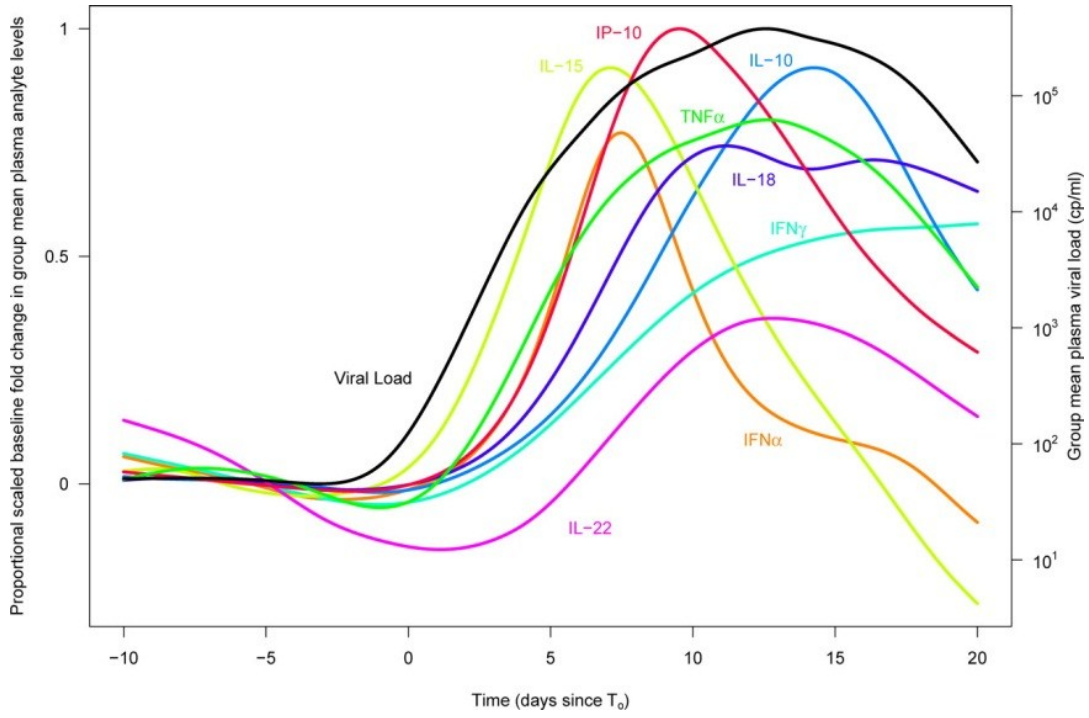


Figure 1.1 The 'Cytokine Storm' during acute HIV-1 infection. The average changes in cytokine levels as associated with time post-infection and viral load in the plasma of HIV-infected subjects during acute infection (Stacey et al., 2009).

After this initial peak of viremia, the viral load declines, and then plateaus at what is referred to as the viral set-point, as do cytokine levels which generally remain elevated compared to uninfected subjects (Stacey et al., 2009). This marks the beginning of the clinically latent phase, although it is now realized that there are many immunological defects associated with

this period, including: aberrant T cell activation, elevated cytokine levels, B cell activation, increased risk of cancers, and decreased *de novo* vaccine responses (Fournier et al., 2002; Khaitan and Unutmaz, 2011; Lane et al., 1983; Moir et al., 2001; Moir et al., 2003). The clinically latent phase can last an average of a decade and although the CD4 counts and viral loads appear to remain relatively stable it is clear that, in the absence of highly active anti-retroviral therapy (HAART), there is active viral replication, and infection and turnover of CD4 T cells especially in secondary lymphoid tissues (Pantaleo et al., 1993). It is hypothesized that the chronic immune activation caused by sustained viral infection and cell death contributes to the decline in the number of CD4 T cells and progression to AIDS. AIDS is defined as having a CD4 cell count below 200/μL, and/or the onset of opportunistic infections, including pneumocystis carinii, Kaposi's sarcoma, cytomegalovirus, and candidiasis. It was the appearance of these infections in otherwise healthy homosexual men in New York City and San Francisco, in 1981 that initially brought HIV/AIDS to the attention of the medical community ((CDC), 1981; du Bois et al., 1981; Gottlieb et al., 1981).

Pathogenesis: Immune Activation

The role of immune activation in HIV-1 disease progression was an idea that first gained traction in the nineties following the observation that activation of CD8 T lymphocytes was a better predictor of disease progression than viral load or CD4 counts (Giorgi et al., 1999). Studies in the non-human primate (NHP) model have further supported the theory that chronic immune activation promotes disease progression in HIV/SIV pathogenesis. The natural hosts for SIV, such as the sooty mangabeys, become infected and propagate virus at high titers but do not progress to immune deficiency (Gordon et al., 2007; Milush et al., 2007; Mir et al., 2011). This is in contrast to the rhesus macaques, which do not have SIV circulating in their wild populations, and suffer a decline CD4 T cell counts and progress to immune deficiency in response to SIV infection. A distinguishing feature between these two infection scenarios is

sustained immune activation in the rhesus macaques. In the SIV-infection of sooty mangabeys, there is evidence of immune activation during acute infection followed by resolution (Mir et al., 2011). It has been suggested that the unresolved immune activation in HIV-infected humans and SIV-infected rhesus macaques is due to the disruption of the gastrointestinal epithelium during acute infection and subsequent translocation of microbial products from the gut lumen across the epithelial barrier (Brenchley and Douek, 2008). Microbial products activate dendritic cells and macrophages, through pathogen recognition receptors and trigger secretion of proinflammatory cytokines. This is evident in the correlation of plasma levels of lipopolysaccharide, LPS, a highly inflammatory bacterial product, and T cell activation in HIV-infected subjects (Brenchley et al., 2006). Furthermore, the HIV/SIV-depletion of TH17 cells in the gut-associated lymphoid tissue of pathogenic HIV and SIV infections has been suggested as the underlying mechanism leading to chronic immune activation (Brenchley et al., 2008; Cecchinato et al., 2008). It is not clear what prevents the depletion of TH17 cells in the non-pathogenic SIV infection of sooty mangabeys.

HIV-1 also directly activates the immune system via pathogen recognition receptors, like toll-like receptors (TLRs). TLRs recognize pathogen-associated molecular patterns (PAMPs), which lead to activation of signaling cascades and ultimately transcription of genes central to the orchestration of the immune response (Kawai and Akira, 2005). TLR activation induces production of type I interferons and proinflammatory cytokines (Bosinger et al., 2004), discussed in more detail in “The Innate Immune Response to HIV” (see below). HIV-1 activates TLR7 on plasmacytoid dendritic cells, inducing type I interferon production (Heil et al., 2004; Meier et al., 2007b), which corresponds to increased plasma levels of IFNα and upregulated interferon-stimulated genes in chronically HIV-infected subjects (von Sydow et al., 1991). In addition, women secrete more IFNα than men in response to HIV-mediated stimulation of TLR7 and this is consistent with higher levels of immune activation and faster disease progression for the

same viral loads (Meier et al., 2009). The direct contribution of HIV-1 viremia to immune activation is further substantiated by the observed decline in immune activation following suppression of viremia by antiretroviral therapy (Baker et al., 2011; Kuller et al., 2008). However, by almost all measures, even with complete suppression of detectable viral load for a sustained period of time, there is remaining evidence of immune activation, suggesting that some but maybe not all immune activation is a direct result of active viral replication.

The Immune Response to HIV-1

The Innate Immune Response to HIV-1

The initial immune response is characterized by innate effectors, both cytokines and cellular. As previously mentioned, detectable waves of cytokines are released in direct response to the ascension of plasma viral load **(Figure 1.1)**. This in turn activates innate and adaptive immune cells. DCs and myeloid cells, like macrophages, constitutively express the anti-viral TLRs: TLR7 and/or TLR8, which recognize and are activated by poly-U or GU-rich regions of the ssRNA HIV-1 genome, signal through the adaptor MyD88 (Beignon et al., 2005; Diebold et al., 2004; Heil et al., 2004) and induce differential cytokine production depending on the receptor and cell type activated. In pDCs, the HIV-1 Env binds the CD4 receptor on the pDC which then engulfs the virion via receptor-mediated endocytosis. Host proteases and endosomal acidification uncoat the virion capsid revealing the HIV-1 RNA genome. The ssRNA HIV-1 genome activates TLR7 initiating signaling cascades resulting in secretion of type I interferon and other proinflammatory cytokines, like TNFα (Beignon et al., 2005; McKenna et al., 2005). Virtually all cell types are able to produce type I interferon (IFN) in response to viral infection, however pDCs produce the most by far.

In humans, the type I IFN family consists of 15 inducible isotypes (13 IFN-α isotypes, 1 IFN-β and 1 IFN-ω), and the IFN-κ isotype, which is constitutively expressed in DCs and keratinocytes

(Diaz et al., 1994; Nardelli et al., 2002). Type I IFNs share common receptors (IFNAR1/IFNAR2) and signal via the JAK-STAT signaling transduction pathway resulting in nuclear translocation of STAT1 homodimers which recognize and bind IFN-stimulated response elements (ISREs) in the promoter regions of genes, thereby inducing their transcription. The induced transcriptional programs include upregulation of antiviral factors, maturation of antigen presenting cells, and activation of both innate and adaptive immune cells, thus promoting the generation of the immune response.

Innate immune cells expand quickly to both: stop or blunt the infection and promote the adaptive immune response. Natural killer (NK) cells are activated by type I IFN as well as by IL-12 and IL-15 which are secreted by myeloid cells in response to TLR activation (Une et al., 2003). Upon activation NK cells proliferate, produce inflammatory anti-viral cytokines, like IFN-γ which is detectable in the plasma during acute infection **(Figure 1.1),** and increase cytotoxicity. NK cells kill virally infected cells, which they recognize through a combination of inhibitory and activating receptors (Lanier, 2008; Yokoyama, 2005). The balance of these receptors determines whether the NK cell will become activated (Lanier, 2008). The inhibitory receptors recognize proteins expressed by healthy cells such as the MHC class I proteins which all nucleated cells express. MHC I proteins serve to present endogenous peptides derived from the cytosol of the cell and thus allow immune cells to survey whether the cell is infected. Many viruses, including HIV-1, down-modulate expression of MHC I proteins in order to evade recognition from cytotoxic T lymphocytes. Since MHC I proteins interact with inhibitory receptors on NK cells, the lack of MHC on the surface of the infected cell removes this inhibition and makes the NK cell easier to activate to be cytolytic (Yokoyama, 2005). NK cells can also recognize virions or infected cells via the Fcγ receptor III, CD16, which binds the constant domain of IgG antibodies, discussed in the “Humoral Response”. There is evidence that NK cells contribute to management of HIV-1 because certain polymorphisms in NK receptors are

associated with control of HIV-1 (Martin et al., 2002; Martin et al., 2007) and there is evidence of NK cell mediated selective pressure on HIV-1 *in vivo* (Alter et al., 2011; Alter et al., 2007a).

The Adaptive Immune Response to HIV-1: Cytotoxic T Lymphocytes

While innate immune responses try to blunt HIV-1 infection, the induction of cytokines and TLR activation results in increased DC-priming of naïve T cells. T cells fall into two major categories: CD8 bearing cytotoxic T lymphocytes (CTLs) and CD4 expressing T helper cells (TH cells) both express antigen-specific T cell receptors (TCRs). CTLs are analogous to NK cells in function, secreting cytokines like IFN-γ and MIP1α, and mediating cytolysis of infected cells. However, CTL recognition of infected cells differs from NK cells. Each CTL expresses a uniquely rearranged TCR encoded from a selection of variable genes, which recognizes a unique peptide presented on a MHC class I molecule on the surface of a cell. A naïve CTL must encounter its cognate peptide/MHCI presented by an antigen-presenting cell and receive co-stimulation via CD80 or CD86 receptors, which signal via CD28 on the T cell. Then the antigen-specific CTL undergoes clonal expansion and upregulates IFN-γ and expression of cytolytic effectors: CD95, perforin and granzyme. Once primed, effector CTLs are believed to contribute directly to HIV-1 suppression via killing of HIV-infected cells by recognition of HIV-derived peptides presented by MHC I molecules on the surface of the infected cells (Harrer et al., 1996).

There are number of lines of evidence which support the hypothesis that CTLs suppress HIV-1 replication. First, there is a temporal association between the rise of HIV-specific CTLs in the periphery and the subsequent decline in plasma viremia (Borrow et al., 1994; Koup et al., 1994; Pantaleo et al., 1994). Furthermore, CTLs inflict selective pressure on the autologous virus as evidenced by detection of escape mutations in the autologous virus as early as 10 days post-infection (Borrow et al., 1997; Goonetilleke et al., 2009). Third, there are strong genetic associations of class I MHC polymorphisms and the spontaneous control of HIV-1 in the

absence of antiretroviral treatment (Carrington et al., 1999; Kaslow et al., 1996; Migueles et al., 2000). Lastly, the emergence of CTL escape mutations in the HIV-1 genome during infection from CTLs restricted by "protective" MHC I alleles can result in a loss of viral control (Dudek et al., 2012; Schneidewind et al., 2007). Within subjects who spontaneously suppress viral replication, there is evidence that both proliferation and polyfunctionality of CTL responses to the more conserved HIV-1 protein, Gag, contribute to viral suppression (Almeida et al., 2007; Harrer et al., 1996; Kiepiela et al., 2007; Migueles et al., 2002; Ogg et al., 1998). Almost all HIV-1 infected subjects have detectable CTL responses, but they are primarily monofunctional, have an "exhausted" phenotype, and unable to control viremia (Almeida et al., 2007; Day et al., 2006; Zhang et al., 2003). The specific mechanisms which allow for some responses to be protective and thus allow some individuals to control viremia remains unclear and inducing such responses by vaccination has been, thus far, unsuccessful (Buchbinder et al., 2008; Gray et al., 2011b).

The Adaptive Immune Response to HIV-1: Helper T Cells

Similar to CTLs, helper T (TH) cells require priming and clonal expansion in order to respond to a *de novo* infection. However, TH cells recognize their peptide ligand in the context of MHC class II molecules, which are only expressed on professional antigen-presenting cells: DCs, monocytes/macrophages and B cells. It is believed that the cytokine milieu, TCR affinity, and co-stimulation modulate the potential effector function of the activated TH cells. TH cells perform many possible functions including: B cell help **(Figure 1.2a),** cytotoxicity **(Figure 1.2b),** help to CTLs **(Figure 1.2c),** and regulatory functions (O'Shea and Paul, 2010; Zhou et al., 2009).

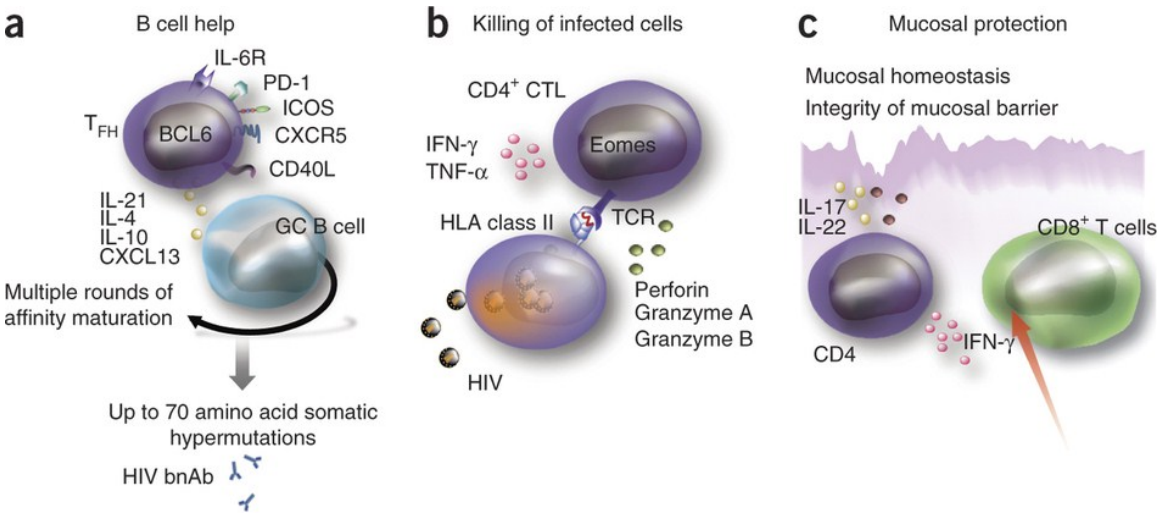


Figure 1.2 TH cells have multiple functions which may contribute to HIV-1 suppression: a) TFH cells are specialized in providing B cell help in B cell follicles and promote the necessary affinity maturation required for developing high affinity antigen-specific antibodies, b) CD4 + T cells can directly kill infected cells or secrete anti-viral cytokines, and c) The largest reservoir of CD4 T cells is in the gut mucosa where they contribute to immune surveillance and help maintain the integrity of the mucosal barrier by cytokine secretion and recruitment of CTLs and neutrophils. Modified from Streeck et al (Streeck et al., 2013).

HIV-specific TH responses have also been associated with viral control (Rosenberg et al., 1997). Specifically, during acute infection, increased frequency of HIV-specific cytotoxic TH cells, as identified by the expression of granzyme A, was associated with slower disease progression (Rosenberg et al., 1997; Soghoian et al., 2012). However it is unclear as to the mechanisms driving cytotoxic activity in TH cells. In addition, TH cells, and especially activated TH cells, are the primary target of HIV-1. Therefore activated HIV-specific TH cells become infected, and propagate virus (Douek et al., 2002; Harari et al., 2002). As a result, it is difficult to detect HIV-specific TH cells in the periphery during chronic infection (Duvall et al., 2006). The loss of HIV-specific TH cells probably contributes to the overall dysregulation of the immune response to HIV during chronic infection. The lack of HIV-specific TH cells probably has implications for the antibody response to HIV-1, since B cells require TH cell help in the generation of high affinity antigen-specific antibody responses. The subset of TH cells

specialized in providing B cell help, follicular T helper cells, are described in detail in the discussion of the humoral response.

The Humoral Response

B Cell Development

B cells were named as such in honor of the bursa Fabricus, the gut-associated gland in chickens, where they were identified as developing, as opposed to T cells which develop in the thymus. However, in mammals, B cells develop in the bone marrow (BM) from hematopoietic stem cells, while T cells still develop in the thymus (Melchers et al., 2000). B cells are the lymphocytes which secrete immunoglobulins or antibodies. With the exception of the transmembrane domain, the antibody secreted by the B cell is identical to membrane-bound B cell receptor (BCR). The BCR is comprised of two identical copies of the heavy chain and two identical copies of the light chain and has two primary regions: the variable region and the constant region. The constant region determines the effector function of the secreted antibody by differentially interacting with Fc receptors. The constant region is expressed as part of the heavy chain, but is composed of one of multiple possible gene segments which are recombined with the variable domain of the heavy chain by class-switch recombination and splicing, which is discussed later. The variable region forms the antigen recognition portion of the molecule and is determined by variable domains of the heavy and light chains. The early stages of BM B cell development revolve around the process of rearrangement of disparate immunoglobulin gene segments in order to generate functional B cell receptors (BCR) **(Figure 1.3)** (Neuberger, 2008; Tonegawa, 1983).

The heavy chain variable region is composed of a gene segment encoded by a variable (V) gene segment which encodes the first approximately 95 amino acids (aa), then the diversity (D) gene segment which encodes about 5 aa, and lastly the junction (J) segment which is the last

10-15 aa (Neuberger, 2008). Initially the pro-B cell undergoes rearrangement of the heavy chain D-J rearrangements on both chromosomes (Melchers et al., 2000). Then the pro-B cell undergoes V-DJ rearrangement on one chromosome (Melchers et al., 2000). If this yields a functional heavy chain, then it pairs with surrogate light chain and is expressed on the surface of the cell as a pre-B cell receptor, signals, and thus prevents the gene rearrangement of the heavy chain on the other chromosome assuring that the B cell will only express a single B cell receptor with a single antigen specificity; this process is called allelic exclusion (Melchers et al., 2000). Otherwise, the pro-B cell undergoes V-DJ rearrangement of the heavy chain on the second chromosome. If this does not result in a functional heavy chain, the pro-B cell undergoes apoptosis (Melchers et al., 2000). The process of gene rearrangement is mediated by a complex of enzymes including RAG1 and RAG2 and yields imprecise junctions and modifications of the nucleotides at the junctions between each pair of gene segments. When the RAG complex cuts at the RAG signal sequence, a hairpin in the DNA is created. RAG cuts this hairpin randomly creating a palindromic sequence which is then replicated in the second strand (P-nucleotides)(Neuberger, 2008). In addition there is random addition of non-templated nucleotides (N-nucleotides) by the enzyme terminal deoxynucleotidyl transferase (TDT), which predominantly affects the heavy chain because it is expressed only during a short time during B cell development (Neuberger, 2008). There is also exonuclease activity which removes nucleotides at the junction. The junction of VDJ forms the third variable loop or complementarity-determining region (CDR3) and is usually crucial in antigen recognition. When a pre-B cell receptor is expressed, the pro-B cell becomes a pre-B cell and undergoes rearrangement of the light chain also one allele at a time (Melchers et al., 2000). The recombination process is the same for the light chain. However, the light chain does not have a D gene segment **(Figure 1.3)**. Expression of a functional B cell receptor (μ heavy and light chain paired) on the cell surface signals to the cell to stop light chain recombination (Melchers et

al., 2000); this is an immature B cell. By alternative splicing a mature naïve B cell will express the germline BCR with both a μ and a δ constant domain (IgM and IgD, respectively).

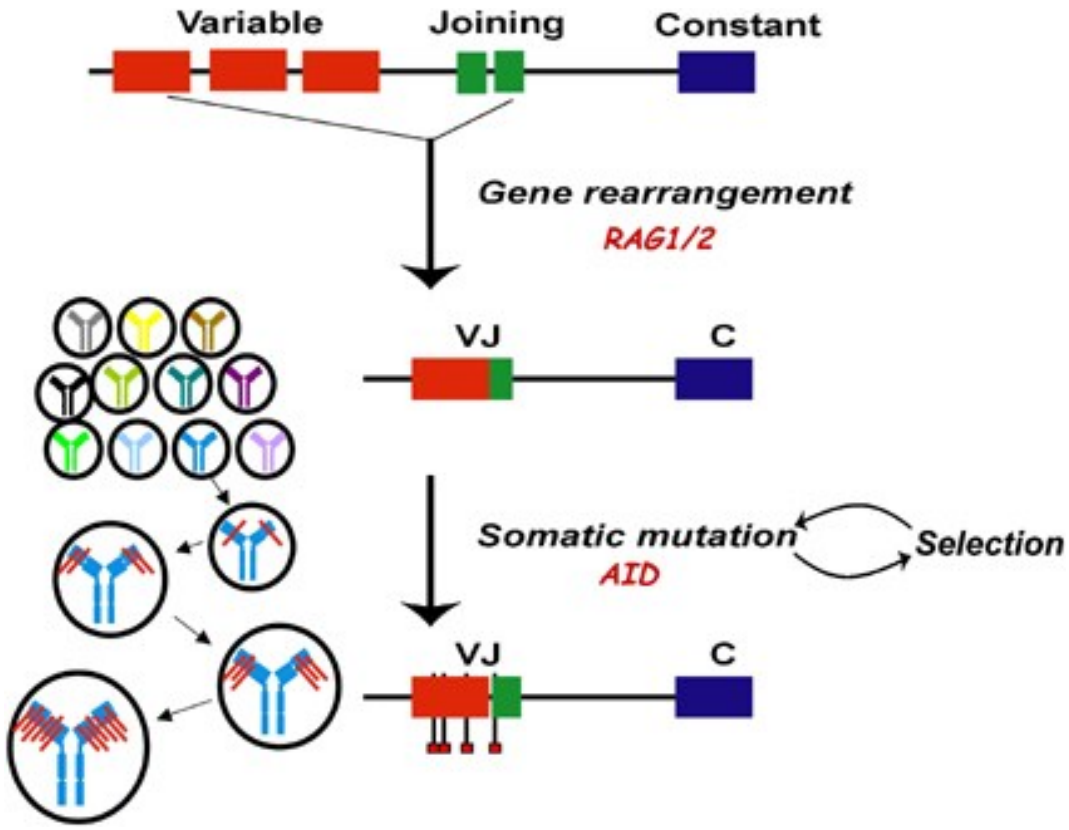


Figure 1.3 The Generation of the B cell Receptor (BCR). There are two primary mechanisms which are responsible for the generation of the variability found in the B cell repertoire. First, gene rearrangement allows for the generation of the germline BCR by combining disparate gene segments to make the variable region for either the heavy and light chain. The light chain is shown here. Second, after the naïve B cell encounters antigen it undergoes a process of somatic hypermutation, which is mediated by the mutagenic enzyme AID. AID mutates the DNA during B cell proliferation resulting in a population of B cell variants. B cells must then compete for antigen and T cell help. B cells with low affinity die, while B cells with higher affinity are selected and undergo further affinity maturation. Adapted from Neuberger (Neuberger, 2008).

The BCR is an unusual protein in that the variable region is extremely diverse as a result of combinatorial diversity acquired from the recombination of different polymorphic gene segments and junctional diversity created by imprecision of the junction. The combined diversity of possible iterations of the variable region of the BCR exceeds 10^{11}.

The Generation of an Antibody Response

After a naïve B cell expresses a functional germline BCR, it can leave the bone marrow. This naïve B cell will circulate through the periphery to secondary lymphoid organs until it meets its cognate antigen. Resident follicular dendritic cells (FDC) capture antigen and present bound antigen to B cells. This initial antigen-BCR interaction is usually of low affinity, but sufficient to activate the B cell. The BCRs binding the antigen undergo receptor aggregation and initiate signaling, activation, and receptor mediated internalization of the antigen. The antigen is processed in endosomes by cellular proteases. Antigen-derived peptides are loaded onto MHC II proteins and shuttled to the cell surface. The B cell migrates into a germinal center in response to a chemotactic gradient of CXCL13. Germinal centers (GC) arise from secondary lymphoid organ follicles and collect three types of cells: follicular dendritic cells (FDC), B cells, and follicular T helper (TFH) cells. The FDCs secrete CXCL13 to recruit B and TFH cells which both express CXCR5, the reciprocal receptor for CXCL13 (Allen and Cyster, 2008). B cells present peptides to an activated TFH cell, initiating an immune synapse with additional receptor ligand interactions facilitating mutual activation. The B cell receives stimulation from the TFH cell through CD40/CD40L and ICOS/ICOSL, as well as the cytokines: IL-21, IL-4, and CXCL13.

In the germinal center, activation of the B cell by the TFH cell results in induction of the enzyme activation-induced cytidine deaminase (AID) expression. AID generates additional variation through random mutagenesis of the variable regions of the germline BCR by somatic hypermutation (**Figure 1.3**) (Neuberger, 2008). AID functions by deaminating a cytidine to a uracil producing a U/G mismatch which can be repaired by the cellular DNA repair machinery, resulting in replacement of the original C/G nucleotide with an A/T upon replication called a transition mutation. During this process the activated B cell undergoes clonal expansion, but the induction of AID increases the mutation frequency of the variable region one million fold meaning that all of the progeny B cells vary from one another. This variation in the variable

region of the BCR has functional consequences in the affinity for the antigen for better or worse, resulting in a pool of highly related antigen-specific B cells with differing affinities. The B cells then must compete with one another for antigen from the FDCs. The B cells with higher affinity are able to bind and internalize antigen for processing and presentation to TFH cells. In contrast, B cells with reduced affinity are out-competed for antigen and therefore do not receive the signals required for survival and instead undergo apoptosis (Anderson et al., 2009). While the process of somatic hypermutation is stochastic, the process of selection ensures that germinal center B cells can undergo multiple rounds of expansion, somatic hypermutation and selection resulting in the generation of high-affinity antigen-specific antibodies. It is, therefore, not surprising that the high affinity, potent and broadly neutralizing monoclonal antibodies which have been isolated from HIV-1 infected subjects are extensively hypermutated (Kwong and Mascola, 2012).

Follicular T Helper (TFH) cells:

As stated previously, TFH cells are found in the B cell follicles of secondary lymphoid tissues and are the subset of T cells specialized to provide help to B cells through receptor-ligand interactions and secretion of IL-21 and CXCL13. TFH cells are crucial for generation of high-affinity antigen-specific antibodies. During the first wave of the humoral response low affinity antibodies are produced. TFH cells are essential in the formation of GCs (Jacobson et al., 1974), and providing the signals to B cells to undergo somatic hypermutation and class-switch recombination, which focus the antibody response. TFH cells are defined by their location in follicles, the expression of CXCR5 and their ability to provide help to B cells, particularly inducing secretion of class-switched antibodies *in vitro* in the absence of antigen or additional stimulation (Breitfeld et al., 2000; Schaerli et al., 2000). The transcriptional repressor, BCL-6, is considered to be the master regulator of TFH differentiation, as in its absence TFH cells do not

form and its forced expression is sufficient to induce a TFH cell phenotype (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009).

Naïve CD4 T cells interact with antigen presenting cells (APCs) presenting antigen-derived peptides on MHC II molecules, and upon TCR recognition and activation can differentiate into many effector types including TFH cells. TCR affinity and strong TCR signaling may contribute to driving TFH differentiation (Fazilleau et al., 2009). Additional receptor-ligand interactions of OX40/OX40L and CD40/CD40L on APCs and T cells are important for generating TFH cells. OX40 signaling on CD4 T cells upregulates CXCR5 expression and therefore aids in the migration of TFH cells into germinal centers (Flynn et al., 1998). Soluble factors also contribute to the milieu that promotes TFH differentiation. IL-6 has been implicated in mice, in particular, as has type I interferon signaling presumably through promoting IL-6 secretion by DCs (Cucak et al., 2009). IL-12 induces TFH phenotypes in human naïve T cells *in vitro,* suggesting that it may contribute to TFH differentiation *in vivo* (Nakayamada et al., 2011). Autocrine IL-21 secretion also contributes to TFH differentiation (Nurieva et al., 2008). However, once TFH cells are generated, murine studies suggest that constant antigen exposure is required to maintain them and therefore the dose of antigen is proportional to the duration and extent of the TFH response (Baumjohann et al., 2013; Deenick et al., 2010).

In addition to expressing high levels of CXCR5, TFH cells express CD40L, a key co-stimulatory molecule, which activates CD40 on B cells and APCs. Deficiency in either CD40 or CD40L results in a deficit in GC development and antibody production (Foy et al., 1994). TFH cells have also been characterized by expressing high levels of programmed cell death receptor-1 (PD-1) (Chtanova et al., 2004). PD-1 expression is induced by persistent TCR signaling and in turn negatively regulates proliferation upon engagement with its ligand (PD-L1 or PD-L2) (Freeman et al., 2000). GC B cells express high levels of both PD-1 ligands. The blocking of PD-1 signaling results in increased frequencies of TFH cells, but decreases in cytokine

production, resulting in increased GC B cell apoptosis and decreased long-lived plasma cells (Good-Jacobson et al., 2010). Another co-stimulatory molecule essential for TFH function is the inducible T cell costimulator (ICOS) which interacts with its ligand (ICOSL) expressed on B cells contributing to TFH cell activation and reciprocally on GC B cell activation (Akiba et al., 2005). In humans, ICOS deficiency manifests as defects in GCs and class-switching, and a loss of memory B cells (Warnatz et al., 2006). TFH cells are also defined by their ability to secrete IL-21 (Chtanova et al., 2004). IL-21 signaling is crucial to antibody production, affinity maturation and IgG class-switching, but also shares some functional redundancy with IL-4 (Ozaki et al., 2002). Depending on the context, IL-21 can induce expression of either BLIMP-1, which is important for plasma cell differentiation, or its antagonist, BCL-6, which contributes to AID expression (Ozaki et al., 2004).

TFH Memory Cells

In the original paper proposing TFH cells as a unique TH lineage the authors suggested that TFH cells did not form memory cells, as they express high levels of pro-apoptotic markers and probably undergo apoptosis in the absence of antigen (Breitfeld et al., 2000). However subsequent studies in the murine model suggested that TFH-like memory cells are derived from a BCL6-dependent CXCR5+CCR7+ population (Pepper et al., 2011). Adoptive transfer experiments using antigen-experienced TFH cells have demonstrated that upon rechallenge in a naïve mouse these cells gave rise to a heterogeneous effector population but preferentially acquired a TFH cell phenotype, entered GCs and secreted IL-21 (Luthje et al., 2012). These memory TFH cells had down-modulated expression of canonical TFH markers (Weber et al., 2012).

In humans, there are also circulating CD4 T cells which express CXCR5 (Breitfeld et al., 2000). They are predominantly also positive for CD45RO, a memory marker, and CCR7, allowing for

reentry into secondary lymphoid organs, which they down-modulate in response to stimulation (Chevalier et al., 2011). Multiple groups have found that peripheral CXCR5+ CD4 T cells preferentially provide help to B cells upon restimulation *in vitro* in comparison to CXCR5- CD4 T cells (Chevalier et al., 2011; Morita et al., 2011; Simpson et al., 2010). Morita et al further delineated the B cell helper function within the heterogenous circulating CXCR5+ CD4 T cell compartment to the CXCR3 negative fraction, and showed that it was increased in the periphery of patients suffering from chronic autoimmune disorders (Morita et al., 2011). A recent report from Locci et al confirmed this but further asserted that the PD-1+ CXCR3- CXCR5+ CD4 T cells were best representative of memory TFH cells in healthy donors (Locci et al., 2013). Therefore, memory TFH cells probably exist, and express moderate levels of CXCR5. However, CXCR5 expression alone is probably not sufficient to identify memory TFH cells, but instead memory TFH cells are enriched within the CXCR5+ CD4 T cell population. Furthermore, the frequencies of TFH cells and peripheral memory TFH-like cells may be related. For example, the severe reduction of GC-TFH cells detected in ICOS or CD40L-deficient humans or mice corresponds to parallel decreases in the numbers of peripheral CXCR5+ CD4 T cells (Bossaller et al., 2006; Warnatz et al., 2006).

The Humoral Response to HIV-1

B Cells: HIV-1 Infection

While B cells do not get infected by HIV, it is clear that the humoral response is directly affected by HIV-1 infection. Early in infection, B cells in the GALT undergo polyclonal activation and class-switching (Levesque et al., 2009). In chronic infection, there is noted dysregulation of B cells and antibody responses characterized by hypergammaglobulinemia (Kekow et al., 1988; Lane et al., 1983; Moir et al., 2001), decreased *de novo* responses (Malaspina et al., 2005), and altered B cell phenotypes (Moir et al., 2001). While total B cell frequencies are not dramatically

altered, specific B cell subsets are skewed. There is a loss of naïve and resting memory B cells and an expansion of immature B cells, activated memory, tissue-like memory B cells and plasmablasts. The immature B cells, which seem to expand in conjunction with lymphopenia, are inert and prone to apoptosis due to low expression of the anti-apoptotic protein, Bcl-2, and are associated with serum levels of IL-7 (Ho et al., 2006; Malaspina et al., 2007). The tissue-like memory B cells that rise as a result of chronic viremia have an exhausted phenotype characterized by increased inhibitory receptors and are enriched for HIV-specific B cells (Kardava et al., 2011; Moir et al., 2008a). The activated memory B cells are highly activated as denoted by expression of Ki-67, and CD80 and CD95. Increased plasmablasts are associated with increased Ig secretion and hypergammaglobulinemia of low affinity antibodies (Buckner et al., 2013). It is believed that these changes in B cell phenotypes during chronic infection may contribute to the decline in *de novo* responses to vaccination in chronically HIV-infected subjects.

TFH cells: HIV/SIV Infection

In HIV-1 infection, it is well established that the germinal centers represent a major reservoir of viral replication and the lymphoid architecture is severely disrupted during progressive infection (Pantaleo et al., 1993). TFH cells have been isolated from lymph node (LN) biopsies from chronic HIV-infected subjects and phenotypically and functionally characterized. TFH cells were identified as CD4 T cells expressing high levels of BCL6, CXCR5 and PD-1, and enriched among the HIV-infected CD4 T cells from the LNs (Perreau et al., 2013). They were also enriched within LN HIV-specific TH cells, which corresponds with previous studies that found that peripheral HIV-specific CD4 T cells were preferentially infected (Douek et al., 2002; Harari et al., 2002). Not only are TFH cells preferentially HIV-specific and HIV-infected, but they also support higher levels of HIV replication (Perreau et al., 2013). Surprisingly, TFH cell frequencies are maintained or even expanded in LN of chronic HIV-infected subjects (Cubas et

al., 2013; Lindqvist et al., 2012; Perreau et al., 2013). This seems to be a result of CD4 T cells actively being recruited and differentiating into TFH cells as a result of viremia because the proportion of TFH cells contracts after sustained ART and suppressed viremia (Perreau et al., 2013). The frequency of TFH cells in LNs correlated with the frequency of GC B cells and viral load (Lindqvist et al., 2012; Perreau et al., 2013). While the TFH cells had heterogeneous cytokine secretion, they were enriched for IL-21 production compared to PD-1- CD4 T cells and supported Ig secretion *in vitro* (Perreau et al., 2013). Surprisingly, the PD-1+ CXCR5- CD4 T cells most closely resembled the TFH cells in functional qualities as opposed to the CXCR5+, PD-1- CD4 T cells (Perreau et al., 2013). The CXCR5- CD4 T cells are located outside of the germinal center and it is possible that the PD-1+ CD4 T cells include a TFH transitioning population. In separate studies, the TFH cells from chronic viremic individuals were inferior in supporting antibody secretion by autologous B cells as compared to TFH cells from uninfected subjects (Cubas et al., 2013). The proposed mechanism was activation-induced upregulation of PD-L1 on GC B cells, which in turn activate the inhibitory receptor PD-1 on TFH cells and limit the ability of TFH cells to secrete IL-21 (Cubas et al., 2013). This was corroborated by blocking PD-1 or supplementing exogenous IL-21, which recovered antibody secretion from autologous B cells *in vitro* (Cubas et al., 2013).

In SIV infection of macaques, flow cytometric analysis of these markers on T cells has been more problematic, instead TFH cells have been delineated by high expression of PD-1 and Bcl-6 (Onabajo et al., 2013; Petrovas et al., 2012). These cells also express CXCR5, IL-21 mRNA, and support antibody secretion by B cells (Onabajo et al., 2013; Petrovas et al., 2012; Xu et al., 2013). The frequency of infected TFH cells was the same or greater than of other CD4 T cell subsets from the LN (Petrovas et al., 2012; Xu et al., 2013). The frequencies of TFH cells among CD4 T cells were maintained or enriched in macaques during chronic SIV infection (Hong et al., 2012b; Petrovas et al., 2012; Xu et al., 2013). TFH cell numbers increased

significantly in the LN during chronic, but not acute SIV-infection, and TFH cell numbers were correlated with follicle size, memory B cell frequencies and IgG production, but not IgM (Hong et al., 2012b). The frequency or number of TFH cells also correlated with anti-SIV antibody titers in chronic SIV-infected rhesus macaques (Hong et al., 2012b; Petrovas et al., 2012). Since there was no evidence that the TFH cells were resistant to virus-induced cytolysis, the increase or maintenance of TFH cells was hypothesized to be a result of CD4 T cells actively being recruited and differentiating into TFH cells as a result of viral-induced immune activation and IL-6 production, as IL6 is elevated in chronic SIV infection (Petrovas et al., 2012).

Recently, Locci et al. reported that the PD-1+ CXCR3- CXCR5+ peripheral CD4 T cells represent memory TFH cells, and that HIV-infected subjects who develop cross-reactive neutralizing antibody activity have a higher proportion of this phenotype among CXCR5+ CD4 T cells starting early in infection (Locci et al., 2013). Since TFH cells have been shown to be deficient in providing help to B cells in chronic HIV-1 infection (Cubas et al., 2013), initiating effective affinity maturation early may be crucial to developing a high-affinity antigen-specific antibody response that later becomes cross-reactive.

HIV-1 Envelope

The HIV envelope protein (Env) is the only virally encoded protein exposed on the surface of the virion and as such is the sole target of the neutralizing antibody response. The Env proteins form a trimeric spike composed of trimers of heterodimers of non-covalently associated gp120 and gp41 subunits. The gp120 subunit includes the CD4 and co-receptor binding sites, crucial for binding and entry of the virion into the host cell. The gp41 subunit is an integral protein which tethers the gp120 subunits to the virion, and mediates fusion of the virus with the host cells during entry. The *env* mRNA transcript contains an N-terminus signal peptide and therefore is targeted to the rough endoplasmic reticulum (ER) and into the ER by co-translational

translocation (Checkley et al., 2011). The signal peptide is removed in the ER lumen by host signal peptidases. Env is also co-translationally glycosylated with N-linked glycans (Checkley et al., 2011). Env is expressed initially as a gp160 precursor and is cleaved by the host protease, furin, in the Golgi complex, into the gp120 and gp41 subunits (Hallenberger et al., 1992). Gp120 is composed of five constant domains (C1-C5) with five variable domains (V1-V5) interspersed between constant domains (Starcich et al., 1986). A number of highly conserved cysteine residues and the di-sulfide bonds between them, help form the structure of the variable loops (Leonard et al., 1990). Gp120 and gp41 subunits remain associated as heterodimers by non-covalent interactions and oligermerize primarily as trimers. The Env complex is trafficked to the plasma membrane by vesicles and is expressed on the surface of the cell before a virion buds off taking both Env spikes and some host plasma membrane with it. The native trimer is believed to have a pre-fusion structure described as a “compact mushroom” with the apex formed by the V1 and V2 variable loops (Bartesaghi et al., 2013; Julien et al., 2013).

As the sole target of neutralizing antibodies, there are a number of features of Env which contribute to immune evasion. As already discussed the Env sequence is highly variable, while the variable loops are immunogenic and exposed, the elicited responses are strain-specific and easily escaped by the virus (Pinter et al., 2004). The V1 and V2 loops are especially hypervariable. These loops vary significantly in length and number of glycosylation sites (Palmer et al., 1996), and there is evidence that the length of these loops and glycosylation sites increases with time post infection (Sagar et al., 2006), suggesting that they are mechanisms that contribute to the evasion of the autologous immune response (Pinter et al., 2004; van Gils et al., 2011). Glycans are not limited to the V1/V2 loops, in fact the Env protein is so heavily glycosylated that glycans are estimated to contribute about half of the mass of the final protein (Leonard et al., 1990). The glycans are derived from host-machinery and therefore are less immunogenic and mask the underlying conserved epitopes (Reitter et al., 1998; Wei et al.,

2003; Wyatt et al., 1998). Additionally, the Env trimer fluctuates between conformations and is able to mask conserved epitopes (Kwong et al., 2002). Lastly, due to Vpu-mediated internalization and the dissociation of heterodimers or oligomers, there are very few (~10/virion) functional Env trimers on the surface limiting any avidity effects of the B cell binding to the virion (Zhu et al., 2003). The presence of alternate forms of the Env protein may also serve as a decoy for the immune system.

HIV-specific Antibodies

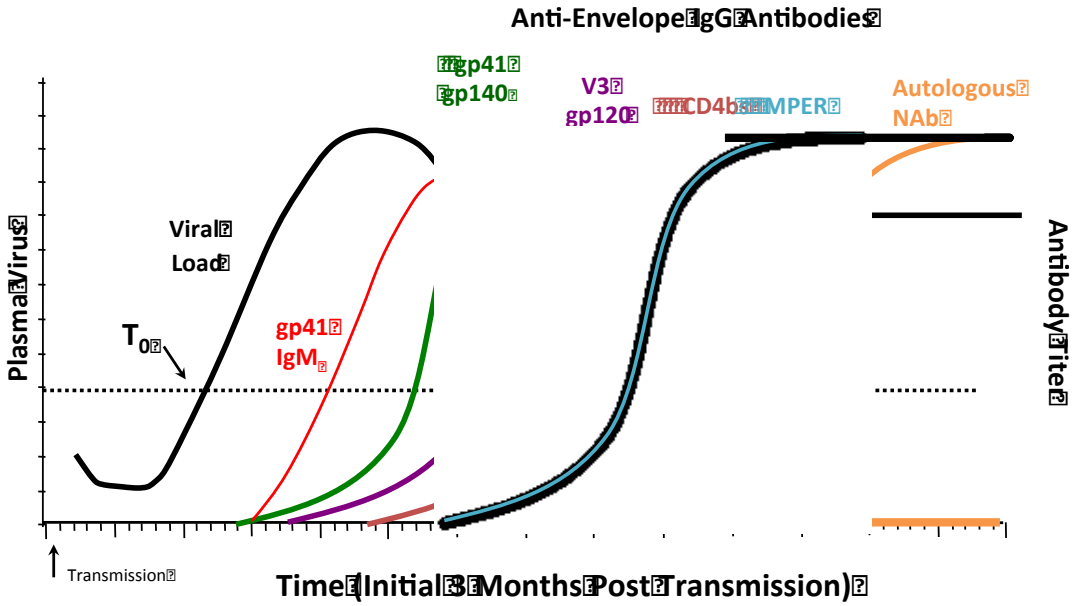


Figure 1.4 Sequential detection of plasma antibodies to HIV-1 proteins. This figure depicts the average time until detection of plasma antibodies in acutely HIV-1 clade B infected individuals. Anti-gp41 IgM antibodies are the first detectable HIV antibodies, using either autologous gp140-transmitted Env or clade B consensus Env gp140 proteins. The median time for appearance of IgG anti-gp41 antibody was 13.5 days, while the median time for appearance of IgG gp120 antibody was 28 days, and the first autologous neutralizing antibodies took 3 months. Adapted from Tomaras and Haynes, 2009. (Tomaras and Haynes, 2009; Tomaras et al., 2008).

The antibody response to HIV-1 is noticeably delayed. In the plasma, the first detected antibodies to HIV-1 are viral immune complexes about 8 days post-infection followed by non-neutralizing IgM responses to the gp41 subunit detected 2-3 weeks post-infection followed by

class-switching to IgG and IgA (Tomaras et al., 2008). Subsequent antibody responses to gp120 arise approximately 28 days post-infection. The initial gp120 response is targeted to the V3 loop and is non-neutralizing followed by weakly neutralizing V3 antibodies (Tomaras et al., 2008). There is no evidence suggesting that these early binding antibodies affect early viral kinetics or exert any immune pressure on the autologous virus (Tomaras et al., 2008). The first autologous neutralizing antibodies emerge about three months post-infection in clade B infection **(Figure 1.4)** and 1-2 months post-infection in clade C infection, followed by rapid viral escape (Davis et al., 2009). This delay is particularly apparent in comparison to other infection models. For instance, in humans immunized with attenuated yellow fever vial strain 17D, neutralizing antibodies were detected by day 31 post-administration (Belmusto-Worn et al., 2005). In addition, in mice infected with vesicular stomatitis virus, the neutralizing IgM antibody response is detectable at 4 days post-infection (Fehr et al., 1998). Therefore, the delay in generating neutralizing antibodies in HIV-1 infection may preclude the antibody response from contributing to early control of viral replication.

It is unclear what role non-neutralizing antibody Fc-mediated functions may play during early infection (**Figure 1.5)**. Antibodies from acute infection can mediate antibody-dependent cellular cytotoxicity through the Fcγ receptor III, CD16, expressed on NK cells *in vitro* (**Figure 1.5)**. Antibody-dependent complement-mediated neutralization of primary HIV-1 viruses has also been detected during primary infection (Aasa-Chapman et al., 2005) (**Figure 1.5)**. In contrast antibody-dependent complement-mediated enhancement of HIV-1 infection through interaction with the complement receptor, CR2 has also been demonstrated (Willey et al., 2011). Whether these alternate antibody effector functions are meaningful *in vivo* is unknown. Similarly, it is not understood why the early antibody responses against HIV-1 are not neutralizing, whether there is a period of affinity maturation required for these early antibody responses to evolve in affinity or whether *de novo* responses arise later in infection against new neutralizing epitopes.

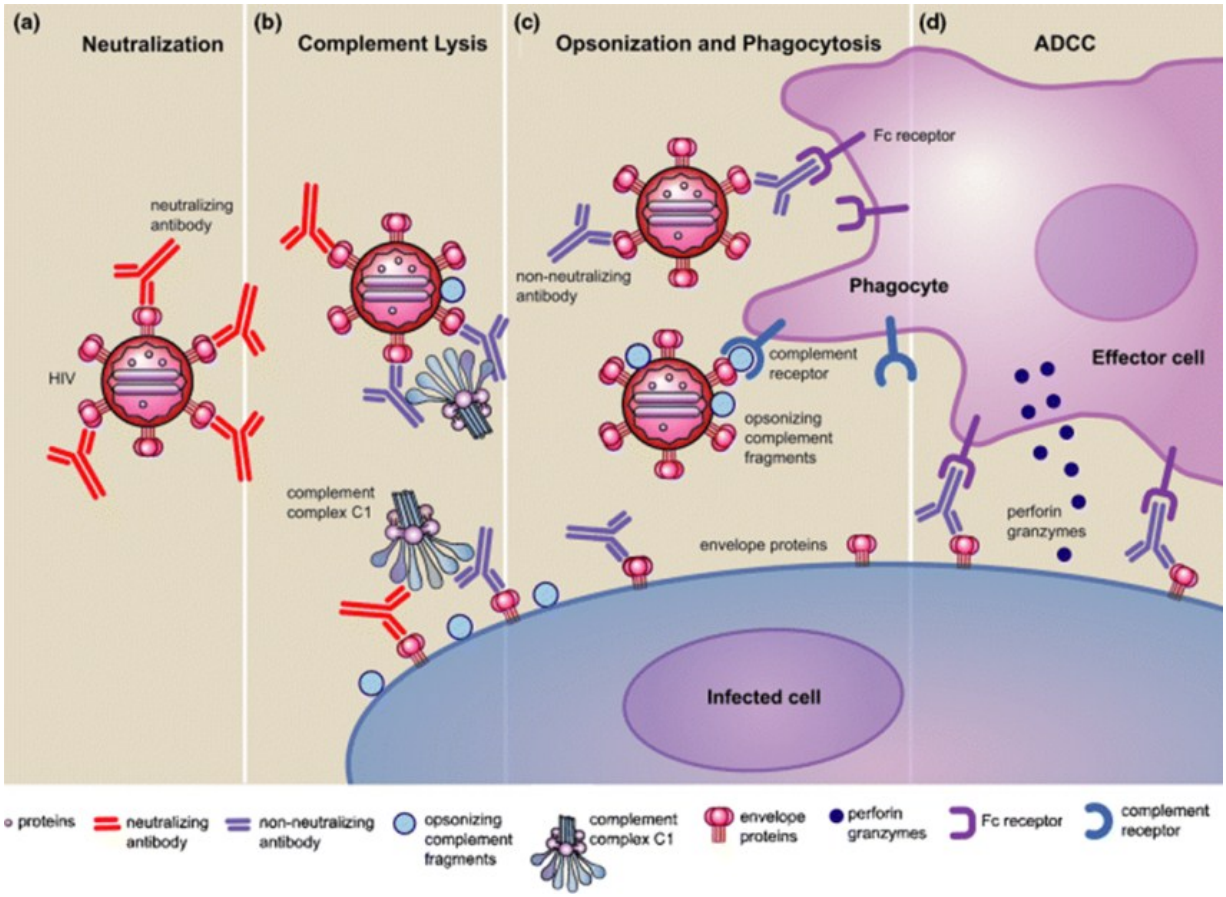


Figure 1.5 Humoral immunity to HIV-1: neutralization and antibody effector functions. a) Neutralization by antibodies binding virions and blocking their ability to infect target cells. b) Complement-mediated lysis of virions triggered by antibodies. c) Phagocytosis of opsonized virions and infected cells. d) Antibody-dependent cellular cytotoxicity (ADCC) against infected cells by release of cytolytic effector molecules (perforin and granzyme). Modified from Huber et al. (Huber and Trkola, 2007).

It remains unclear whether the antibody response to HIV-1 confers any benefit to HIV-infected persons. There is conflicting evidence as to whether an antibody response during HIV-1 infection can prevent infection with another strain of HIV-1. Overall being infected with an HIV-1 virus does not preclude one from becoming superinfected, i.e., infected with an additional strain of HIV-1 (Piantadosi et al., 2007). However, in a recent study of high-risk Kenyan women, the incidence of superinfection was significantly reduced, about half the incidence of primary infection in the surrounding population (Ronen et al., 2013). This difference was only evident after 6 months post-infection which suggests a temporal association with the development of

HIV-specific immunity, potentially antibody responses (Ronen et al., 2013). In addition, some studies suggest that having a particularly potent neutralizing antibody response does confer some protection in preventing superinfection (Smith et al., 2006). In addition, in a patient undergoing B cell depletion for lymphoma treatment, there was a dip in autologous neutralizing antibody titers followed by a viral rebound, suggesting that the antibody response may contribute to viral control at least in that subject (Huang et al., 2010).

HIV-1 Cross-Reactive Neutralizing Antibodies

Cross-Reactive Neutralizing Activity

Cross-reactive neutralizing antibodies (CRNA) are defined as the ability of serum antibodies to neutralize heterologous primary HIV-1 isolates of different clades. CRNA have been routinely detected in the plasma of a minority of HIV-infected subjects *in vitro* (Binley et al., 2004; Doria-Rose et al., 2010; Euler et al., 2010; Gray et al., 2011a; Li et al., 2009; Sather et al., 2009; Simek et al., 2009). Most researchers use a Hela-derived cell-line, TZM-bl, expressing the CD4 receptor and both main co-receptors: CCR5 and CXCR4, and an integrated luciferase gene under tight regulatory control of an HIV-1 LTR (Wei et al., 2003). TZM-bl cells are exposed to standardized panels of single round pseudoviruses pre-incubated with or without diluted heat-inactivated plasma or serum (Li et al., 2005; Mascola et al., 2005). Cell-associated luminescence is directly correlated with the extent of cells infected, and neutralization is expressed as plasma dilution which decreases in luminescence by at least 50% (IC50). In cross-sectional studies of HIV-infected cohorts, ~10-30% of subjects have plasma with CRNA (Binley et al., 2008; Doria-Rose et al., 2010; Piantadosi et al., 2009; Simek et al., 2009; van Gils et al., 2009).

While CRNA are readily detectable in some people, it is still a minority of individuals who develop these CRNA responses and it takes 2-4 years for CRNA to become detectable (Gray et

al., 2011a; Mikell et al., 2011b; Moore et al., 2011). In at least one individual, CRNA have been shown to induce escape mutations in the autologous virus, which decreased its viral fitness (Sather et al., 2012). However, in the majority of cases, CRNA do not seem to provide any particular benefit during natural infection or prevent superinfection (Blish et al., 2008). In fact, superinfection potentially promotes development of CRNA at least in some individuals (Cortez et al., 2012). Whether CRNA contribute to prevention of mother-to-child transmission via breastfeeding is also unclear; some reports suggest benefits where others do not (Barin et al., 2006; Scarlatti et al., 1993). Indeed, development of CRNA have actually been positively associated with early viral load in multiple cohorts and tends to wane after suppressive ART (Doria-Rose et al., 2010; Mikell et al., 2011b; Piantadosi et al., 2009; Sather et al., 2009). CRNA have also been associated with early envelope diversity although this was not independent of viral load (Piantadosi et al., 2009), and has been shown to evolve in parallel to viral evolution (Liao et al., 2013b). In contrast, CRNA are not exclusive to viremic individuals as it has been detected in elite controllers (Medina-Ramirez et al., 2011). CRNA have not been associated with any particular MHC haplotype (Doria-Rose et al., 2010), but has been associated with the frequency of peripheral CD4 T cells with follicular T helper phenotype (Locci et al., 2013; Mikell et al., 2011b), described in more detail in “Follicular T Helper Cells”.

CRNA have been mapped to Env-specific IgG1 antibodies with differing epitopes (Binley et al., 2008; Sather et al., 2009). They are primarily polyclonal responses meaning that multiple antibody specificities contribute to the breadth of neutralization detected (Bonsignori et al., 2012; Mikell and Stamatatos, 2012). However, some extremely potent and broad monoclonal antibodies have been isolated from some individuals, and the breadth in these individuals can be greatly recapitulated by only one or two specificities (Zhou et al., 2010).

Broadly Neutralizing Antibodies

Monoclonal antibodies, isolated from HIV-infected subjects, capable of broad and potent cross-clade neutralization of HIV-1 viral primary isolates have been termed broadly neutralizing antibodies (BnAbs). Until 2009, there were only four BnAbs isolated: B12, 4E10, 2F5, and 2G12 (Burton et al., 1994; Muster et al., 1994; Trkola et al., 1995). B12 was isolated by phage display, included random pairing of heavy and light chains (Burton et al., 1994), and recognizes the CD4 binding site through only the heavy chain (Zhou et al., 2007). Both 4E10 and 2F5 are highly polyreactive and bind the membrane proximal external region (MPER) of gp41, and 2G12 has an unusual domain-swapped structure and binds a conserved cluster of high mannose glycans on gp120 (Trkola et al., 1996). Therefore, it was very controversial whether any such antibodies could or should (in the context of the autoreactive antibodies) be elicited by immunization. However since 2009, there have been dramatic advances in isolating broader and more potent BnAbs from HIV-infected subjects, namely the development of high-throughput methods of identifying and isolating Env-specific B cells from infected subjects with broad and potent serum responses. Single-cell FACS sorting with fluorescently labeled recombinant modified Envs has been used to isolate Env-specific B cells with certain epitope specificities (Scheid et al., 2011; Wu et al., 2010). Subsequently, the variable regions of the heavy and light chains of single B cells were PCR amplified and sequenced, allowing for the production of recombinant monoclonal antibodies and testing for neutralization (Scheid et al., 2011). In fact, so many new BnAbs have been isolated that they will be summarized by epitope. There are 4 main epitopes of BnAbs that have been characterized: 1 on gp41 and 3 on gp120 **(Figure 1.6**).

Gp41-Membrane Proximal External Region (MPER)

Gp41 is the transmembrane subunit of Env, which also mediates fusion. The first gp41 specific BnAbs, 4E10 and 2F5, were poly- and autoreactive. In a humanized mouse expressing the BCR for 4E10, the B cells expressing 4E10 were deleted by tolerance mechanisms because of the autoreactivity of the BCR (Doyle-Cooper et al., 2013). Therefore, it was believed that gp41

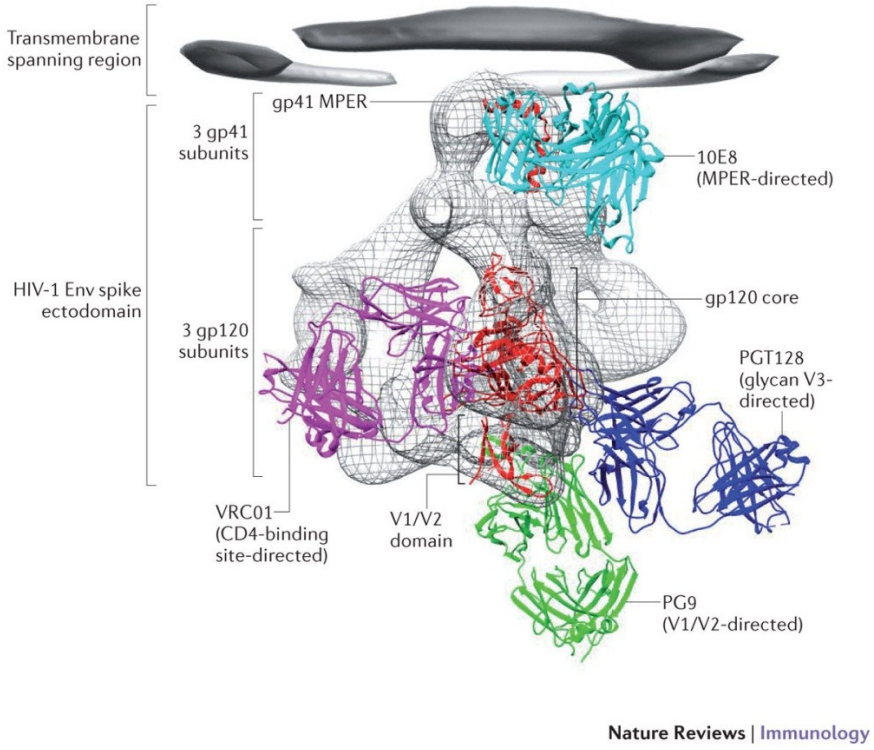


Figure 1.6 The Four Pivotal Epitopes of BnAbs on the HIV-1 Env Trimer. A cryo-electronmicroscopy immage of the HIV-1 Env trimeric spike (in light grey). The plasma membrane is at top. Atomic level ribbon models of 3 Env domains are shown (in red): gp41 MPER, gp120 core, and V1/V2. Four represenative BnAbs are illustrated by their crystal structures bound to their respective epitopes. 10E8 (light blue) binds the MPER or gp41. PGT128 (dark blue) binds the V3 of gp120. PG9 (green) binds the V1/V2-domain. VRC01 (purple) binds the CD4-binding site. Adapted from Kwong et al. (Kwong et al., 2013).

was only targeted by BnAbs that were able to also interact with the lipids in the membrane and thus autoreactive. However, in 2012, a new MPER-directed antibody was isolated and described, 10E8 **(Figure 1.6)** (Huang et al., 2012). 10E8 exceeded the previous MPER BnAbs in potency and breadth and is not autoreactive (Huang et al., 2012). While 10E8 offers new hope for targeting of gp41 by BnAbs, the lack of other BnAbs with this specificity, suggest that it is a less promising target for immunization designs.

Gp120- CD4-Binding Site

The CD4-binding site (CD4-bs) is highly conserved as it is functionally restricted to maintain binding with host cell CD4 to mediate entry and infection (Kwong et al., 1998; Wyatt et al., 1998). There are a number of BnAbs, which potently target the CD4-bs neutralizing 80-90% of heterologous viral isolates. These include: VRC01 **(Figure 1.6)**, NIH45-46, 12A12, 3BNC117 VRC-PG04 and VRC-CH31 (Scheid et al., 2011; Wu et al., 2010; Wu et al., 2011; Zhou et al., 2010). Even though they were isolated from different individuals, many of these very potent and broad CD4-bs antibodies share restricted heavy (VH1-2*02) and limited light chain usage and similar modes of interaction with Env, imitating the CD4 interaction with its binding site (Diskin et al., 2011; Wu et al., 2011; Zhou et al., 2010). These heavy chain and light chain variable regions are commonly found in the naïve B cell repertoire (Zhang et al., 2013). There is great hope that if an immunogen can activate a germline version of these antibodies and be sequentially matured that these would be good targets for a vaccination strategy.

A number of additional antibodies have been isolated that target the CD4-bs with less breadth of heterologous neutralization and lower potency, including: b12, HJ16, VRC03, 1B2530 and 8ANC131 (Burton et al., 1994; Corti et al., 2010; Scheid et al., 2011; Wu et al., 2010). These antibodies differ from the VRC01 antibodies in lineage and mode of action but may have sufficient breadth and potency if present at high titers in an individual before exposure from a sensitive viral strain. Therefore, there are multiple potential pathways in which the CD4-bs may be a feasible target for vaccination strategies.

Gp120- V1/V2

The fact that the highly conserved CD4-bs is the target of broadly neutralizing antibodies is not unexpected. However there are multiple antibodies that target the conserved epitopes on the variable loops. The first (V1) and second loop (V2) virion-associated spike form an epitope that is preferentially exposed on the virion-associated trimer as opposed to recombinant monomeric

gp120 or gp140 modified-trimers. Antibodies which potently target the V1/V2 include: PG9 **(Figure 1.6)**, PG16, PGT141-145, and CH01-04 (McLellan et al., 2011; Pancera et al., 2010; Pejchal et al., 2010; Walker et al., 2009). In the V1/V2 directed BnAbs, the mechanism of recognition tends to rely on unusually long CDR3 regions of the heavy chain which are very uncommon in the naïve repertoire (Briney et al., 2012), and contact with glycans (McLellan et al., 2011; Pejchal et al., 2010). While there is a preference for the virion-associated trimer, PG9 can bind certain gp120 monomers with a glycan at position 160. The glycan at 160 seems to be highly conserved and although V1 and V2 are dispensable for entry, they contribute to immune evasion. The conservation of the glycan at 160 is probably important for immune evasion (Kwong and Mascola, 2012). These antibodies have been predominantly isolated by limiting dilution cloning, *in vitro* stimulation and screening of supernatants by micro-neutralization assays. The preference of these antibodies for the virion-associated trimer has implications for immunogen design, as well as monitoring BnAb development in vaccination and infection.

Gp120- Glycan V3-directed

The last identified BnAb epitope is formed by glycans on the V3 loop. As stated before, the V3 loop is highly immunogenic, but generally the antibodies generated are highly strain-specific, and easily evaded. However, there is a conserved cluster of glycans in the V3 region which have probably evolved ironically as a method of viral immune evasion. Highly potent and broad neutralizers PGT121 and PGT128 **(Figure 1.6)**, as well as 2G12 and PGTs: 122, 123, 125-127, 129-131, and 135-137, target this epitope (Trkola et al., 1995; Walker et al., 2011). As stated earlier, this site was first identified after isolation of 2G12 through mutation analysis, but more recently in multiple donors with broadly neutralizing sera (Kong et al., 2013; Trkola et al., 1996). While these V3-glycan binding antibodies have various modes of binding, they all recognize one or more glycans and have distinct but overlapping epitopes. Again it seems that immune evasion by glycosylation has resulted in an epitope that is highly conserved across viral isolates.

Characteristics of BnAbs

While the epitopes targeted by sera with CRNA and of BnAbs have been well-characterized, how these responses develop during infection is not well-understood, nor is how to recapitulate such responses by vaccination. While the VRC01 family of BnAbs seem to share a similar evolution beginning with similar gene rearrangements and evolving convergent modes of Env recognition, these antibodies are also extensively hypermutated from germline (Scheid et al., 2011). For example, VRC01 is 32% mutated in the variable region of the heavy chain, and 20% mutated in the variable region of the light chain. This suggests that even if an immunogen engages the preferred gene rearrangement, the B cell would have to undergo substantial somatic hypermutation in order to achieve the breadth detected with the VRC01-like CD4-bs BnAbs. As explained previously, AID is the enzyme which mediates somatic hypermutation and usually targets specifically the hypervariable regions of the variable regions of the heavy and lights chains. However, in BnAbs, the framework regions are also mutated and these mutations contribute to the affinity and functionality of these antibodies (Klein et al., 2013). In addition, when these BnAbs are reverted to predicted germline versions, they are unable to bind to recombinant Env proteins: monomers or trimers, as detected by ELISA, surface plasmon resonance, calcium flux, or flow cytometry (Hoot et al., 2013; McGuire et al., 2013; Xiao et al., 2009).

The BnAbs that are more moderately mutated tend to be structurally unusual. For instance, PG9 is less mutated but has an unusually long CDR3, which seems to have been generated during the VDJ gene recombination step. While long CDR3s do exist in the naïve BCR repertoire in the general population, they are at a lower frequency in the memory repertoire, suggesting that they are selected against (Briney et al., 2012). Therefore, it is likely that even if an immunogen engages a BnAb precursor BCR in a naïve person it is likely that an immunization strategy will need to promote that antibody response through maturation and

selection and even into differentiation into long-lived plasma cells and memory B cells. It is not understood how to achieve this via vaccination.

Evidence for Protective Role of Neutralizing Antibodies

There is substantial evidence that BnAbs will be protective if they are present in subjects at high enough titers before exposure. In multiple macaque studies using chimeric SHIV viruses, pre-exposure passive transfer of either individual or combined BnAbs conferred sterilizing protection from intravenous or mucosal SHIV challenge (Burton et al., 2011; Hessell et al., 2009a; Hessell et al., 2009c; Mascola et al., 1999b; Mascola et al., 2000; Veazey et al., 2003b). Similar studies have been conducted in the humanized mouse studies with high efficacy (Gauduin et al., 1997; Horwitz et al., 2013; Klein et al., 2012). Importantly in these studies broadly neutralizing antibodies conferred protection whereas non-neutralizing antibodies did not (Burton et al., 2011). Furthermore BnAbs have been used in human treatment interruption interventions of HIV-infected subjects temporarily suppressing viral replication (Mehandru et al., 2007; Trkola et al., 2005). These studies support the contention that induction of BnAbs by immunization could contribute significantly to protection from acquisition of HIV-1.

Vaccines

The question remains: How do we generate BnAbs by immunization? Vaccines in general serve to prime the immune system to a pathogen in the absence of infection so that either titers of antibodies are induced which block infection from occurring or when infection occurs a rapid recall response is triggered which can quickly clear the pathogen before the infection becomes irreversibly established. A vaccine traditionally has been composed of an attenuated or killed pathogen which can present the appropriate danger signals and antigens to generate an immune response (Plotkin, 2008). Natural occurring clearance of infection tends to inform vaccine design. However, in HIV-infection that is not applicable since no one has been known to

naturally clear infection and the study of subjects who naturally suppress viral replication have been shown to be likely not antibody mediated (Harrer et al., 1996).

Initially HIV-vaccine design focused on use of recombinant Env gp120 monomer as the immunogen, but the antibody responses induced were poor at neutralization and highly-strain specific (Mascola et al., 1996). Subsequently there was a shift in the field to eliciting CTL responses in light of evidence that CTLs likely mediated suppression of viremia in absence of ART in a minority of HIV-infected subjects. However, although T cell based vaccines were immunogenic, subsequent vaccine efficacy trials yielded no efficacy at protection from HIV-infection and no impact on viral load in vaccinees (Buchbinder et al., 2008; McElrath et al., 2008). Unexpectedly in 2009, at the conclusion of a vaccine efficacy trial based in Thailand (RV144), analyses demonstrated that the vaccination had moderate efficacy in this low-risk heterosexual population (Rerks-Ngarm et al., 2009). Through additional post-hoc analyses of potential mediating mechanisms, detection of binding antibodies to the V2 of Env correlated with reduction in risk of infection (Haynes et al., 2012). While these antibody responses were not neutralizing, these results have provided substantial hope to the field that a vaccine against HIV-1 could be created which is capable of inducing immune responses which could provide sterilizing protection from HIV-1 infection.

The Goals of the Thesis Project

A key component of an effective HIV-1 vaccine will likely be a neutralizing antibody response capable of blocking infection of diverse viral isolates. Most of the studies evaluating cross-reactive neutralizing antibodies (CRNA) have been cross-sectional studies focused on the frequency and epitope specificity of such responses. Little is known of the immunological factors associated with the development of these responses during natural HIV-1 infection. Identifying those factors is central to our ability to elicit similar responses by immunization. **The overall goal of this project was to determine the immunological factors associated with**

cross-reactive neutralizing antibodies in natural HIV-1 infection. The specific aims were to determine the phenotype of peripheral T and B cells associated with development of cross-reactive neutralizing antibodies during HIV-1 infection and the mechanisms contributing to these phenotypes. In addressing these aims we utilized a cohort of HIV-infected subjects, who were identified in acute infection, followed prospectively, and whose neutralization breadth has been previously characterized by our lab (Mikell et al., 2011b).

Chapter II of this work contains a thorough characterization of plasma cytokines, and phenotypic and functional profiling of peripheral T follicular helper-like T (pTFH) cells and Env-specific B cells in subjects who develop CRNA and those who do not. We found a significant positive correlation between the development of CRNA in HIV sera with the frequency of pTFH cells and the plasma levels of the TFH and B cell-specific chemokine, CXCL13, especially early in infection (<1yr), independent of plasma viral load. The frequency of pTFH cells also correlated with B cell expression of AID, which mediates the process of somatic hypermutation. In chapter III, the potential mechanisms responsible for increased levels CXCL13 during HIV-1 infection are elucidated. The results suggest that HIV-1 induces CXCL13 secretion in monocytes by two mechanisms: directly through HIV-ssRNA activation of TLR8, and via TLR7 induced pDC-secreted type I interferon. The role of increased CXCL13 induction in development of CRNA is not clear, but may be related to efficient recruitment of B cells and TFH or pTFH cells into germinal center reactions thus promoting affinity maturation. Taken together these findings suggest that B cells require early increased TFH cell help for development of cross-neutralizing antibody responses during HIV-1 infection.

Chapter II:

Frequency of Peripheral T Follicular Helper-Like Cells Correlates with the Development of Cross-Reactive Neutralizing Antibodies HIV-1 Infection

Abstract

Cross-reactive neutralizing antibodies (CRNA) have been routinely detected in a fraction of HIV-1 infected subjects, with broad and potent monoclonal antibodies having been isolated from some. Although the epitopes targeted by many such cross-neutralizing antibodies have been identified, little is known about immunological factors associated with the development of these responses during infection. Here we show a significant correlation between the frequency of peripheral T follicular helper-like T (pTFH) cells, plasma levels of CXCL13, and development of CRNA, independent of plasma viral load. Early in infection the subjects who later developed CRNA had a higher frequency of pTFH cells, reflecting levels found in HIV-1 naïve subjects. The pTFH cells from these individuals were also more effective at inducing class-switching in autologous B cells *in vitro*. In addition, we show that B cells from these subjects expressed more activation-induced cytidine deaminase (AID) within the first year post-infection, and that AID transcript levels correlated with the frequency of pTFH cells. Therefore, our results directly link the development of CRNA against HIV-1 with pTFH cell frequency and function, and identify an early 'signature' with potential for predicting the development of cross-reactive neutralizing antibodies following HIV-1 infection and vaccination.

Introduction

A primary objective in the development of an effective vaccine against HIV-1 is the elicitation of antibodies able to neutralize heterologous viral isolates (Kwong et al., 2013; Mascola and Montefiori, 2010; McElrath and Haynes, 2010; Stamatatos et al., 2009). However despite intensive efforts over the past three decades, cross-reactive neutralizing antibodies (CRNA) have not been generated by current immunization strategies (Belshe et al., 1994; Mascola et al., 1996; Pitisuttithum et al., 2004). In contrast, CRNA are readily detectable in some HIV-1 infected subjects (see review (Stamatatos et al., 2009)). The majority of sera from HIV-1-infected subjects display narrow breadth of heterologous neutralization (the serum neutralizes fewer than 50% of heterologous strains tested against), but approximately 20% display broad neutralization of heterologous isolates (neutralization of 75% or more of heterologous strains) (Binley et al., 2004; Doria-Rose et al., 2010; Euler et al., 2010; Gray et al., 2011a; Li et al., 2009; Sather et al., 2009; Simek et al., 2009), and a very small percentage (approximately 1%) display exceptionally broad and potent CRNA (Euler et al., 2012; Sather et al., 2012; Simek et al., 2009).

CRNA become detectable approximately 2-3 years after infection (Gray et al., 2011a; Mikell et al., 2011b; Moore et al., 2011), and do not appear to offer a clinical benefit to the infected subject (Blish et al., 2008; Euler et al., 2010; Sather et al., 2012). Monoclonal antibodies (MAbs) displaying broad and potent anti-HIV-1 neutralizing activities have been isolated from HIV-1 infected subjects and have been shown to offer protection from infection in experimental animal models (Baba et al., 2000; Hessell et al., 2009b; Mascola et al., 1999a; Moldt et al., 2012; Veazey et al., 2003a; Watkins et al., 2011), reduce established plasma viremia in humanized mice infected with HIV-1 (Horwitz et al., 2013; Klein et al., 2012), and delay the viral-rebound in chronically infected humans undergoing structured ART interruption (Mehandru et al., 2007;

Trkola et al., 2005). It is believed therefore that vaccine-elicited CRNA will prevent HIV-1 infection (Mascola and Montefiori, 2010; Stamatatos et al., 2009). The development of CRNA during HIV-1 infection have been associated with plasma viremia and the time since infection (Doria-Rose et al., 2010; Piantadosi et al., 2009; Sather et al., 2009), but the precise immunological stimuli required for the development of broadly neutralizing antibodies remain unknown. Determining whether specific immunological factors are involved with the development of broad CRNA against HIV-1 is critically important for the eventual elicitation of such responses by vaccination.

Antibody affinity maturation leads to the emergence of antibodies with greater binding affinities than those produced early following infection or vaccination (Eisen and Siskind, 1964; French et al., 1989; Siskind and Benacerraf, 1969; Weiss et al., 1992), and is dependent on the help B cells receive from specialized CD4 T cells (Jacobson et al., 1974), T follicular helper (TFH) cells. TFH cells are found in the B cell follicles of secondary lymphoid organs and are characterized by high expression of ICOS, PD-1, CXCR5, Bcl-6, IL21 and CXCL13 (Breitfeld et al., 2000; Schaerli et al., 2000; Yu et al., 2009). The expression of CXCR5 by TFH cells and mature B cells allows for their co-migration into germinal centers (GC) via a CXCL13 gradient (Kim et al., 2001; Schaerli et al., 2000). Interestingly, a population of CXCR5+ CD4 T cells are also found in the periphery and are believed to encompass memory TFH cells which have down-modulated expression of many of the molecules characteristic of TFH cells (Chevalier et al., 2011; Hale et al., 2013; Kim et al., 2001; MacLeod et al., 2011; Morita et al., 2011; Schaerli et al., 2000). Upon restimulation, peripheral T follicular helper-like cells (pTFH) take on more pronounced TFH cell phenotype and provide help to B cells (Chevalier et al., 2011; Hale et al., 2013; Kim et al., 2001; MacLeod et al., 2011; Morita et al., 2011; Schaerli et al., 2000; Simpson et al., 2010).

Although TFH cells are preferentially infected during chronic HIV-1 infection of humans or SIV infection of macaques, the frequency of TFH cells is generally maintained or even expanded

(Cubas et al., 2013; Perreau et al., 2013; Petrovas et al., 2012; Xu et al., 2013). While TFH cells from chronic HIV-1 infected subjects are capable of B cell help (Perreau et al., 2013), there is evidence that the interaction between TFH and B cells in the lymph nodes is impaired (Cubas et al., 2013). Similarly, impaired pTFH cell responses have been associated with reduced efficacy of influenza vaccination in HIV-1 infected subjects (Pallikkuth et al., 2012). Our group and others have suggested that higher frequencies of peripheral CD4 T cells with TFH-like phenotypes may be associated with development of CRNA during HIV-1 infection (Locci et al., 2013; Mikell et al., 2011b). However, these studies have not investigated the functionality of these peripheral TFH-like cells during infection. Therefore, it remains unknown what role TFH cells or peripheral TFH-like cells have in the development of CRNA during HIV-1 infection. Understanding the mechanism(s) by which TFH cells participate in the development of broad serum neutralizing antibody responses during HIV-1 infection may assist the development of vaccination protocols that will lead to the elicitation of similar antibody responses by immunization. Due to the limitations associated with accessing lymph node samples, we examined how peripheral TFH-like cells may be involved in the development of CRNA during HIV-1 infection.

Materials and Methods

Plasma and PBMC Collection and Isolation: HIV-1 infected subjects were enrolled in the Harvard Cohort at Massachusetts General Hospital. Plasma and PBMCs were isolated from HIV naïve human subjects enrolled at Seattle Biomedical Research Institute. All subjects were enrolled in IRB-approved protocols and provided written informed consent. PBMCs were isolated within 4 hours of venipuncture by Histopaque centrifugation.

Cytokine Analysis: CXCL13, BAFF and IL21, were measured in plasma by ELISA (CXCL13 and BAFF ELISA kits from R&D Systems; IL-21 ELISA kit from Ebioscience). Plasma levels of

26 cytokines and chemokines (Eotaxin, G-CSF, GM-CSF, IFN-α2, IFN-γ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, TNF-β) were measured by luminex (Millipore).

T and B Cell Phenotyping: Cryopreserved and thawed PBMCs were used for flow cytometric analysis. For T cell phenotyping, 500,000-1,000,000 PBMCs were stained for Live/Dead dye, CD3, CD4, CD8, CXCR5, PD-1 and ICOS to identify peripheral TFH cells. Approximately 5 million PBMCs were sorted by magnetic bead negative selection B cell isolation kit (STEMCELL). Isolated B cells were split: half were preserved in Trizol and immediately placed at -80°C. The other half was used for flow cytometric analysis. B cells were stained with Live/Dead dye, biotinylated SF162 K160N gp120 plus streptavidin Qdot 605, CD3, CD19, CD38, CD27, and CXCR5.

RNA Extraction, cDNA Synthesis, and Gene Expression: RNA was extracted from B cells using a modified Trizol/Chloroform extraction combined with Qiagen RNAeasy MinElute columns. In brief, cells were sorted directly into 500μl chilled Trizol and frozen at -80°C. 100μl chloroform was added. The mixture was centrifuged. The aqueous phase was transferred to a separate tube and combined with RLT buffer and EtOH. The sample was transferred to Qiagen MinElute spin column in a 2ml collection tube. The rest of the Qiagen RNAeasy protocol was followed. Eluted RNA was transferred immediately to -80°C for storage. cDNA was synthesized using the QIAGEN QuantiTect Reverse Transcription Kit following the manufacturer's instructions. Expression levels of 48 genes were evaluated using the appropriate TaqMan® Gene Expression Assay (validated primer/probe sets) for each gene and TaqMan® Gene Expression Master Mix (Life Technologies) using the Fluidigm BioMark 48-well nano-chip system following Fluidigm BioMark protocols. Expression levels were analyzed using ΔCT method relative to GAPDH.

T and B Cell Co-Culture: CXCR5+ and CXCR5- CD4+ CD3+ T cells, and CD27- CD19+ CD3- B cells were sorted using a FACS Aria II. 25,000 naïve B cells were plated with varying numbers of CXCR5+ or CXCR5- CD4 T cells in 200μL media, in a 96-well plate. Co-cultures were stimulated with 1μg/mL SEB. On day 7 post-stimulation plates were spun down and supernatants were collected and immediately transferred to -80°C until analyzed for IL-21 (ELISA from R & D Systems) and Ig levels (human isotyping kit from Bioplex).

Statistical analysis: Data are presented as individual means or the medians per group of different donors. Two-tailed Mann-Whitney or Wilcoxon-paired nonparametric tests were used to determine statistical significance. Significance of p-values from gene expression data was determined by computing Q-values, using an FDR level of 0.06 (Storey and Tibshirani, 2003). Spearman correlations were used to determine significance of associations.

Results

To examine the role(s) of pTFH cells in the development of CRNA, we examined a cohort of clade B HIV-1 infected subjects who have been monitored regularly during their infection starting soon after diagnosis for up to several years (an average of 3.7 years post-infection [YPI]). The development of serum CRNA in this cohort was documented in detail previously (Mikell et al., 2011b), and is summarized here (**Figure 2.1**). All subjects remained ART naïve through the period of observation with peripheral CD4 lymphocyte counts of >200 /μl and had varying levels of plasma viremia (Mikell et al., 2011a; Mikell and Stamatatos, 2012). Five out of seventeen subjects examined developed neutralizing antibodies against at least 75% of the heterologous strains tested at some point during the period of observation (we term this group as 'broad'; **Figure 2.1**). Two subjects were excluded from further analysis as they were not followed sufficiently long enough (less than 2 YPI) to conclusively determine the breadth of CRNA. The remaining ten subjects did not develop broad CRNA at any time of observation;

their sera neutralized less than 50% of heterologous strains tested at any time during the period of observation (we term this group as 'narrow'; **Figure 2.1**). These two groups of subjects did not differ significantly in viral load either within the first year post-infection, or at the time when CRNA became detectable (approximately 2.5 YPI).

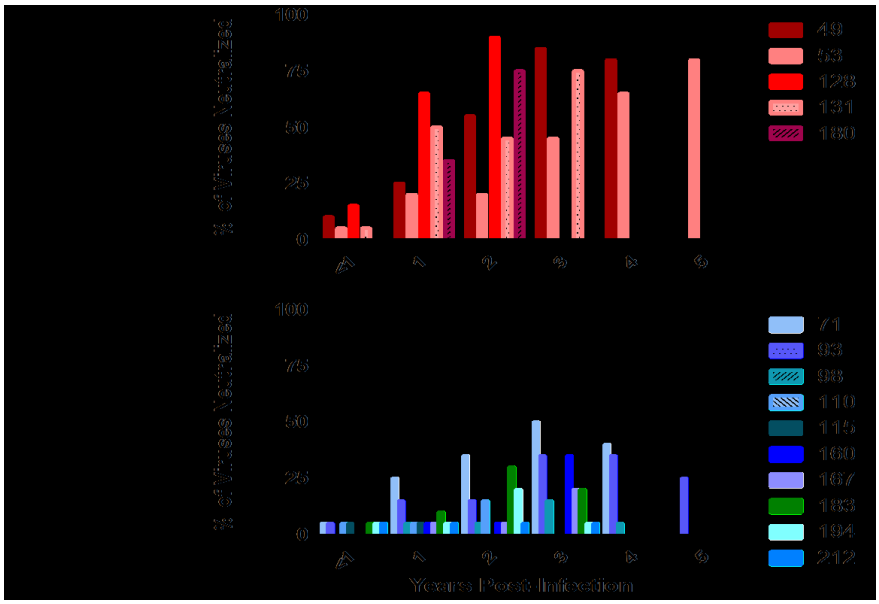


Figure 2.1 Summary of development of CRNA in cohort. The subjects who developed CRNA ('Broad') only did so after 1-3 years of infection (top). The subjects who did not develop CRNA ('Narrow') have some broadening of their neutralizing antibody response over time but it remained narrow (bottom).

Frequency of Peripheral TFH-like Cells Correlates with Breadth of Neutralization

The expression of CXCR5, ICOS, PD-1, Bcl-6, CXCL13 and IL-21 are commonly used to characterize TFH cells in secondary lymphoid organs (Breitfeld et al., 2000; Schaerli et al., 2000; Yu et al., 2009). In contrast, peripheral TFH memory cells demonstrate a much less polarized phenotype and tYPIcally only express CXCR5 of the TFH-associated markers (Luthje et al., 2012; Morita et al., 2011). However, the peripheral CXCR5+ CD4 T cells, but not CXCR5- CD4 T cells, likely contain memory TFH cells which upon re-stimulation preferentially support antibody secretion, class-switching, affinity maturation and differentiation by plasma B cells (Chevalier et al., 2011; Hale et al., 2013; Kim et al., 2001; MacLeod et al., 2011; Morita et al., 2011; Schaerli et al., 2000). We evaluated the frequencies of CD4 T cells expressing the

TFH markers: CXCR5, ICOS, PD-1 and Bcl-6, from the peripheral blood. We observed significantly higher frequencies of CXCR5 CD4+ T cells in the 'broad' than the 'narrow' CRNA groups (**Figure 2.2A**), not only at the time when CRNA became evident (at approximately 2.5 YPI; P<0.05, **Figure 2.2E**), but even more so in the first year post-infection (P=0.003, **Figure 2.2B**), before the CRNA became detectable in the 'broad' group.

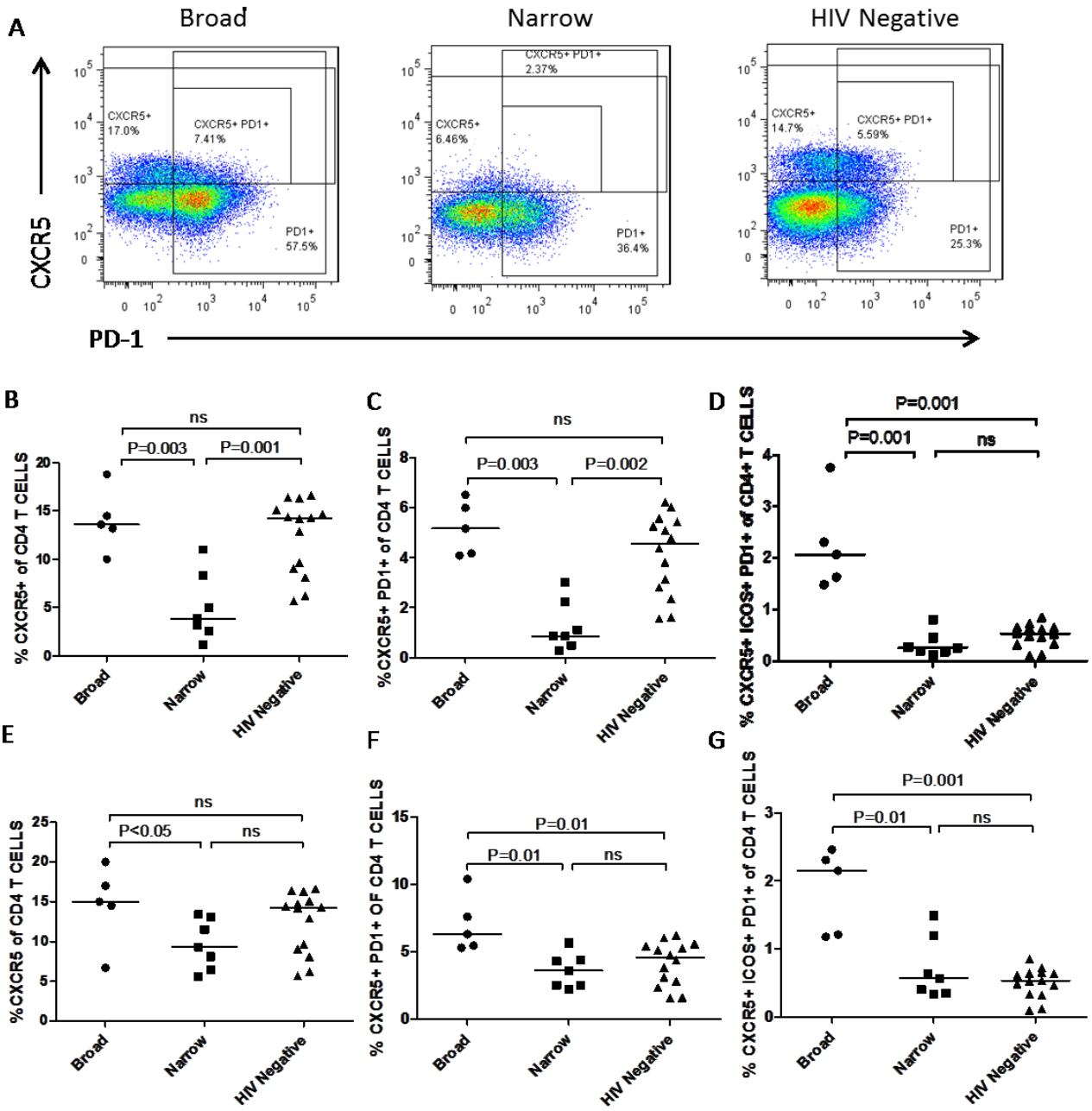


Figure 2.2 Subjects with CRNA ('Broad') have a higher frequency of peripheral TFH-like cells. A) Flow cytometry scatter plots showing CXCR5 and PD-1 expression of CD4 T cells from a representative

subject from each group: Broad (left), Narrow (middle), and HIV Negative (right). B) Frequencies of CXCR5+ CD4 T cells within the first year post-infection (YPI). C) Frequencies of CXCR5+ PD-1+ CD4 T cells within the first YPI. D) Frequencies of CXCR5+ PD-1+ ICOS+ CD4 T cells within the first YPI. E) Frequencies of CXCR5+ CD4 T cells for all subjects approximately 2.5 YPI. F) Frequency of CXCR5+ PD-1+ CD4 T cells at approximately 2.5 YPI. G) Frequency of CXCR5+ PD-1+ ICOS+ CD4 T cells at approximately 2.5 YPI. The horizontal lines represent the median. The stated p-values were calculated using Mann-Whitney test for nonparametric data and considered significant if less than 0.05.

The frequencies of CXCR5 CD4+ T cells during the first year of infection in the 'broad' group were similar to those of HIV-naïve subjects, suggesting that this population of CD4 T cells is preserved during early HIV-1 infection in those subjects who later develop CRNA. In contrast, the frequencies of CXCR5+ CD4 T cells in the 'narrow' breadth group were significantly lower than in HIV-naïve subjects (P=0.001**, Figure 2.2**). Although there was a partial rebound in the frequency of CXCR5+ CD4 T cells in the narrow group (P<0.05, **Figure 2.4**) at 2.5 YPI, the frequency in the 'broad' group was stable (**Figure 2.4**) and remained significantly increased compared to the 'narrow' group.

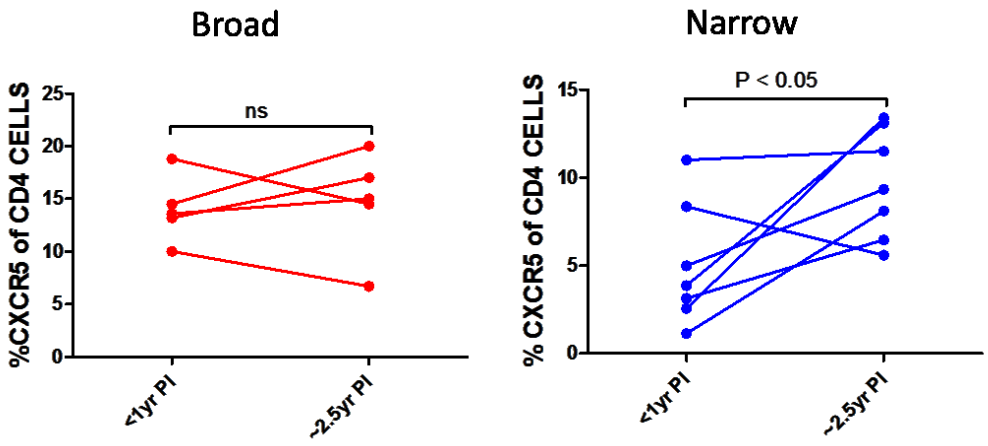


Figure 2.3 Subjects who develop CRNA ('Broad') maintain a normal frequency of peripheral TFH-like cells. In the 'broad' group, the frequency of CXCR5+ CD4 T cells remained stable across both time-points tested (left panel). In contrast, the 'narrow' group had a rebound in the frequency of CXCR5+ CD4 T cells by ~2.5 YPI. The stated P-values were calculated using Wilcoxon matched pairs test for nonparametric data and considered significant if less than 0.05.

The frequency of CD4 T cells that were both CXCR5+ and PD-1+ was higher in the 'broad' group than the 'narrow' group during the first year of infection (P=0.003, **Figure 2.2C**), and remained increased at 2.5 YPI, although less so (P=0.01, **Figure 2.2F**). The frequencies of

CD4 T cells that resemble more activated TFH cells by expressing ICOS and PD-1 (Greenwald et al., 2005; Weber et al., 2012), in addition to CXCR5, were increased in the 'broad' group at both time-points (P=0.001, **Figure 2.2D**, and P=0.009, **Figure 2.2G**). The frequency of PD-1+ CD4 T cells did not differ between the two groups during the first year of infection, but was increased later in infection in the subjects in the 'broad' group, as previously reported (Mikell et al., 2011b) (data not shown).

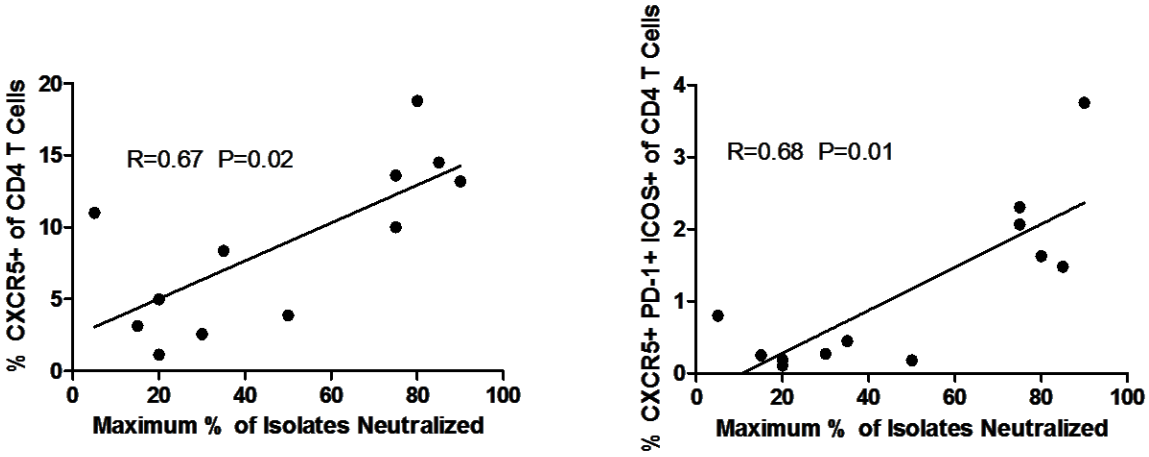


Figure 2.4 Frequency of peripheral TFH-like cells correlates with maximum breadth of serum CRNA. The frequency of CXCR5+ CD4 T cells during the first year of infection predicts the percent of heterologous isolates neutralized in chronic infection when CRNA peaks (left panel). Similarly the frequencies of pTFH cells that express CXCR5, PD-1, and ICOS during the first year of infection also predict the breadth of CRNA in chronic infection (right panel). The line is a linear regression curve. The stated R and P-values were calculated using Spearman correlation for nonparametric data and considered significant if less than 0.05.

The reasons for the reduction in the frequencies of TFH in the 'narrow' group after HIV-1 infection as compared to the 'broad' group or uninfected controls are unknown. Importantly, the frequency of CXCR5+ CD4 T cells or CXCR5+ PD-1+ ICOS+ CD4 T cells during the first year of infection predicted the breadth of CRNA (i.e., the percent of viruses neutralized by the sera (Mikell et al., 2011b)) later in infection (**Figure 2.4**), independent of viral load (multiple regression analysis: T ratio=3.4, P=0.008; T ratio=3.7, P=0.005, respectively).

B Cells of Subjects with CRNA have Higher Activation Transcription Profile

Median Relative Transcription (<1 YPI)				Broad vs Narrow	Broad vs HIV-	Narrow vs HIV-
	Broad	Narrow	HIV Negative	P-Value	P-Value	P-Value
AICDA	**0.01**	**0.002**	**0.04**	**0.02**	0.96	**0.005**
AKT	0.17	0.03	0.08	0.15	0.79	**0.002**
BAFFR/TNFRSF13C	0.51	0.29	0.56	0.53	0.79	0.22
BCL-2	0.21	0.09	0.33	0.53	0.43	**0.048**
BCL2A1	0.39	0.21	0.31	0.27	0.37	0.25
BCL2L1	0.15	0.03	0.09	0.20	0.87	**0.002**
BCL6	0.19	0.04	0.08	0.27	0.96	**0.01**
BCMA/TNFRSF17	0.13	0.01	0.07	0.20	0.96	**0.003**
BLIMP1/prdm1	0.22	0.02	0.08	0.07	0.57	**0.02**
BLNK	0.15	0.04	0.13	0.27	0.71	**0.002**
CD11b/ITGAM	**0.09**	**0.01**	**0.07**	**0.048**	0.96	**0.002**
CD19	0.24	0.06	0.16	0.20	0.87	**0.01**
CD21/CR2	0.12	0.04	0.12	0.34	0.43	**0.01**
CD22	0.48	0.29	0.68	0.88	0.32	0.07
CD23a/FCER2	0.22	0.19	0.42	1.00	0.05	**0.04**
CD27	0.11	0.01	0.08	0.07	0.87	**0.005**
CD32/FCGR2B	0.17	0.04	0.14	0.34	0.79	**0.01**
CD38	**0.16**	**0.02**	**0.08**	**0.048**	0.71	**0.00**
CD40	0.18	0.12	0.18	0.53	0.49	0.06
CD80	**0.14**	**0.01**	**0.05**	**0.03**	0.37	**0.02**
CD86	0.14	0.04	0.08	0.15	0.71	**0.03**
CD95/fas	**0.16**	**0.03**	**0.07**	**0.01**	0.23	**0.03**
CHUK	0.13	0.02	0.06	0.34	0.87	**0.01**
CXCL13	**0.01**	**0.001**	**0.04**	**0.03**	0.87	**0.02**
CXCR5	0.18	0.10	0.32	0.53	0.71	0.14
DUSP2	0.34	0.19	0.31	0.53	0.87	0.33
FCRL4	**0.06**	**0.00**	**0.04**	**0.02**	0.49	**0.02**
GAPDH	1.00	1.00	1.00	1.00	1.00	1.00
IFI27	**0.24**	**0.01**	**0.05**	**0.003**	**0.01**	**0.048**
IghG1	0.23	0.05	0.15	0.15	0.79	**0.01**
IL17A	0.39	0.16	0.36	0.11	0.79	**0.05**
IL1F1/IL1A	**0.02**	**0.004**	**0.04**	**0.048**	0.79	**0.01**
IL21r	0.19	0.06	0.14	0.43	0.96	0.08
IL6	0.13	0.06	0.16	0.20	0.57	**0.004**
IRF7	0.01	0.001	0.04	0.07	0.82	**0.02**
ISG15	**0.20**	**0.07**	0.20	**0.04**	0.43	0.06
LTA	0.09	0.04	0.07	0.27	0.87	0.06
LTB	0.25	0.06	0.17	0.27	0.64	**0.001**
MAPK1	0.14	0.03	0.07	0.27	0.79	**0.01**
NFKB1	0.16	0.09	0.09	0.34	0.96	0.33
PAX5	0.29	0.07	0.26	0.15	0.64	**0.01**
PTPN11	0.14	0.04	0.08	0.27	0.87	**0.03**
RGS13	**0.12**	**0.01**	0.04	**0.03**	0.32	0.06
STAT3	**0.11**	**0.01**	**0.07**	**0.048**	0.96	**0.005**
TLR7	0.16	0.04	0.10	0.15	0.96	**0.01**
TNFSF2/TNF	**0.19**	**0.05**	0.10	**0.048**	0.27	0.14

Table 2.1 Gene expression profile at <1 year post-infection of *ex vivo* total B cells. Blue bars indicate median transcript levels relative to GAPDH of bulk B cells *ex vivo* with significant P-values denoted in red/bold. Individual per-comparison P-values were calculated using Mann-Whitney test for

nonparametric data and considered significant if less than 0.05, at a FDR of 0.06 to account for multiple comparisons.

In parallel to the analysis on the frequencies of pTFH cells, we evaluated the frequencies of naïve, memory, and HIV-1 envelope (Env)-specific B cells by flow cytometry at <1 YPI and at ~2.5 YPI. In contrast to the observed differences in the CD4 T cell populations discussed above, there were no significant differences between the two groups in the proportion of naïve (CD3- CD19+ CD27-), memory (CD3- CD19+ CD27+), or Env-specific memory B cells (CD3- CD19+ CD27+ gp120+) of total B cells (CD3- CD19+) at either time-point (**Supplementary Figure 2.1 and Supplementary Figure 2.2**), in agreement with previous findings (Doria-Rose et al., 2009). However, we also isolated bulk B cells to evaluate from the same samples and time-points. We evaluated a specific set of genes that report on multiple facets of B cell-activation (**Supplementary Table 2.1**). We hypothesized that we would observe increased activation in the B cells of the 'broad' subjects, which we did observe in the early time-point. However, these differences were only detected at the early time-point. At ~2.5 YPI, the expression levels of these genes did not vary significantly between groups. During the first year of infection, we detected significantly higher expression of activation-associated genes (IL1A, TNF, CD11b, CD38, CD80, CD95 and FCRL4), IFN-stimulated genes (IFI27, ISG15), genes associated with germinal center B cell chemotaxis (CXCL13 and RGS13), and genes associated with somatic mutation and differentiation [AICDA **(Figure 2.5A)**, and STAT3 **(Figure 2.5B)**], in the 'broad' subjects (**Table 2.1**). Activation-induced cytidine deaminase (AID or AICDA) is an enzyme required for somatic hypermutation and class switch recombination and can be induced in B cells by TFH cells via IL-21 or ligand-receptor signaling. STAT3 expression is both induced by cytokine signaling and regulates the cellular response to cytokine signaling, including AID expression and B cell to plasma cell differentiation induced by IL-21 (Avery et al., 2010). The expression levels of AID and STAT3 were highly correlated ($R=0.87$, $P<0.001$; **Figure 2.5A**), and each correlated with the frequency of CXCR5+ CD4 T cells ($R=0.44$, $P=0.04$;

R=0.43, P<0.05, **Figure 2.5B and Figure 2.5C**). Interestingly, the gene expression profile of the 'broad' group resembled that of HIV-uninfected controls except the interferon stimulated gene, IFI27, which was upregulated in the 'broad' subjects compared to the HIV negative controls (**Table 2.1**). In contrast, the 'narrow' group had significantly reduced expression of most of the genes we evaluated compared to HIV-uninfected controls (**Table 2.1**), suggesting a deficit in B cell activation. The differences in gene expression between the 'narrow' subjects and the 'broad' subjects were no longer apparent at the later time-point (~2.5 YPI) (**Supplementary Table 2.2**).

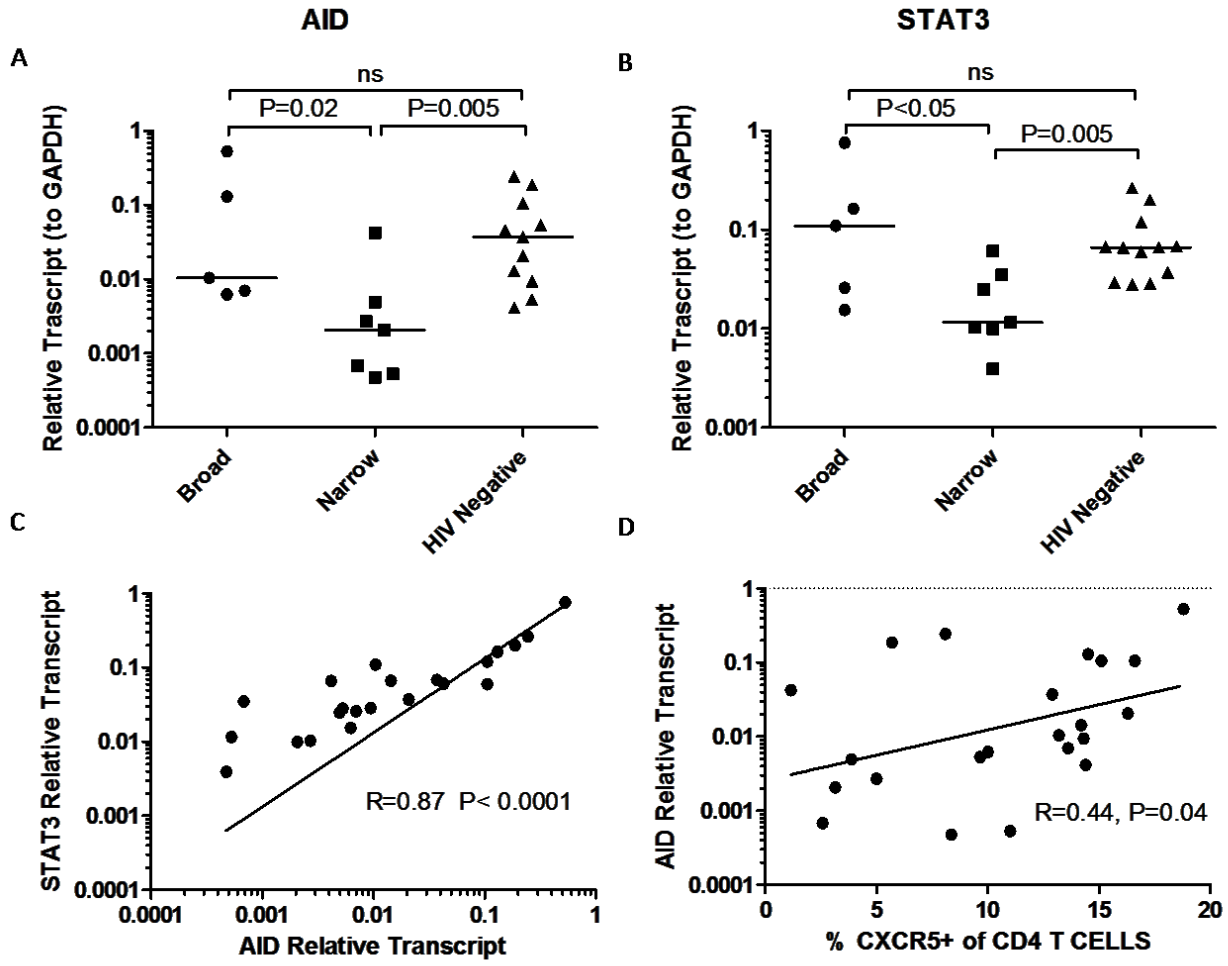


Figure 2.5 STAT3 and AID gene expression in B cells is highly correlated and each correlates with the frequency of CXCR5+ CD4 T cells. Transcript levels relative to GAPDH were used to quantify gene expression of STAT3 and AID in bulk B cells from a single time-point for HIV-1 infected and uninfected subjects. A) AID and STAT3 gene expression are significantly correlated. B) The frequency of CXCR5+ CD4 T cells correlates with the expression levels of AID. C) The frequency of CXCR5+ CD4 T cells

correlates with the expression levels of STAT3. The line is a linear regression curve. The stated R and P-values were calculated using Spearman correlation for nonparametric data and considered significant if less than 0.05.

Plasma Levels of CXCL13 Uniquely Predict Breadth of CRNA

We compared the plasma concentration levels of three B cell-tropic cytokines (CXCL13, BAFF and IL-21). Cytokine plasma levels were determined at the above-mentioned two time points of HIV-1-infection: during the first year of infection and at approximately 2.5 years of infection. We observed higher plasma concentrations of CXCL13 in the 'broad' group at both time-points (P=0.005 **Figure 2.6A,** and P=0.003 **Figure 2.6B**, respectively). CXCL13 is a chemokine, chemotactic for CXCR5 expressing cells, including B and TFH cells, and thus facilitates the recruitment and interaction between B and TFH cells in the CXCL13-rich B cell follicles and germinal centers where B cells undergo T-dependent affinity maturation and differentiation (Kim et al., 2001; Moser et al., 2002; Shi et al., 2001). Plasma levels of CXCL13 within the first year of infection correlated significantly with the maximum breadth of CRNA detected (R= 0.65, P=0.005, **Figure 2.6C**), independent of viral load. In addition, plasma CXCL13 concentrations were uniquely correlated with the frequencies of pTFH cells (CXCR5+ CD4 T cells) (R=0.67, P=0.02, **Figure 2.6D**) and of activated pTFH cells (CXCR5+, ICOS+, and PD1+ CD4 T cells) (R=0.79, P=0.002, **Supplementary Figure 2.3**).

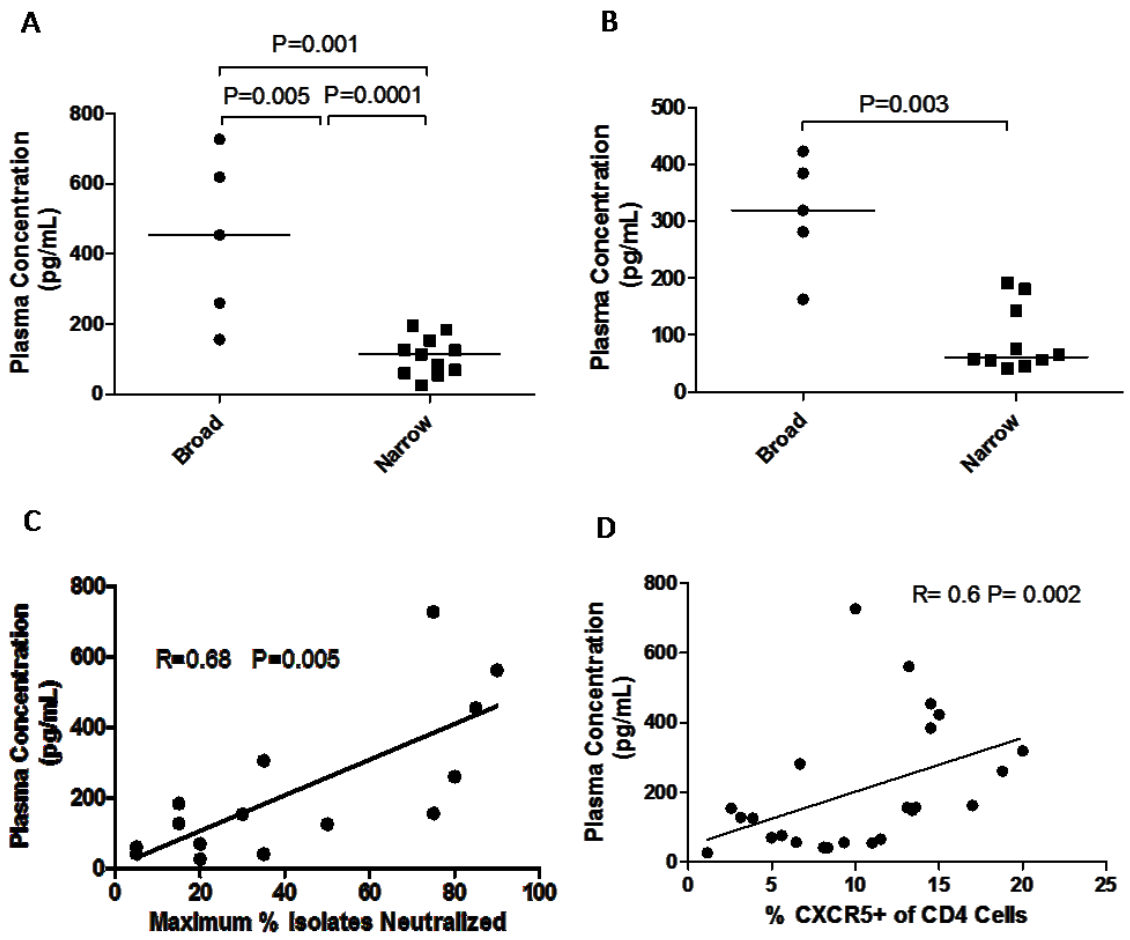


Figure 2.6 Plasma levels of CXCL13 are increased in subjects with CRNA and correlate with the maximum breadth of serum cross-neutralizing activities and the frequency of pTFH cells. A) The plasma concentrations of CXCL13 with during the first year post-infection. B) The plasma concentrations of CXCL13 ~2.5 years post-infection. Horizontal lines are at the median plasma concentration values. The stated P-values were calculated using Mann-Whitney test for nonparametric data and considered significant if less than 0.05. C) Plasma concentration of CXCL13 during the first year post-infection correlates with the maximum 'breadth' of cross-neutralizing activities (i.e., the maximum percentage of heterologous HIV-1 isolates neutralized by any given serum during the period of observation D) The plasma concentration of CXCL13 also correlates with the frequency of CXCR5+ CD4 T cells. The line is a linear regression curve. The stated R and P-values were calculated using Spearman correlation for nonparametric data and considered significant if less than 0.05.

In contrast, no association was detected between the plasma levels of IL-21 or BAFF and the breadth of CRNA or pTFH frequencies. Potentially, the higher levels of CXCL13 in the plasma of the 'broad' group may be indicative of increased systemic immune activation. To address this point, we evaluated an additional 26 cytokines and chemokines in this cohort, and in HIV negative subjects, included as controls (**Figure 2.7; Supplementary Figure 2.4**). Within the

first-year of infection, we observed significantly higher plasma levels of IP-10 (P=0.03, **Figure 2.8A**) in the 'broad' compared to the 'narrow' groups.

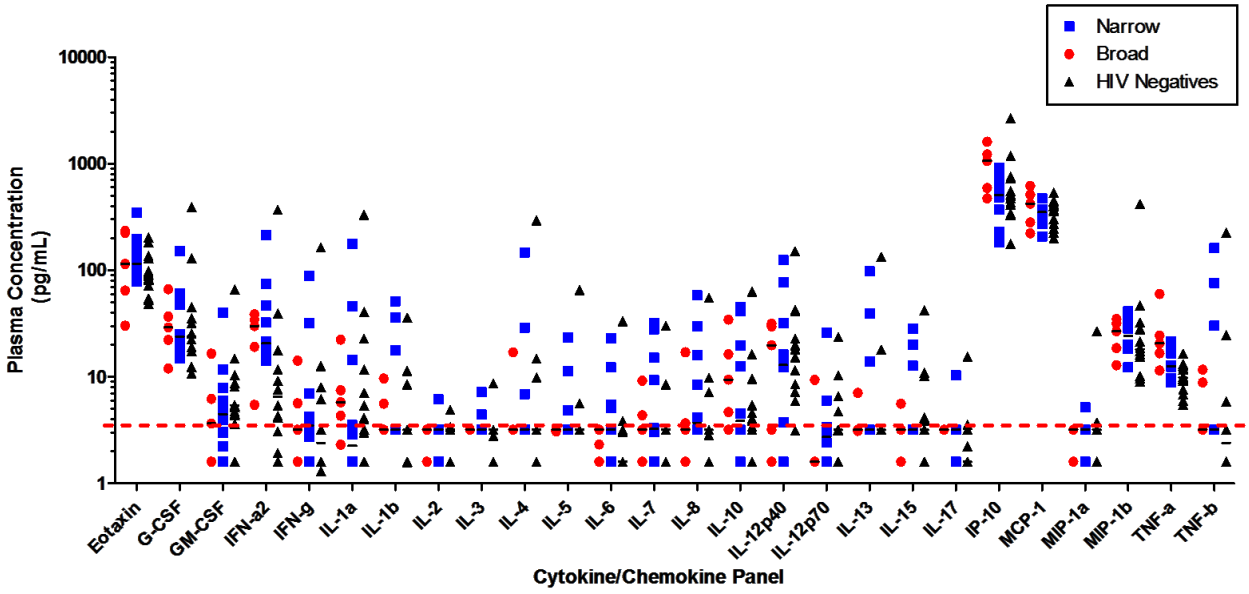


Figure 2.7 Plasma levels for 26 cytokines and chemokines did not vary significantly overall between 'Broad' and 'Narrow' groups. Values are from the first year of infection for HIV+ subjects. The red dash line indicates the detection limit (3.2 pg/mL). The horizontal lines are at the median. The stated p-values were calculated using Mann-Whitney test for nonparametric data and considered significant if less than 0.05.

However, in contrast to the observation that CXCL13 levels in first year of infection correlate with (and are predictive of) the maximum breadth of CRNA detected later in infection, no such correlation was apparent in the case of IP-10 **(Figure 2.8C)**. In addition, no difference was detected in the plasma IP-10 concentrations between the two groups at ~2.5 years post-infection **(Figure 2.8B).** Overall, mean IP-10 levels were not associated with CRNA, but were associated with mean plasma viral load (R=0.61, P=0.01; **Figure 2.8D).** Therefore, the chemokine, CXCL13, is uniquely associated with development of CRNA and the frequency of pTFH cells.

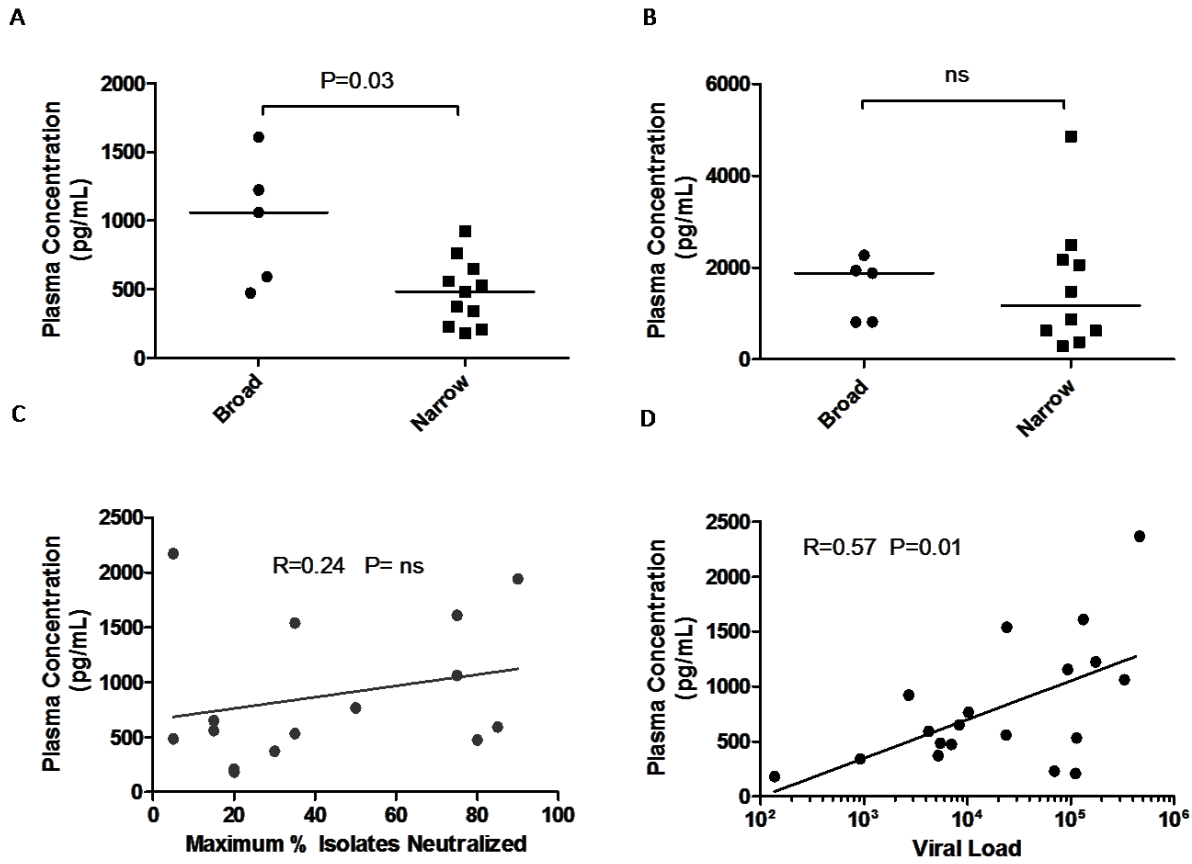


Figure 2.8 Plasma levels of IP-10 are increased in subjects with CRNA in the 1st year, but correlate with viral load not with CRNA. A) The plasma concentrations of IP-10 with in the first year post-infection. B) The plasma concentrations of IP-10 ~2.5 years post-infection. The horizontal lines are at the median. The stated p-values were calculated using Mann-Whitney test for nonparametric data and considered significant if less than 0.05. C) Plasma concentrations of IP-10 during the first year post-infection do not correlate with the maximum breadth of CRNA (i.e., the maximum percentage of heterologous HIV-1 isolates neutralized during the period of observation). D) The mean plasma concentrations of IP-10 correlate with the mean plasma viral loads. The line is a linear regression curve. The stated R and P-values were calculated using Spearman correlation for nonparametric data and considered significant if less than 0.05.

CXCR5+ CD4 T Cells from 'Broad' Subjects Induce Greater Antibody Class-Switching

Various murine immunization studies of recall responses to model antigens, and LCMV infection studies in mice along with *in vitro* studies of re-stimulated human peripheral blood CD4 T cells, demonstrated that peripheral CXCR5+ CD4 T cells, while heterogeneous, preferentially provide B cell help in comparison to their CXCR5- counterparts (Chevalier et al., 2011; Hale et al., 2013; Kim et al., 2001; MacLeod et al., 2011; Morita et al., 2011; Schaerli et al., 2000; Simpson et al., 2010). It has also been shown that CXCR5 is transiently up-regulated on many T cells subsets

after activation (Ansel et al., 1999; Langenkamp et al., 2003; Moser et al., 2002; Schaerli et al., 2001). Recent reports demonstrated reduced functionality of TFH cells during chronic HIV infection (Cubas et al., 2013). Since we demonstrated a pronounced increase in the frequency of pTFH cells during the first year of infection in the 'broad' subjects compared to the 'narrow' group **(Figure 2.2)**, we wanted to confirm that those cells were indeed specialized in providing B cell help. We also wanted to determine whether the frequency of pTFH cells had consequences in the outcome of B cell function. Lastly, we wanted to determine whether there were differences in the functionality of the pTFH cells from the 'broad' as compared to the 'narrow' subjects.

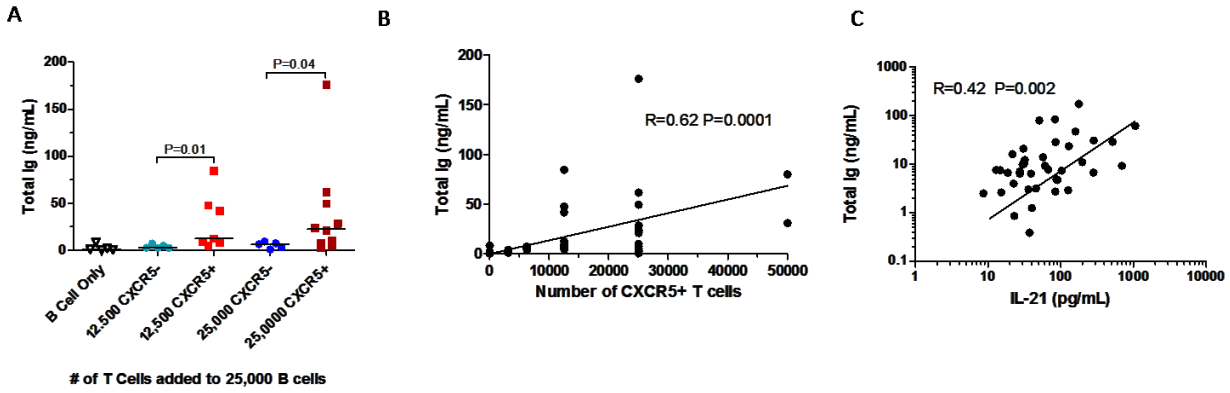


Figure 2.9 CXCR5+ CD4 T cells are specialized in providing B cell help. A) The amount of Ig detected in the supernatant of co-cultures after 7 days, from mixed subjects of both groups containing 25,000 naïve B cells co-cultured with either: 0, 12,500, or 25,000 CXCR5- or CXCR5+ T cells, and stimulated with 1µg/mL SEB. CXCR5+ CD4 T cells more effectively support antibody secretion from autologous naïve B cells, than the CXCR5- CD4 T cells in a dose-dependent manner. The horizontal line is at the median. The stated P-values were calculated using Mann-Whitney test for nonparametric data and considered significant if less than 0.05. C) Both the number of CXCR5+ CD4 T cells and C) the amount of IL-21 detected in the supernatant in the T/B cell co-cultures were correlated with the total amount of antibody detected in the supernatant across all subjects. The lines are linear regression curves. The stated R and P-values were calculated using Spearman correlation for nonparametric data and considered significant if less than 0.05.

To address these points, we isolated CXCR5+ and CXCR5- CD4 T cells from all subjects during the first year of infection and co-cultured them with autologous naïve B cells in the presence or absence of Staphococcal Endotoxin B (SEB; a T cell stimulant). Antibody and IL-21

concentrations in the supernatant were determined after 7 days. The CXCR5+ CD4 T cells secreted significantly more IL21 than CXCR5- CD4 T cells (P=0.01, **Figure 2.9A**). Likewise, the CXCR5+ CD4 T cells provided significantly better support to autologous naïve B cells for antibody-production than the CXCR5- CD4 T cells from the same subjects (P=0.0006, **Figure 2.9B**). Both the amount of secreted IL-21, and the number of CXCR5+ CD4 T cells in the co-cultures correlated with the amount of antibody produced by the autologous B cells **(Figure 2.9C).** For a given matched concentration of CXCR5+ CD4 T cells in the co-cultures, the amount of antibody secreted did not differ significantly between the two groups of subjects (**Figure 2.10A**). Taken together these observations suggest that the CXCR5+ CD4 T cells are more effective than CXCR5- CD4 T cells in providing help to B cells in a dose-dependent manner. Despite the fact that similar amounts of antibodies were produced by B cells in the presence of the same number of CXCR5+ CD4 T cells from both groups of subjects, a higher proportion of the antibodies produced by B cells from the 'broad' group were class-switched (P=0.03, **Figure 2.10B; Figure 2.10C**). Since the co-cultures contained only naïve B cells, these observations suggest that the CXCR5+ CD4 T cells from the 'broad' subjects are more effective at inducing class-switching than these cells from the 'narrow' group.

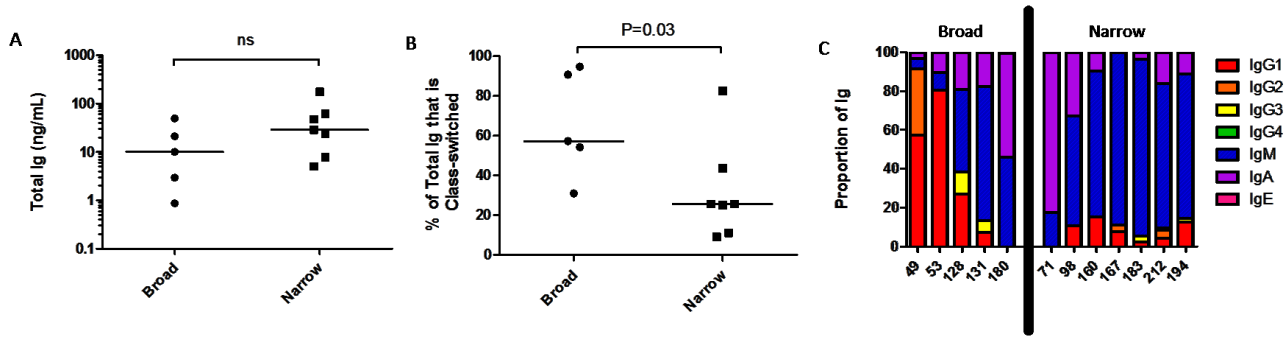


Figure 2.10 pTFH cells from the 'broad' group induce more class-switching. For equal numbers of CXCR5+ CD4 cells (25,000) co-cultured with equal numbers of naïve B cells (25,000), the amount of antibody secreted did not differ significantly between the two groups. B) However, there was a difference in the proportion of the antibody detected that was class-switched. The horizontal line is at the median. The stated P-values were calculated using Mann-Whitney test for nonparametric data and considered significant if less than 0.05. C) The proportion of the antibody detected in supernatant of each isotype for each subject.

Discussion

We report a unique correlation between the frequency of peripheral TFH-like (pTFH) cells, plasma concentrations of CXCL13 **(Figure 2.6),** and the subsequent development of broad cross-reactive neutralizing antibodies (CRNA) in HIV-1 infection **(Figure 2.4 and Figure 2.6),** independent of plasma viral load. These correlations were most pronounced within the first year post-infection, before CRNA became evident, suggesting that they may provide a predictive immunologic 'signature' of the development of CRNA during natural HIV-1 infection. Potentially, the underlying mechanisms by which pTFH, CXCL13 and CRNA are linked may be relevant and could be exploited during the evaluation of vaccine approaches to elicit CRNA.

While the frequencies of pTFH cells in the subjects who develop CRNA were maintained at physiological levels during the first year of infection, they were drastically reduced in those subjects who did not develop CRNA. Higher frequencies of pTFH cells were linked with greater production of antibodies during *ex vivo* experiments during which pTFH cells were co-cultivated with autologous naïve B cells **(Figure 2.9)**. Additionally, the pTFH cells from subjects who develop CRNA induced more extensive antibody class-switching in these co-culture experiments, than pTFH cells from subjects that did not generate CRNA **(Figure 2.10)**. Class-switching is mediated by the enzyme, AID, which is also responsible for somatic hypermutation. Indeed, we recorded higher AID transcripts in B cells from subjects who developed CRNA than subjects who did not. Therefore the increased induction of AID by TFH cells may contribute to the high rates of somatic hypermutation observed in broadly neutralizing anti-HIV-1 antibodies (Klein et al., 2013; Scheid et al., 2011; Wu et al., 2011). In addition to higher AID levels, B cells from those who developed CRNA also showed higher levels of STAT3 gene expression during the first year of infection **(Table 2.1; Figure 2.5)**. STAT3 is a crucial regulator of B cell differentiation into plasma cells and is induced via signaling by various interleukins including IL-21, which is secreted by TFH cells (Avery et al., 2010). The increased expression of STAT3

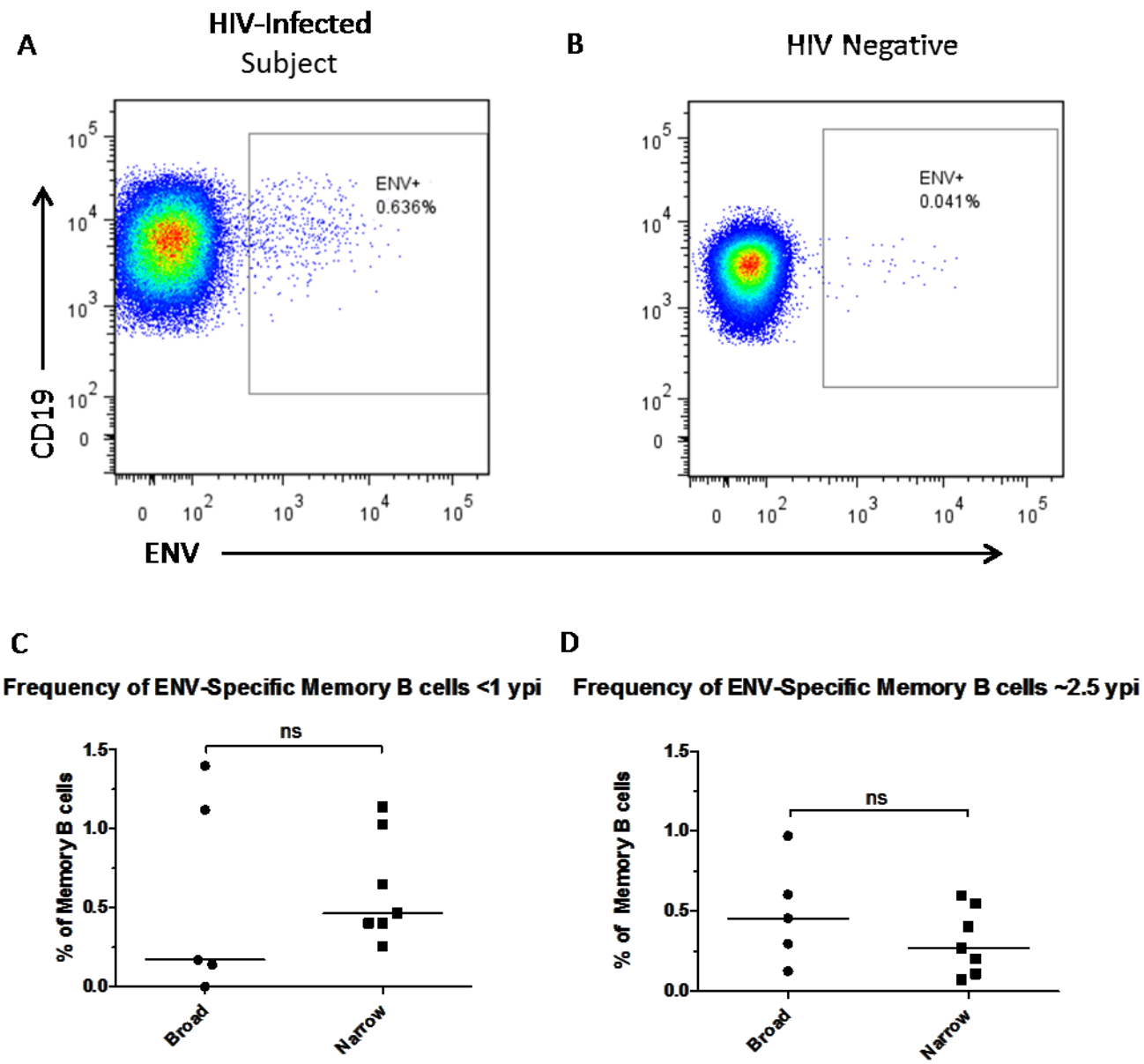
observed *ex vivo,* may be indicative of more effective IL-21 stimulation and B cell differentiation *in vivo* (Avery et al., 2010) in subjects who developed CRNA than those who did not. IL-21 and STAT3 mediated signal transduction induce AID expression, which is supported by the strong correlation between AID and STAT3 gene expression **(Figure 2.5)**. The fact that increased frequency of pTFH cells correlates with higher STAT3 and AID transcription further suggests that they are mechanistically linked **(Figure 2.5)**. The higher AID expression in B cells from subjects in the 'broad' compared to the 'narrow' group is suggestive of increased somatic hypermutation in subjects who develop broad CRNA. Lastly, we hypothesize that the higher levels of CXCL13 detected in the plasma may be a result of, or contribute to the higher frequency and activation of pTFH cells.

Based on these observations, we propose a model in which the development of CRNA in HIV-1 infection is dependent on the early preservation of TFH cells, as indicated by the generation and circulation of TFH-like cells in the periphery. This is similar to LCMV infection where the ability of the neutralizing antibody response to broaden and evolve in chronic infection is limited by the availability and functionality of CD4 T cell help (Ciurea et al., 2001). The data presented here suggest that in early HIV-1 infection the subjects from the 'narrow' group suffer a deficit in B cell activation potentially related to the loss of CXCR5+ CD4 T cells. Therefore maintaining physiological frequencies of TFH cells early following infection appears to be crucial for the development of high-affinity antigen-specific antibody response. Affinity maturation is considered essential for the generation of broadly neutralizing antibodies, as the ones that have been isolated have evidence of extensive somatic hypermutation (Kwong and Mascola, 2012). However, antibody lineages which later become broadly neutralizing have been detected as early as 14 weeks post-infection, highlighting the importance of this early window in infection in the development of these responses (Liao et al., 2013a).

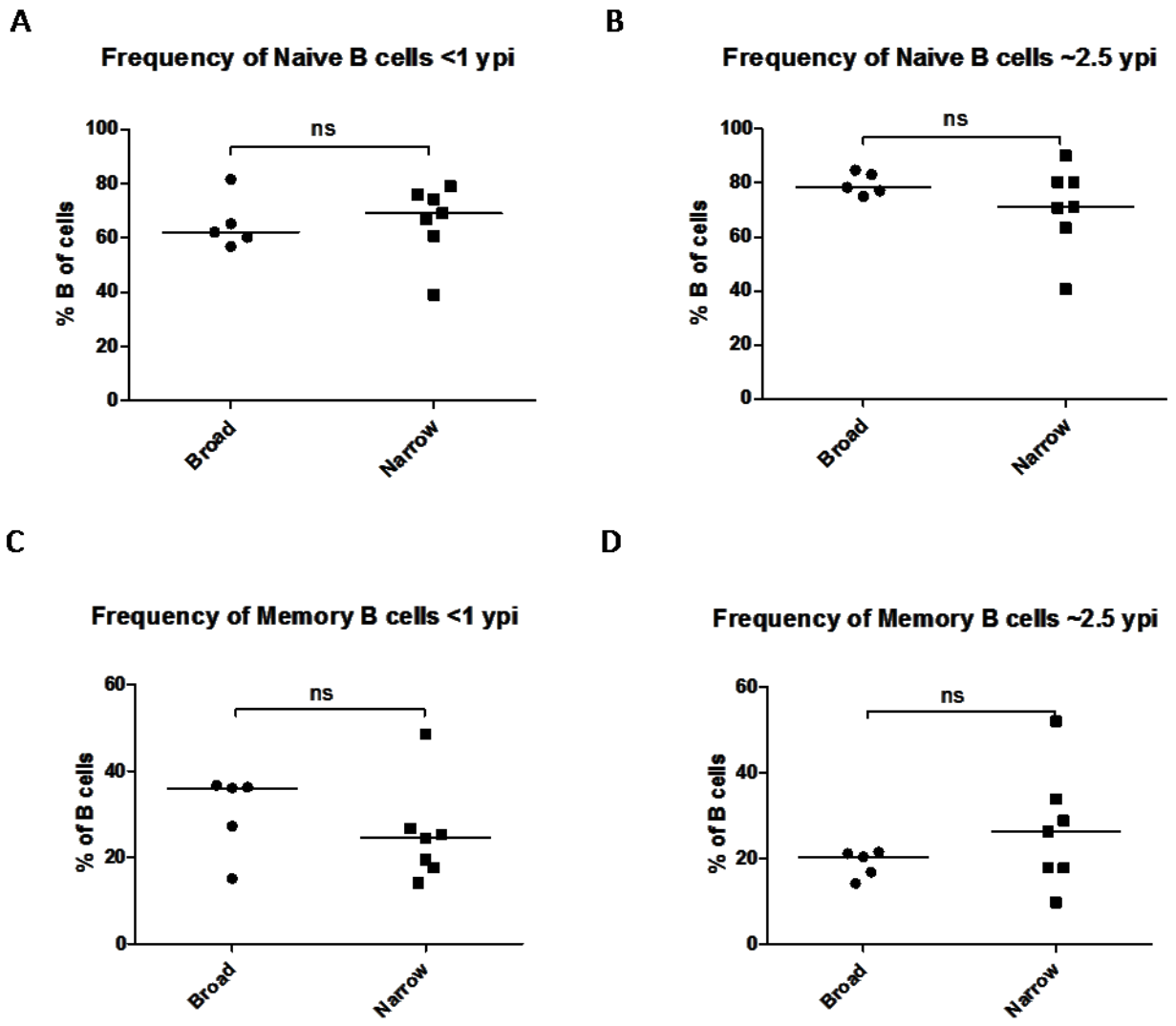
The mechanisms allowing for the preservation of pTFH or TFH cells during early HIV-1 infection are unknown. Potentially, the TFH cells in the 'narrow' group are preferentially destroyed, either by HIV-1-mediated cell-lysis (HIV is known to infect TFH (Perreau et al., 2013)), or by anti-HIV-1 CTL cells, which emerge during the first few weeks after infection (Koup et al., 1994). It is also possible that the majority of TFH cells in the 'narrow' group are retained in the lymph nodes after HIV-1 infection, but in the case of the 'broad' group the traffic between the periphery and lymph nodes of TFH cells is not disrupted. Possibly, the TFH cell pool is more readily replenished in the 'broad' group through DC/cytokine-mediated generation from naïve CD4 T cells or increased proliferation of TFH cells. While we were not able to evaluate the frequency or functionality of the TFH cells from the secondary lymphoid organs from the subjects studied here, there have been previous studies to suggest that the frequencies of these two populations are inherently linked. For example, the severe reduction of GC-TFH cells detected in ICOS or CD40L-deficient humans or mice corresponds to parallel decreases in the numbers of peripheral CXCR5+ CD4 T cells (Bossaller et al., 2006; Warnatz et al., 2006).

Broadly neutralizing antibodies against HIV-1 are extensively somatically hypermutated (Kwong and Mascola, 2012), and our results directly implicate the CXCR5+ CD4 T cells in this process. We suggest that peripheral TFH-like cells and plasma CXCL13 levels should be further investigated to understand their relationship with development of CRNA, and may provide a useful 'signature' for evaluating antibody responses by vaccination.

Supplementary Materials:



Supplementary Figure 2.1 The frequency of Env-specific memory B cells did not differ significantly between groups. A) A flow plot of a representative HIV-infected subject gated on CD27+ CD19+ CD3- live lymphocytes. B) A flow plot of a representative HIV-uninfected subject gated on CD27+ CD19+ CD3- live lymphocytes. C) The frequencies of Env-specific memory B cells did not differ significantly at <1 year post-infection. D) The frequencies of Env-specific memory B cells did not differ significantly at 2-3 years post-infection. P-values were calculated using Mann-Whitney test for nonparametric data and considered significant if less than 0.05.

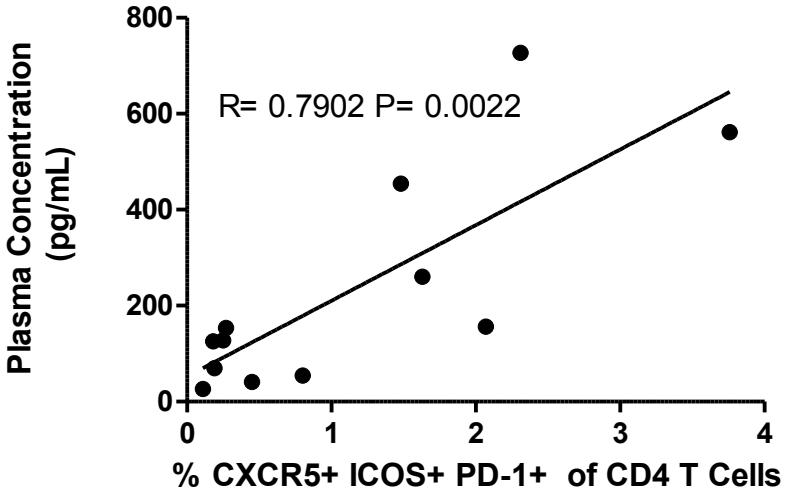


Supplementary Figure 2.2 Frequency of B cell subsets were not significantly different between groups. A) The frequencies of naïve B cells (CD27- CD19+ CD3-) were not significantly different at <1 year post-infection. B) The frequencies of naïve B cells (CD27- CD19+ CD3-) were not significantly different at 2-3 years post-infection. A) The frequencies of memory B cells (CD27+ CD19+ CD3-) were not significantly different at <1 year post-infection. B) The frequencies of memory B cells (CD27+ CD19+ CD3-) were not significantly different at 2-3 years post-infection. P-values were calculated using Mann-Whitney test for nonparametric data and considered significant if less than 0.05.

Gene	Type	Details
AICDA	Cytokine	RNA-editing deaminase, somatic hypermutation, and class-switching.
AKT	Transcription Factor	Regulates cell survival (bcl2), regulation of NF-kappa-B-dependent gene transcription.
BAFFR/TNFRSF13C	Receptors	Binds BAFF, activates NFKB and BCL2, cell survival and prolifferation.
BCL-2	Transcription Factor	Anti-apoptotic.
BCL2A1	Transcription Factor	BFL1; induced by NFKB transcription; Promotes survival.
BCL2L1	Transcription Factor	BCLXL; ANTI-Apoptosis
Bcl-6	Transcription Factor	Transcriptional repressor which is required for germinal center formation and antibody affinity maturation
BCMA/TNFRSF17	Receptors	Binds BAFF/APRIL, activates NFKB & JNK, cell survival and prolifferation.
BLIMP1/prdm1	Transcription Factor	Plasma cell differentiation.
BLNK	Transcription Factor	Important for the activation of NF-kappa-B and NFAT; BCR signaling
CD11b/ITGAM	Receptors	CR3, associated with SLE.
CD19	Receptors	BCR co-receptor.
CD21/CR2	Receptors	CR2, binds C3d and IFNa; BCR co-receptor.
CD22	Receptors	Binds sialyated glycoproteins including CD45; Pos/Neg BCR regulator.
CD23a/FCER2	Receptors	FcERII mostly expressed on GC B cells.
CD27	Receptors	Binds CD70; signals NFKB activation; memory marker.
CD32/FCGR2B	Receptors	FcGRII low affinity, inhibitory.
CD38	Receptors	Ectoenzyme essential for Ca regulation; activation marker; expressed on plasma cells.
CD40	Receptors	Binds CD40L.
CD80	Receptors	Activation; interacts with cd28 on T cells
CD86	Receptors	Activation; interacts with cd28 on T cells
CD95/fas	Receptors	(FasR, APO, APT-1, TNFRSF6) induces apoptosis; activation.
CHUK	Transcription Factor	IKKA; degradation of the inhibitor via the ubiquination pathway, thereby activating NFKB.
CXCL13	Cytokine	Chemokine important for TFH/GC migration.
CXCR5	Receptors	CXCL13 chemokine receptor, important for TFH/GC migration.
DUSP2	Transcription Factor	Protein phosphatase; regulate members of the mitogen-activated protein (MAP) kinase superfamily.
FCRL4	Receptors	Fc receptor-like 4; inhibitory receptor, binds IgG. Expressed on epithelium memory B cells.
GAPDH	Housekeeping	
IFI27	Cytokine	Interferon, alpha-inducible protein 27.
IghG1	Cytokine	Antibody-secretion.
IL17A	Cytokine	Pro-inflammatory cytokine

IL1F1/IL1A	Cytokine	IL1a; proinflammatory cytokine.
IL21r	Receptors	Interleukin receptor.
IL6	Cytokine	Pro-TFH cytokine; TFH and B cell modulate cytokine through IL6R.
IRF7	Transcription Factor	Regulator of type I interferon (IFN)-dependent immune response.
ISG15	Cytokine	Ubiquitin-like protein; induced by interferon-alpha.
LTA	Cytokine	(TNFβ) inducible inflammatory, immunostimulatory, and antiviral responses.
LTB	Cytokine	(TNFC) inducible inflammatory, immunostimulatory, and antiviral responses.
MAPK1	Transcription Factor	Mitogen-activated protein kinase 1: proliferation, differentiation, transcription regulation and development.
NFKB1	Transcription Factor	Pleiotropic transcription factor; cellular responses to stimuli such as cytokines and stress and plays a key role in regulating the immune response to infection.
PAX5	Transcription Factor	B-cell differentiation; Involved in the regulation of the CD19 gene, a B-lymphoid-specific target gene.
PTPN11	Transcription Factor	SHP2; negative regulator of signaling.
RGS13	Transcription Factor	Regulator of G protein signaling.
STAT3	Transcription Factor	Mediates cellular responses to interleukins (including IL-21), and other growth factors.
TLR7	Receptors	Innate anti-viral; Upregulated in response to type I IFN.
TNFSF2/TNF	Cytokine	TNFα; pro-inflammatory cytokine.

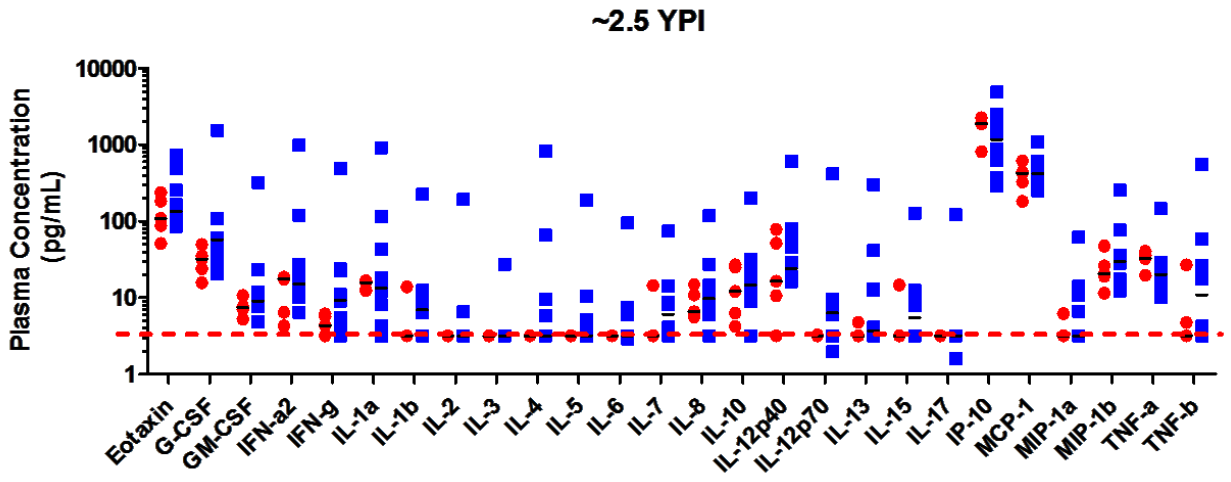
Supplementary Table 2.1 Summary of genes evaluated for transcription levels of bulk B cells *ex vivo*.



Supplementary Figure 2.3 Plasma levels of CXCL13 correlate with maximum breadth of serum neutralization and frequency of pTFH cells. A) Plasma concentration of CXCL13 during the first year post-infection correlates with the frequency of CXCR5+ ICOS+ PD-1+ CD4+ T cells. The line is a linear regression curve. The stated r and p-values were calculated using Spearman correlation for nonparametric data and considered significant if less than 0.05.

	Median Relative Transcription (~2.5 YPI)			Broad vs Narrow	Broad vs HIV-	Narrow vs HIV-
	Broad	Narrow	HIV Negative	P-Value	P-Value	P-Value
AICDA	0.0235	0.0504	0.0372	0.9307	0.5711	0.9599
AKT	0.0633	0.0944	0.0808	0.8763	0.734	0.5869
BAFFR/TNFRSF13C	0.6071	0.6354	0.5606	1.0000	1	1
BCL-2	0.1096	0.1745	0.3335	0.7551	0.174	**0.0373**
BCL2A1	0.3054	0.3089	0.3075	0.7551	0.5711	0.7859
BCL2L1	0.0662	0.1028	0.0883	0.7551	0.5711	0.4687
BCL6	0.0992	0.1348	0.0814	0.7551	0.4278	1
BCMA/TNFRSF17	0.0353	0.0261	0.0691	0.7551	0.2573	0.0794
BLIMP1/prdm1	0.0737	0.1236	0.0815	0.8763	1.0000	0.6009
BLNK	0.0783	0.1063	0.1345	0.8763	0.5711	0.2048
CD11b/ITGAM	0.0417	0.0306	0.0693	0.8763	0.4967	0.4687
CD19	0.1834	0.2621	0.1555	1.0000	0.6504	1
CD21/CR2	0.0519	0.1194	0.1179	1.0000	0.3648	0.5869
CD22	0.5189	0.8111	0.6842	0.6389	0.4967	0.9278
CD23a/FCER2	0.1734	0.4792	0.4171	0.2020	**0.0127**	0.8563
CD27	0.0450	0.0344	0.0768	0.8763	0.4967	0.3191
CD32/FCGR2B	0.1267	0.0974	0.1446	0.7551	0.5711	0.2048
CD38	0.0438	0.0602	0.0832	0.8763	0.5711	0.3191
CD40	0.0998	0.2020	0.1822	0.8763	0.4278	0.7172
CD80	0.0542	0.1002	0.0540	1.0000	0.8208	0.8563
CD86	0.0836	0.0935	0.0828	0.7551	0.9098	0.6507
CD95/fas	0.1016	0.1007	0.0677	0.6389	0.2127	0.5869
CHUK	0.0631	0.0898	0.0561	0.9484	0.9599	0.9671
CXCL13	0.0237	0.0164	0.0369	1.0000	0.4278	0.415
CXCR5	0.1679	0.2348	0.3230	0.2303	0.2671	0.7859
DUSP2	0.4443	0.5899	0.3136	0.5303	0.3079	**0.0463**
FCRL4	0.0283	0.0426	0.0363	0.6623	0.9098	0.9599
GAPDH	1.0002	1.0001	1.0001	1.0000	1.0000	1.0000
IFI27	0.1612	0.0462	0.0473	0.2020	0.1408	0.9278
IghG1	0.1144	0.1431	0.1473	0.7922	0.734	0.6511
IL17A	0.2903	0.2906	0.3569	0.9273	0.7441	0.5869
IL1F1/IL1A	0.0282	0.0243	0.0437	1.0000	0.5711	0.6507
IL21r	0.1412	0.1512	0.1394	0.8763	0.6504	0.8563
IL6	0.1198	0.2224	0.1615	0.5273	0.3961	1
IRF7	0.0277	0.0188	0.0419	0.7879	0.8447	0.5869
ISG15	0.2949	0.2320	0.1989	0.2677	0.5711	0.9278
LTA	0.0581	0.1114	0.0741	0.8763	1	0.9278
LTB	0.1060	0.1956	0.1686	1.0000	0.2573	0.5869
MAPK1	0.0532	0.1015	0.0725	0.8763	0.4967	0.415
NFKB1	0.1719	0.1536	0.0948	0.7551	1	0.7172
PAX5	0.1676	0.3077	0.2628	1.0000	1	0.5869
PTPN11	0.0792	0.1125	0.0829	0.8763	0.734	1
RGS13	0.0835	0.0610	0.0432	0.4318	0.3648	0.7859
STAT3	0.0512	0.0527	0.0661	0.8763	0.9098	0.7859
TLR7	0.0755	0.1100	0.0994	0.5303	0.6504	0.3191
TNFSF2/TNF	0.1386	0.1577	0.0968	0.7551	0.2684	0.3191

Supplementary Table 2.2 Gene expression profile at ~2.5 years post-infection of total B cells ex vivo. Blue bars indicate median transcript levels relative to GAPDH of bulk B cells *ex vivo* with significant P values denoted in red/bold. P-values were calculated using Mann-Whitney test for nonparametric data and considered significant if less than 0.05.



Supplementary Figure 2.4 Plasma levels for 26 cytokines and chemokines did not vary significantly overall between 'Broad' and 'Narrow' groups. Values are from ~2.5 years post-infection for HIV-1+ subjects. The red dash line indicates the detection limit. The horizontal lines are at the median. The stated p-values were calculated using Mann-Whitney test for nonparametric data and considered significant if less than 0.05.

Chapter III:

HIV-1 ssRNA Induces CXCL13 Secretion in Human Monocytes via TLR7/8 Activation and Plasmacytoid Dendritic Cell-Derived Type I Interferon

Abstract

Objective: Elevated levels of the chemokine, CXCL13, have been observed in the plasma of chronic HIV-1 infected subjects and have been correlated with plasma viremia, which in turn has been linked to progressive dysregulation of humoral responses. Therefore we sought to study the mechanisms of CXCL13 induction in response to HIV-1 infection.

Methods: Plasma levels of CXCL13 in HIV-1 infected ART-naïve and ART-treated individuals were determined. To elucidate the relationship between HIV-1 viremia and CXCL13 plasma levels, peripheral blood mononuclear cells from uninfected donors were stimulated with ssRNA derived from HIV-1, TLR7/8 agonists or interferon alpha. The cellular sources of CXCL13 were determined by sorting cell populations before and after stimulation.

Results: CXCL13 plasma concentrations were associated with HIV-1 viremia. CXCL13 was secreted in response to stimulation with TLR7/8 ligands and ssRNA derived from HIV-1. Monocytes produced significant amounts of CXCL13 after *in vitro* stimulation with TLR7/8-ligands and HIV-1-derived ssRNA. However, TLR7 induced plasmacytoid dendritic cell-derived type I interferon was required for maximal CXCL13 secretion. Interferon alpha alone was sufficient for induction of CXCL13 in isolated human monocytes.

Conclusion: We identified two potential mechanisms of HIV-1-induced CXCL13 secretion; one due to direct TLR7/8 activation in monocytes by ssHIV-1 RNA and another due to TLR7 induction of type I interferon by pDCs and subsequent IFNα stimulation of monocytes. TLR7/8 and IFNα induction of CXCL13 in monocytes have not been previously demonstrated.

Introduction

A characteristic immunological defect of HIV-1 infection is chronic and progressive humoral dysregulation (Reviewed in (Amu et al., 2013; Moir and Fauci, 2013)). This dysregulation includes changes in the frequencies of specific B cells subsets (Ho et al., 2006), hypergammaglobulinemia (De Milito et al., 2004), and impaired induction of *de novo* vaccine responses (Malaspina et al., 2005). While the specific mechanisms of B cell dysregulation have yet to be elucidated, there is evidence that some of these defects are associated with viremia (Moir et al., 2008b; Pensieroso et al., 2013; Sciaranghella et al., 2013). Elevated levels of the chemokine, CXCL13, have been observed in the plasma of chronic HIV-1 infected subjects and have been correlated with viral load (Cagigi et al., 2008; Regidor et al., 2011; Widney et al., 2005). Furthermore, increased CXCL13 plasma levels were associated with reduced chemokine receptor CXCR5 expression on B cells and with alterations in the chemotactic potential of B cells during HIV-1 infection (Cagigi et al., 2008).

CXCL13 is a chemokine crucial for the development of secondary lymphoid structures, where it is secreted by stromal cells and follicular dendritic cells in response to lymphotoxin receptor activation (Legler et al., 1998; Ngo et al., 1999). CXCL13 is chemotactic for cells expressing the receptor, CXCR5 (Gunn et al., 1998; Legler et al., 1998), including mature B cells and follicular helper T cells (TFH) (Breitfeld et al., 2000; Forster et al., 1994; Schaerli et al., 2000), and is expressed at high levels in the B cell follicles of lymphoid organs (Legler et al., 1998). Importantly, CXCL13 facilitates the co-migration of B cells and TFH cells into B cell follicles and germinal centers, where high-affinity antibody-secreting memory B and plasma cells are generated (Allen et al., 2004; Jacob et al., 1991). Conversely, aberrant CXCL13 secretion has been implicated in the pathogenesis of many chronic inflammatory conditions, including various infections and autoimmune disorders associated with dysregulated lymphoid genesis and

humoral responses (Carlsen et al., 2004; Shomer et al., 2003; Vermi et al., 2006; Wong et al., 2010).

It is unclear what is responsible for the increase in peripheral CXCL13 during HIV-1 infection. While increased transcriptional expression of CXCL13 was detected in B cells from HIV-infected subjects, secretion of CXCL13 by those B cells was only detectable at low levels and only after sustained stimulation *ex vivo*. In lymph node biopsies from these subjects, the majority of CXCL13 was co-localized with macrophages and immature dendritic cells (Cagigi et al., 2008). Whether these two cell types secrete CXCL13 in the periphery is unknown. It also not well understood whether HIV-1 itself directly induces an increase of CXCL13 expression by certain cells, or whether the observed increase in CXCL13 levels during HIV-1 infection is due to a bystander effect of a general immune activation that takes place during HIV-1 infection.

HIV-1 triggers cytokine secretion directly through activation of toll-like receptors 7 and 8 (TLR7/8) (Diebold et al., 2004; Heil et al., 2004). TLR7 and TLR8 are expressed in antigen presenting cells, predominantly, and are located in the endosomes where they are activated by internalized guanosine and uridine-rich single-stranded RNAs derived from the HIV-1 genome. Several studies have addressed the potential contribution of TLR7-mediated activation to chronic immune activation and immune cell dysfunction through induction of type I interferon (IFN) by plasmacytoid dendritic cells (pDCs) (Alter et al., 2007b; Chang et al., 2013; Hardy et al., 2013; Meier et al., 2007a; Meier et al., 2008; Simmons et al., 2013). Additionally, monocytes, macrophages, and monocyte-derived dendritic cells have been identified as inducible producers of CXCL13 by TLR2 and TLR4 activation (Carlsen et al., 2004; Moreth et al., 2010; Perrier et al., 2004; Rupprecht et al., 2007; Shomer et al., 2003; Vermi et al., 2006). TLRs 2, 4, 7 and 8 share overlapping signaling pathways and downstream transcription factors. Furthermore, myeloid cells express TLR7 and TLR8 and therefore we hypothesized that they

might produce CXCL13 in response to TLR7/8 activation and that this may contribute to the increased CXCL13 observed during HIV-1 infection.

Here, we investigated potential mechanisms of CXCL13 induction and secretion in the periphery during HIV-1 infection. Using human PBMCs, we found that CXCL13 is secreted in response to HIV-1-derived ssRNA and the TLR7/8 ligand, R848, by monocytes, and that maximal CXCL13 production required pDC-secretion of type I IFN. Overall our study reveals two novel mechanisms of CXCL13 induction in human monocytes; one, in direct response to TLR7/8 activation and a second via the TLR7 stimulation of pDCs and secretion of type one IFN.

Materials and Methods

Study Participants

HIV-1 infected subjects were enrolled in protocols at the Massachusetts General Hospital approved by the Institutional Review Board. HIV negative human subjects were enrolled in a protocol at Seattle Biomedical Research Institute approved by the Institutional Review Board. Each participant gave written informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated within 4 hours of venipuncture by density-gradient centrifugation. *In vitro* stimulation experiments were performed on fresh PBMCs from HIV negative donors. Plasma samples were aliquoted and frozen at -80°C. Concentrations of CXCL13 in thawed plasma samples were determined by ELISA according to the manufacturers' instructions (R & D systems).

Isolation of Cell Subsets

Plasmacytoid dendritic cells (pDCs) were depleted from human PBMCs using a CD123 Microbead Kit (Miltenyi Biotec). Since CD123 is expressed on both pDCs and on basophils, pDCs were positively selected for with CD304 magnetic bead isolation (Miltenyi Biotec).

Monocytes were isolated from PBMCs using the monocyte negative selection enrichment kit and depleted using the CD14 positive selection kit (Stem Cell Technologies). All PBMCs and cell subsets were cultured in RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin (Life Technologies). Cell purity was consistently greater than 98% (data not shown), and confirmed by surface staining with mAbs against CD19, CD3, CD14, CD11c and CD123 (BD Biosciences) and analyzed using an LSR II flow cytometer (BD Biosciences).

In vitro Stimulation of PBMCs with Toll-like Receptor Ligands

Two million freshly isolated PBMCs or 2 x 10^5 monocytes incubated with or without 25,000 purified pDCs, were cultured in 1 ml of complete media and treated for 1-4 days with the TLR7/8 agonist R848 (Invivogen) at 1 μg/ml. Increasing concentrations of HIV-1 LTR derived GU-rich ssRNA40/LyoVec (5'GCCCGUCUGUUGUGUGACUC-3') and negative control RNA, where U nucleotides were replaced with adenosine, ssRNA41/LyoVec (5'GCCCGACAGAAGAGAGACAC-3') (Heil et al., 2004) (Invivogen), and recombinant human IFNα2A (PBL Interferon) were added to cells. The soluble IFN receptor, B18R (Affymetrix), was added (at 0.1μg/mL) to cells directly preceding stimulation with the above TLR7/8 agonists in order to neutralize type I interferons in the supernatant. 500μL of supernatant were removed and replaced with fresh media at 24 hr intervals. Concentrations of CXCL13 and IFNα in culture supernatants were determined by ELISA according to the manufacturers' instructions (IFNα kit from PBL Biomedical Laboratories; CXCL13 kits from R & D systems).

Quantification of mRNA by Real-Time PCR

Total RNA was isolated from monocytes and monocyte-depleted PBMCs using the Qiagen RNAeasy Kit (Qiagen). Total RNA was reverse transcribed into cDNA using QuantiTect Reverse

Transcription Kit. Briefly, genomic DNA was removed by DNAse digestion by incubation with gDNA wipeout buffer. First-strand cDNA synthesis was carried out for 30 min at 42°C in 20 μl solution containing Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix (Qiagen), followed by denaturation for 3 min at 95°C. 4 μL of cDNA templates were used for real-time PCR reactions to quantify CXCL13 (assay number Hs00757930_m1) in a 96-well plate. Each reaction was carried out in 20 μL solution containing 10 μL TaqMan reaction mix and 1 μL TaqMan FAM dye–labeled MGB probe using a 7500 Fast Real-Time PCR machine (Applied Biosystems). Target gene expression was normalized against the level of GAPDH (assay number Hs02758991_g1).

Statistical Analysis

Data are presented as the median of 4 independent experiments carried out using PBMCs from different donors unless stated otherwise. Two-tailed Mann-Whitney or Wilcox-paired nonparametric tests were used to determine statistical significance. Correlations were determined using Spearman's Rank. P values of less than 0.05 were considered significant.

Results

Plasma CXCL13 Correlates with Plasma HIV-1 Viral Load

In order to determine the relationship between HIV viremia and induction of CXCL13, we compared levels of CXCL13 and viral load in the plasma of chronically HIV-1-infected subjects. We found a significant correlation between viral load and CXCL13 plasma concentrations **(Figure 3.1A;** R=0.58, P=0.001), in agreement with previous reports (Cagigi et al., 2008; Regidor et al., 2011; Widney et al., 2005). Similarly, we found that subjects with suppressed viral load by ART-treatment had significantly lower levels of plasma CXCL13 **(Figure 3.1B)**, suggesting that viral replication drives the production of CXCL13 and not the reverse. The

presence of higher than normal CXCL13 concentrations in the periphery of those chronically-infected with HIV-1 may contribute to the well-documented dysregulation of B cell responses during HIV-1 infection (Reviewed in (Amu et al., 2013; Moir and Fauci, 2013)). We wanted to determine whether HIV-1 induces CXCL13 directly and to define potential mechanism(s) by which viremia contributes to elevated CXCL13 expression.

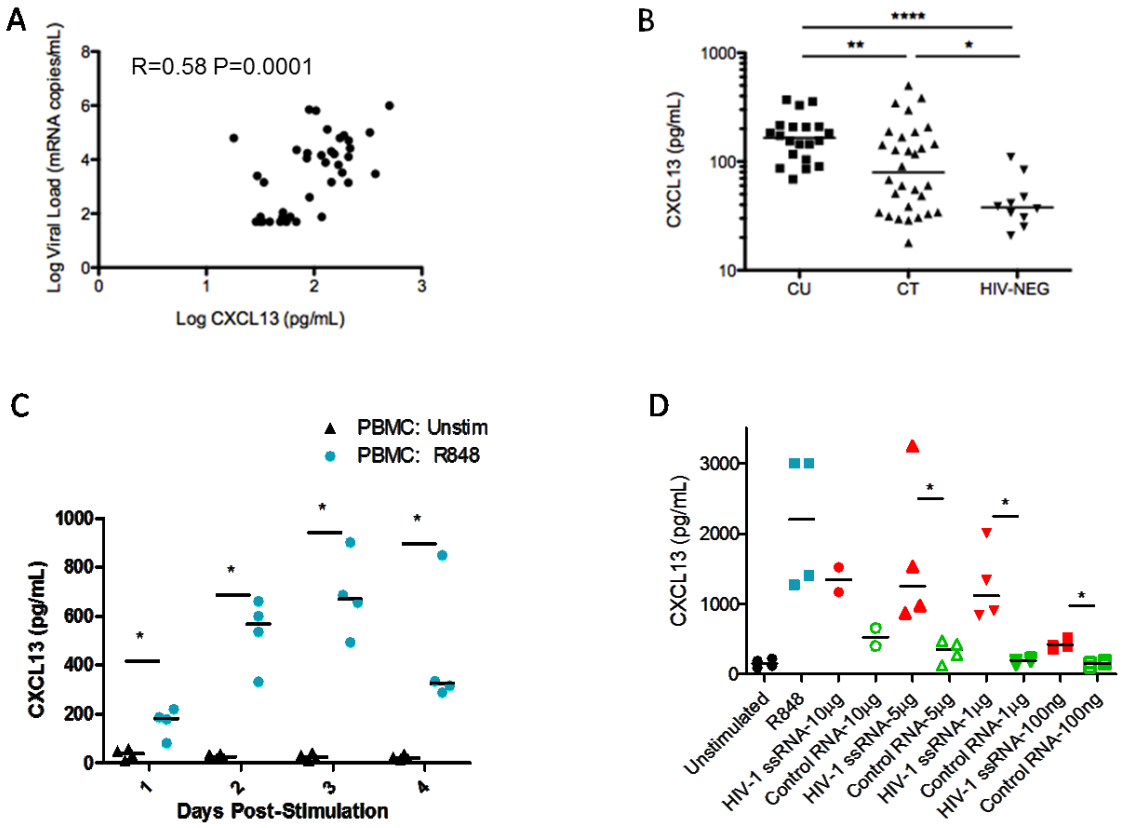


Figure 3.1 CXCL13 plasma levels are significantly elevated in chronic HIV-1 infected subjects and TLR7/8 agonists stimulate CXCL13 secretion in PBMCs. A) CXCL13 levels correlate with corresponding plasma viremia. Correlation coefficient and significance were calculated using a Spearman's rank-order correlation for nonparametric data. B) CXCL13 levels in plasma of HIV-1 infected patients: Chronic Untreated (CU), and Chronic Treated (CT), compared to HIV negative donors (HIV-NEG). The horizontal lines are at the median. The stated P-values were calculated using Mann-Whitney test for nonparametric data and considered significant if less than 0.05 (*), 0.01(**), or 0.0001(****). C) PBMCs from four HIV negative donors were stimulated with the TLR7/8 agonist, R848 (1 μg/mL) and supernatant was removed and replaced at the indicated time points, and the secreted CXCL13 was determined. The horizontal lines are at the median. P-values were calculated using Mann-Whitney test for nonparametric data and considered significant if less than 0.05 (*). D) PBMCs from HIV negative donors were stimulated *in vitro* with ssRNA derived from HIV-1 (ssRNA40) or control RNA (ssRNA41) at multiple doses (μg/mL) and CXCL13 levels in the supernatants were determined after two days post-stimulation.

TLR7/8 Induction of CXCL13

Previously, TLR2 and TLR4 ligands have been shown to induce CXCL13 secretion by myeloid cells (Carlsen et al., 2004; Moreth et al., 2010; Rupprecht et al., 2007). However, since HIV-1 RNA activates TLR7/8 (Alter et al., 2007b; Heil et al., 2004) and is involved in inducing immune activation, we hypothesized that HIV-1 may similarly induce CXCL13 through TLR7 and/or TLR8 activation. To investigate this possibility, we stimulated PBMCs from HIV-1 negative donors with the TLR7/8 agonist, R848, and measured the secretion of CXCL13 in the cell supernatants at days 1, 2, 3, and 4 post-stimulation. We observed a significant induction of CXCL13 as early as 1 day post-stimulation ($p=0.03$), although CXCL13 secretion peaked at day 3 **(Figure 3.1C**). Then, we treated PBMCs from HIV-negative subjects with ssRNA derived from the long terminal repeat of HIV-1 (ssRNA40), and also observed CXCL13 secretion in a dose-dependent manner **(Figure 3.1D**). CXCL13-expression was not observed with a control ssRNA in which uridines have been replaced with adenosines (ssRNA41) **(Figure 3.1D**). Because HIV-1 derived ssRNA is known to activate TLR7/8, the above results indicate that HIV-1 ssRNA stimulates the production of CXCL13 from PBMCs through activation of TLR7 and/or TLR8.

Monocytes are Necessary for CXCL13 Secretion

We next performed experiments to determine which cells types in PBMCs were responsible for the observed secretion of CXCL13. While follicular dendritic cells have historically been considered the main producers of CXCL13, myeloid cells have also been shown to secrete CXCL13, and have been shown to be associated with pathogenic lymphoid genesis (Carlsen et al., 2004; Moreth et al., 2010; Rupprecht et al., 2007). Indeed, CXCL13 secretion from PBMCs stimulated with R848 **(Figure 3.2A)** or with HIV ssRNA was completely abrogated when monocytes were depleted **(Figure 3.2B).** This led us to conclude that monocytes were necessary in the PBMC secretion of CXCL13 in response to TLR7/8 stimulation. TLR7/8 stimulation of purified monocytes resulted in CXCL13 secretion, but the amounts of CXCL13

produced were approximately 4 fold lower than those produced by total PBMCs **(Figure 3.2A and 3.2C).** Two possibilities could explain these results: (a) monocytes are the major producers of CXCL13 but that additional cell types are necessary for optimal CXCL13 production by monocytes or (b) monocytes are not the major producers of CXCL13 but, are critical for CXCL13 production by another cell type in PBMCs.

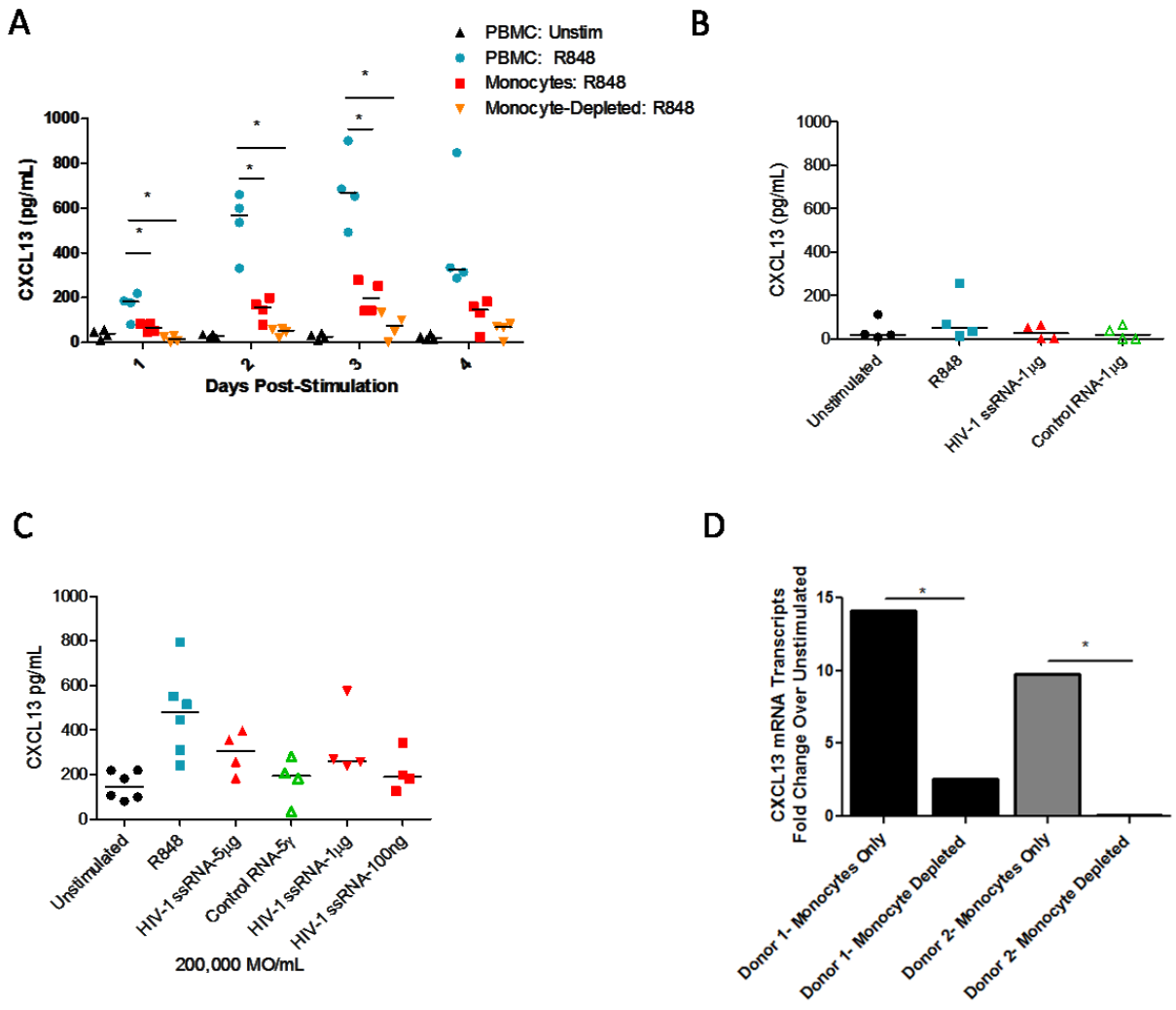


Figure 3.2 Monocytes are necessary, but not sufficient for optimal CXCL13 secretion. A) PBMCs, monocytes, or monocyte-depleted PBMCs from HIV-1 negative donors were stimulated for 4 days with a TLR7/8 agonist, R848 (1 μg/mL), and supernatant was removed and replaced at the indicated time points, and levels of CXCL13 were determined. The horizontal lines are at the median. The stated P-values were calculated using Mann-Whitney test for nonparametric data and considered significant if less than 0.05 (*). B) CXCL13 levels in the supernatant of monocyte-depleted PBMCs from HIV-1 negative donors two days after stimulation with HIV-1-derived ssRNA or control RNA. C) Monocytes isolated from HIV-1 negative donors were cultured at 200,000/mL and stimulated with R848 (1 μg/mL), HIV-1 derived

ssRNA, or control RNA for two days. D) PBMCs were stimulated with R848 for two days. Monocytes were isolated from the PBMCs, RNA was extracted from both the monocytes and the monocyte-depleted PBMCs and CXCL13 transcripts were measured by real-time PCR. Fold change was calculated by ΔΔCT using GAPDH as internal control and compared to unstimulated controls. Values represent mean of triplicates and SEM. The stated P-values were calculated by Wilcoxon matched pairs test and considered significant if less than 0.05 (*).

To distinguish between these two possibilities, we stimulated PBMCs from HIV-negative donors with R848 for 2 days and subsequently isolated the monocytes. Then, we compared CXCL13 mRNA expression in the monocytes and monocyte-depleted PBMCs by real-time PCR **(Figure 3.2D).** We found significant up-regulation of CXCL13 mRNA expression in the monocytes, but not in the monocyte-depleted PBMCs (as compared to the unstimulated controls). These results confirmed that: (a) monocytes were the primary cells in PBMCs secreting CXCL13 under TLR7/8 stimulation, (b) that TLR7/8 stimulation of monocytes is sufficient for the production of CXCL13 and (c) that an intermediary cell type in PBMCs was required for optimal CXCL13 secretion by monocytes.

Type I Interferon is Necessary and Sufficient for CXCL13 Secretion

To determine whether that second cell type responds to TLR7/8 activation, we began by depleting pDCs. Since pDCs, which express TLR7, but not TLR8, are known to secrete significant quantities of cytokines, IFNα in particular, in response to TLR7 stimulation (Beignon et al., 2005; Diebold et al., 2004; Gibson et al., 2002; Simmons et al., 2013), we investigated whether pDCs play a role in CXCL13 secretion by monocytes. A significant decrease in CXCL13 production in PBMC cultures was observed when pDCs were depleted from the PBMCs **(Figure 3.3A).** To further confirm the critical nature of pDC induced type I IFN, we next stimulated PBMCs with TLR7/8 agonists in the presence of the type I IFN soluble receptor, B18R, which neutralizes type I IFNs, and observed a similar reduction of CXCL13 production **(Figure 3.3A).** These results suggested that TLR7 induction of type I IFN by pDCs was required for maximal secretion of CXCL13 by monocytes. In fact, the concentrations of CXCL13 in the

PBMC supernatants correlated with the concentrations of IFNα in the same supernatants (R=0.69, P<0.0001) **(Figure 3.3B).**

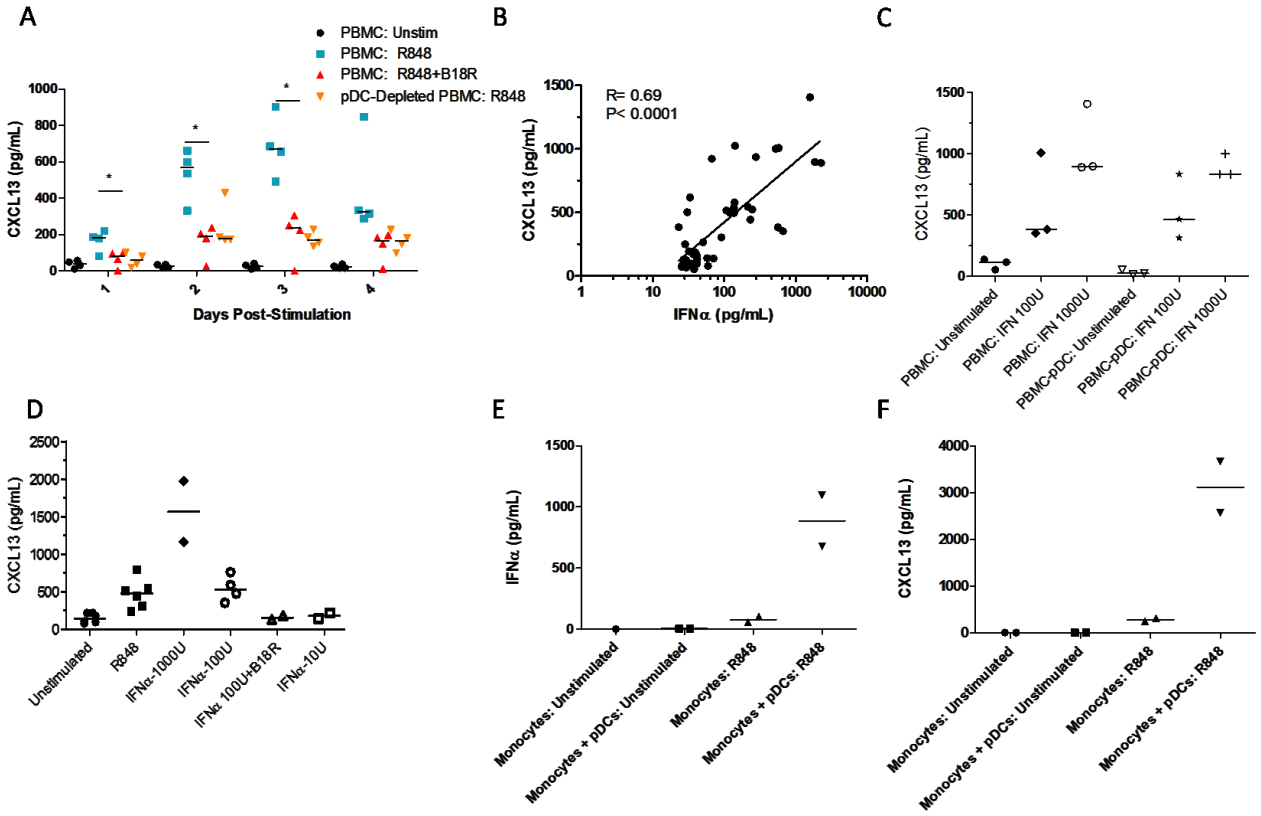


Figure 3.3 Type I IFN is required for maximal induction of CXCL13 in monocytes. A) PBMCs from four HIV-1 negative donors were depleted of pDCs or stimulated in the presence or absence of the type I IFN inhibitory molecule, B18R (0.1ng/mL). CXCL13 concentrations in the cell supernatants were determined on days 1, 2, 3 and 4 post-stimulation. The horizontal lines are at the median. The stated P-values were calculated using Mann-Whitney test for nonparametric data and considered significant if less than 0.05 (*). B) Spearman's rank-order correlation was used to determine the correlation of IFNα levels in supernatants with the corresponding amounts of CXCL13 from the same samples as in 3A. The line is a linear regression curve. C) PBMCs and pDC-depleted PBMCs from three HIV-1 negative donors were stimulated with recombinant IFNα2A (at the indicated amounts per mL) for two days at which point CXCL13 concentrations in the cell supernatants were determined. D) CXCL13 concentrations in the supernatant of purified monocytes stimulated for two days with the indicated amounts of recombinant IFNα2A. E) PBMC-isolated monocytes combined with or without purified autologous pDCs were stimulated for 2 days with R848 and the IFNα levels were determined. F) Supernatant levels of CXCL13 from the same samples as in 3E. The horizontal lines are at the median.

To determine whether IFNα alone was sufficient to induce CXCL13 production by monocytes, we purified monocytes and stimulated monocytes or PBMCs with exogenous recombinant IFNα2A. In both cases, we observed that CXCL13 expression was induced in an IFNα-dose-

dependent manner **(Figure 3.3C).** IFNα-mediated CXCL13-expression was abrogated when the soluble IFN receptor, B18R, was added during stimulation **(Figure 3.3D).** To further confirm the role of pDC-secreted IFNα in the stimulation of monocytes and the subsequent secretion of CXCL13 by these cells, we isolated pDCs and monocytes by negative selection, recombined them, and stimulated them with a TLR7/8 agonist (R848). After 2 days we measured the IFNα and CXCL13 in the supernatant **(Figure 3.3E and 3.3F respectively).** The recombined pDCs and monocytes recapitulated the PBMC response to TLR7/8 stimulation **(Figure 3.1).** The recombined cells actually surpassed the response observed in PBMCs, probably due to differences in the natural cell frequencies in the PBMCs as compared to the *in vitro* experiment and the additional day of cytokine accumulation in the supernatant in the latter experiment.

Discussion

The CXCL13 chemokine is the ligand for the receptor, CXCR5, expressed on B and follicular T helper cells and is critical for follicle development, affinity maturation of B cells, and organization of secondary lymphoid architecture. While follicular dendritic cells have historically been considered the main producers of CXCL13, monocytes, macrophages and myeloid DCs have been shown to secrete CXCL13 in response to bacterial derived TLR2/4 *ligands* (Carlsen et al., 2004; Moreth et al., 2010; Perrier et al., 2004; Rupprecht et al., 2007; Shomer et al., 2003; Vermi et al., 2006). Previous reports have demonstrated an increase in plasma levels of CXCL13 during HIV-1 infection and that a positive correlation exists between the plasma CXCL13 concentration and plasma viremia (Cagigi et al., 2008; Regidor et al., 2011; Widney et al., 2005). In the context of HIV-1 infection a correlation was observed between plasma CXCL13 concentrations and changes in the chemotactic potential of B cells (Cagigi et al.,

2008). However, the mechanistic connections between HIV-1 viral load and CXCL13-production are unknown.

Here, we confirmed the correlation between plasma HIV viremia and CXCL13 plasma concentrations in the context of chronic HIV-1 infection and identified two potential mechanisms of HIV-induced CXCL13 secretion **(Figure 3.4).** HIV-1 can induce production of CXCL13 by monocytes directly through TLR7/8 activation, but more potently via TLR7 induction of IFNα secretion by pDCs and subsequent IFNα stimulation of monocytes. Specifically, we identified HIV-1-derived ssRNA, which is a TLR7/8 agonist, as a potent inducer of CXCL13 secretion from PBMCs. Furthermore, we established that monocytes were the main producers of CXCL13 under these conditions and that maximum CXCL13 secretion was dependent on pDC-secreted IFNα. IFNα was sufficient to induce high levels of CXCL13 secretion from isolated monocytes.

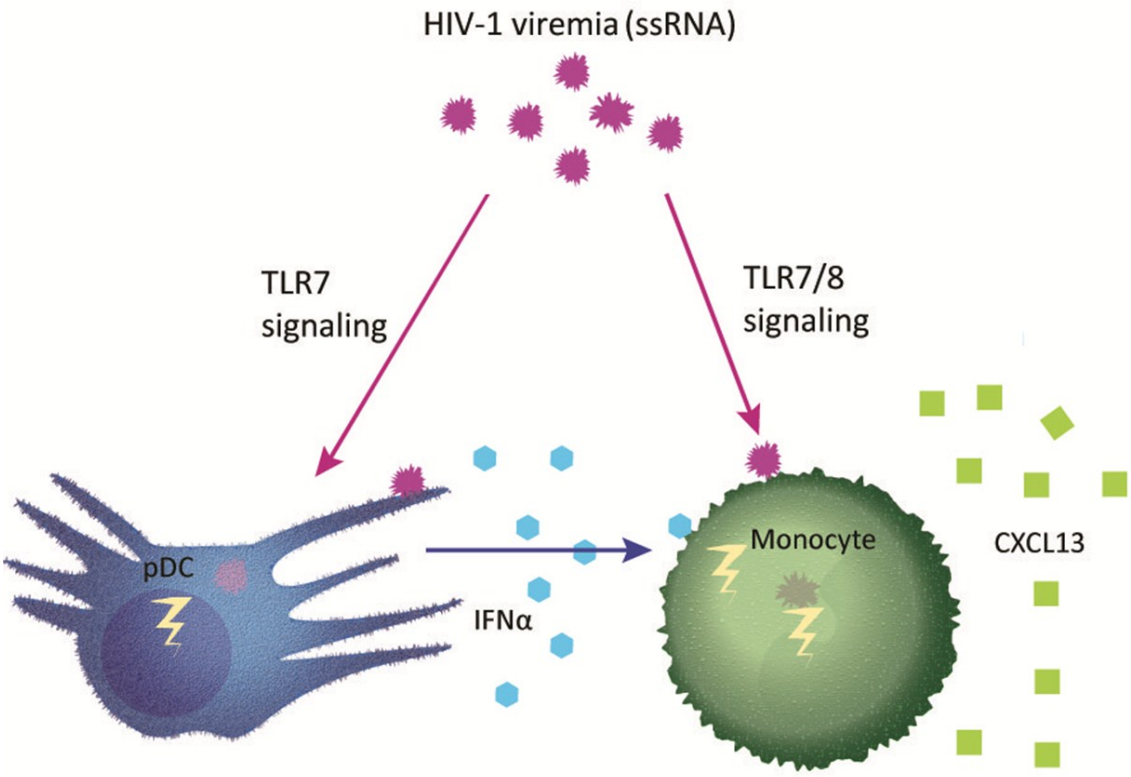


Figure 3.4 Schematic representation of two mechanisms of CXCL13 production by monocytes in HIV-1 infection: 1) HIV-1 ssRNA-mediated TLR7-induced pDC secretion of type I IFN and subsequent IFN-stimulation of CXCL13 secretion by monocytes. 2) HIV-1 ssRNA-mediated TLR7/8 activation of monocytes.

This observation indicates that direct contact between pDCs and monocytes upon TLR7/8 stimulation of these two cell types is not required for secretion of CXCL13 by the monocytes. Therefore, the elevated CXCL13 levels observed during chronic HIV-1 infection may result from HIV-induced IFNα stimulation of monocytes via TLR7 activation of pDCs **(Figure 3.4).** If transcription of CXCL13 in monocytes is IFN-inducible, as we conclude here, then this may explain the previously reported up-regulation of CXCL13 mRNA in B cells of HIV-infected subjects (Cagigi et al., 2008), since all nucleated cells express type I IFN receptors. Although we only detected secretion of CXCL13 by monocytes, it is also possible that the timing or the inflammatory milieu of our assay precluded other cell types from being represented accurately; i.e., under *in vivo* conditions. However, our observation that myeloid cells were the predominant producers of CXCL13 is in agreement with the previous observation that macrophages were the cell type most often associated with CXCL13 staining in the LNs of HIV-1 infected subjects (Cagigi et al., 2008). We also detected direct induction of CXCL13 by TLR7/8 activation of purified monocytes. Additionally, the promoter region of CXCL13 contains the binding sites of transcription factors, ISGF-3 and IRF-7A, which are downstream of type I IFN and TLR7/8 signaling, respectively, further corroborating the induction of CXCL13 secretion by IFNα and TLR7/8 signaling (Deng et al., 1996).

The IFN-dependent and TLR7/8-mediated up-regulation of CXCL13 in monocytes may explain the elevated levels of CXCL13 found in the periphery during chronic HIV-1 infection. In addition, it may indicate a similar mechanism in lymphatic tissues where substantial HIV-1 replication occurs (Pantaleo et al., 1993). pDCs have been shown to accumulate in the lymph nodes (LN) of HIV-infected subjects and secrete IFNα (Lehmann et al., 2010; O'Brien et al., 2011).

Likewise, IFN-inducible genes have been reported as being increased in the LN of SIV-infected macaques and HIV-1-infected humans (Lehmann et al., 2010; Malleret et al., 2008; Sanghavi and Reinhart, 2005). Furthermore, accumulation of TFH cells, which express CXCR5 and migrate in response to CXCL13, has been described in the LN of chronically HIV-1-infected humans and some SIV-infected macaques (Lindqvist et al., 2012; Perreau et al., 2013; Petrovas et al., 2012; Xu et al., 2013). The accumulation of TFH cells in LN during chronic HIV-1 infection was also associated with hypergammaglobulinemia (Hong et al., 2012a; Lindqvist et al., 2012). Therefore, if HIV-1 induction of CXCL13 by innate immune mechanisms occurs in the LNs, as our results suggest, this may contribute to the influx of TFH cells in lymphoid tissues. IFN-induced CXCL13 secretion by myeloid cells may similarly contribute to the pathogenesis of other inflammatory conditions occurring during HIV-1 chronic infection as well.

The mechanisms of CXCL13 induction by monocytes described here may be useful for vaccine development. DNA encoding CXCL13 has been shown to modify vaccine-induced immune responses (Frauenschuh et al., 2004). In addition, TLR7 agonists have been considered as potential vaccine adjuvants and numerous reports suggest that they may benefit the induction of a high-affinity antibody response in murine models (Kasturi et al., 2011; Neighbours et al., 2012). Likewise, type I IFN has been associated with development of antigen-specific neutralizing antibodies against influenza in vaccine studies (Proietti et al., 2002; Stetson and Medzhitov, 2006). Our results suggest that the induction of CXCL13 by type I IFN and TLR7/8 may be of functional relevance in these models. Furthermore, TLR7/8 adjuvants should be thoroughly investigated for modulating the antibody response by induction of CXCL13.

Chapter IV:

Discussion

Summary of Results and Caveats

Despite over three decades of intensive effort, only one HIV-1 vaccine clinical trial (RV144 trial) has demonstrated any efficacy at all, and it was limited and transient (Rerks-Ngarm et al., 2009). The development of a highly effective HIV-1 vaccine will likely require the elicitation of potent and cross-reactive neutralizing antibodies (CRNA; not elicited by the RV144 vaccine) with activity against diverse clinical HIV-1 isolates (Kwong et al., 2013; Mascola and Montefiori, 2010; McElrath and Haynes, 2010; Stamatatos et al., 2009). Although CRNA are generated during natural HIV-1 infection in a subset of those infected with HIV-1, they have not yet been generated by current immunization strategies (Belshe et al., 1994; Mascola et al., 1996; Pitisuttithum et al., 2004). It is important to mention that, broad and potent anti-HIV-1 monoclonal neutralizing antibodies, isolated from HIV-1 infected subjects, have provided sterilizing protection in experimental animal models (Baba et al., 2000; Hessell et al., 2009b; Mascola et al., 1999a; Moldt et al., 2012; Veazey et al., 2003a; Watkins et al., 2011). Therefore, the hope is that if induced, vaccine-elicited CRNA will block HIV-1 infection (Mascola and Montefiori, 2010; Stamatatos et al., 2009).

As mentioned above, CRNA are detectable in a minority of HIV-1 infected subjects, while the majority of HIV-1 infected subjects have neutralizing antibodies with only strain-specific activity (Binley et al., 2008; Binley et al., 2004; Doria-Rose et al., 2010; Euler et al., 2010; Gray et al., 2011a; Li et al., 2009; Sather et al., 2009; Simek et al., 2009). The development of CRNA in the sera of HIV-1-infected subjects takes time (Gray et al., 2011a; Mikell et al., 2011b; Moore et al., 2011), and antigenic stimulation, as subjects who develop CRNA tend to be viremic and responses wane after sustained viral suppression by anti-retroviral therapy (Doria-Rose et al., 2010; Piantadosi et al., 2009; Sather et al., 2009). However, the specific immunological mechanisms required for the development of CRNA remain unknown. Understanding these mechanisms may provide new insights about how to elicit CRNA by immunization. **In this**

thesis, we sought to identify immunological correlates, which distinguish the individuals who develop CRNA during untreated HIV-1 infection from those individuals who do not develop CRNA. Previously it has been suggested that higher frequencies of peripheral CD4 T cells with TFH-like phenotypes during HIV-1 infection may be associated with development of CRNA (Locci et al., 2013; Mikell et al., 2011b). Therefore, in chapter two, I describe our investigation of the involvement of peripheral TFH-like cells during development of CRNA during HIV-1 infection.

In this study, we distinguished virus-driven systemic immune activation in early infection from independent correlates of CRNA. We observed significant relationships between the frequency of peripheral TFH-like (pTFH) cells, plasma concentrations of CXCL13 **(Figure 2.6),** and the subsequent development of broad CRNA in HIV-1 infection **(Figure 2.4 and Figure 2.6),** independent of plasma viral load. These observations were most pronounced within the first year post-infection, suggesting that they may provide a predictive immunologic 'signature' of CRNA as well as indicate mechanisms important for developing such responses, both useful for vaccine design and evaluation of vaccine responses.

In interpreting the results of our study described in chapter two, there are several important caveats worth consideration. One major caveat is that the samples evaluated are all from the periphery. There is likely a relationship between the frequency of TFH cells in the secondary lymphoid tissues and of pTFH cells in the peripheral blood, as is demonstrated by the simultaneous reduction of GC-TFH cells and peripheral CXCR5+ CD4 T cells detected in ICOS- or CD40L-deficient humans or mice (Bossaller et al., 2006; Warnatz et al., 2006). However, the correlation of frequencies of pTFH cells and GC-TFH cells under 'normal' conditions is unknown. Therefore, while we hypothesize that the higher frequency of pTFH cells in the 'broad' group corresponds to more GC-TFH cells, we don't have direct evidence of this. In contrast the decline in the frequency of pTFH cells in the 'narrow' group could be the result of pTFH cells

being recruited out of the periphery into lymph nodes or retention of TFH cells in LNs. An additional caveat of this study is sample size. The cohort we evaluated was small and homogenous (clade B infected, Caucasian, male and middle-aged) therefore the observations we describe may not apply globally to the wider HIV-1 infected population. However as recommendations for beginning anti-retroviral therapy move earlier and earlier, it is difficult to find large cohorts of HIV-infected subjects which are both untreated and followed longitudinally. However, given the thoroughness of our investigation and the multitude of approaches used, we believe that the results presented, while not necessarily generalizable to all HIV-1 infected subjects with CRNA, do identify potential pathways important for development of these responses.

In chapter three, we investigated potential mechanisms of CXCL13 induction and secretion in the periphery during HIV-1 infection. Overall, our study revealed two novel and generalizable mechanisms of CXCL13 induction in human monocytes; one, in direct response to HIV-1 ssRNA TLR7/8 activation and a second via the HIV-1 ssRNA TLR7 stimulation of pDCs and secretion of type I interferon (IFN) **(Figure 3.4).** The IFN-dependent and TLR7/8-mediated up-regulation of CXCL13 in monocytes may explain the elevated levels of CXCL13 found in the periphery during chronic HIV-1 infection and in the individuals who develop CRNA. A major caveat of the investigation described in chapter three is that it relies on *in vitro* stimulation with synthetic TLR agonists of PBMCs from HIV-uninfected donors. Therefore, it is unclear as to the extent that these mechanisms contribute to the induction of CXCL13 in HIV-1 infected subjects. In spite of these caveats, these studies identify novel mechanisms of CXCL13 induction and secretion and may have implications for HIV-1 pathogenesis and vaccine development. The implications of these results as they relate to understanding development of CRNA will be the emphasis of this discussion.

Implications

The results described in chapter two contribute to a growing body of literature which suggest that peripheral CD4 T cells expressing CXCR5 are/or include T cells which preferentially provide help to B cells. In addition, Locci et al recently reported that CXCR5+ CD4 T cells which express PD-1 but not CXCR3 bear the most resemblance to GC TFH cells in healthy donors and that the frequency of CXCR3- PD-1+ cells within the CXCR5+ CD4 T cells in HIV-1 infected subjects was correlated with CRNA. While we did not evaluate CXCR3 expression, we did demonstrate that functionally the CXCR5+ CD4 T cells in these subjects were significantly more effective at providing help to autologous naïve B cells than the CXCR5- counterparts, whereas Locci et al. did not evaluate the functionality of pTFH cell subsets in HIV-infected subjects. Additionally, PD-1 is an inhibitory receptor upregulated on activated T cells in HIV-1 infection (Breton et al., 2013; D'Souza et al., 2007; Greenwald et al., 2005; Prendergast et al., 2012). Therefore without functional analyses in HIV-infected subjects it remains unclear whether the CXCR3- PD-1+ subset of CXCR5+ CD4 T cells, actually delineate a functionally distinct population during HIV-1 infection. In addition, we observed a decrease in the frequencies of total CXCR5+ CD4 T cells in the 'narrow' group during the first year of infection as compared to uninfected and 'broad' subjects, whereas Locci et al did not. The subjects evaluated in our study were a U.S. cohort of Caucasian middle-aged males infected with Clade B viruses, whereas the Locci et al studied the IAVI Clade C infected African cohort with unknown sex distribution. Furthermore, the average frequency of CXCR5+ CD4 T cells in uninfected individuals from the IAVI cohort was about half of what has been previously reported (~7.5% vs ~15%) (Breitfeld et al., 2000) and compared to our cohort **(Figure 2.1)**. Also, the development of CRNA in the IAVI cohort was surprisingly limited, with only 7% developing CRNA compared to 29% in the Ragon cohort. Finally, the potency of serum neutralizing activities in the IAVI cohort was very weak (in most cases the extent of neutralization was approximately 40%).

Taken together these differences suggest that there may be confounding variables affecting the results of the two studies. It will be crucial that functional studies be performed of different pTFH cell subsets in multiple cohorts to better understand the role of pTFH cells in development of CRNA during HIV-1 infection.

It was unexpected that the frequencies of pTFH cells in the 'broad' group most resembled those observed in uninfected individuals and that the subjects in the 'narrow' group suffered a deficiency of pTFH cells in early infection. We hypothesize three potential mechanisms explaining the loss of pTFH cells in the 'narrow' group or maintenance of pTFH cells in the 'broad' group. 1) Since pTFH cells are infectable by HIV-1 (Perreau et al., 2013), perhaps the pTFH cells in the 'narrow' group are eliminated during early infection by CTL-mediated lysis (Koup et al., 1994), and frequencies of pTFH cells rebound **(Figure 2.3)** after the virus escapes the autologous CTL responses (Borrow et al., 1997; Goulder et al., 1997; Price et al., 1997). 2) It is also possible that subjects in the 'broad' group lack similar early CTL responses or the virus in those subjects has already escaped these responses. 3) Alternately, the frequencies of pTFH cells in the 'broad' group are maintained by the active recruitment and differentiation of naïve T cells into a TFH cell phenotype. At the systemic level, we did not see any evidence to support this. The only observed differences in cytokines in the plasma between the 'broad' and 'narrow' groups were higher IP-10 and CXCL13. However, we can't rule out that pertinent differences in chemokine/cytokine production between the 'broad' and 'narrow' groups are restricted to microenvironments and/or tissues, and are not observable in the periphery. CXCR5 is the chemokine receptor specific for CXCL13, and is also the dominant receptor used to distinguish TFH cells from other CD4 T cell subsets (Breitfeld et al., 2000; Schaerli et al., 2000). It is possible that there is a mechanistic relationship between the observed decrease in the frequency of pTFH cells in the 'narrow' group during early infection and the decreased levels of CXCL13 in this group. Since pTFH cells can themselves secrete CXCL13, we are unable to

conclude whether the increase in CXCL13 is promoting the maintenance of physiological frequencies of pTFH cells in the 'broad' group, or that the increased activation (as evidenced by more ICOS and PD-1 expression) combined with physiological frequencies of pTFH cells contributes to higher plasma CXCL13 in that group.

Alternately, the increased plasma levels of CXCL13 observed in the 'broad' subjects (chapter two) may be a result of increased HIV-1 mediated TLR 7/8 and type I IFN activation of monocytes or increased responsiveness of monocytes to these stimuli. We didn't observe a difference in plasma levels of IFN2α between these two groups, however there are many isotypes of type I IFN that we did not measure. We evaluated frequencies of monocytes and pDCs and observed no difference in pDC frequencies (data not shown). However, we did observe higher frequencies of monocytes in the 'narrow' group compared to the 'broad' group during early infection (data not shown), suggesting that the higher levels of CXCL13 in the 'broad' group were not due to simply more monocytes. Therefore, we are not able to conclude whether the mechanisms of CXCL13 secretion that we identified in chapter three make any direct contributions to the higher levels of CXCL13 we observed in chapter two. However, we hypothesize that the mechanisms of HIV-1 induced CXCL13 secretion described in chapter three may contribute to both: the phenotypes associated with CRNA observed in chapter two, and disruption of humoral responses in chronic HIV-1 infection. Previous studies demonstrated that higher CXCL13 plasma levels corresponded to increased chemotaxis of B cells in response to CXCL13 *in vitro* (Cagigi et al., 2008). Therefore possibly the increased secretion of CXCL13 by monocytes activates CXCR5-expressing B cells and pTFH cells making them prepared to enter secondary lymphoid organs, follicles and germinal centers. We hypothesize that HIV-1 induction of CXCL13 secretion by monocytes likely occurs in lymphatic tissues where substantial HIV-1 replication occurs (Pantaleo et al., 1994), which corresponds to previous reports that monocytes stained positive for CXCL13 in LN from HIV-infected subjects (Cagigi et

al., 2008). In which case, effective recruitment of B cells and TFH cells during HIV-1 early infection may be beneficial to the development of CRNA. However we hypothesize that the same mechanisms during chronic HIV-1 infection may contribute to generalized immune activation and accumulation of TFH cells (Lindqvist et al., 2012; Perreau et al., 2013; Petrovas et al., 2012; Xu et al., 2013), and hypergammaglobulinemia (Hong et al., 2012a; Lindqvist et al., 2012).

TFH cells are essential for germinal center formation and generation of high-affinity antibodies (Victora and Nussenzweig, 2012). Indeed, TFH cells have been shown to be the limiting factor in generation of high-affinity antibodies (Ciurea et al., 2001; Johnston et al., 2009). Likewise, effective pTFH cell responses have been associated with influenza vaccination efficacy in HIV-infected subjects (Pallikkuth et al., 2012). We hypothesize that the functionality and frequency of pTFH cells correlates with that of GC TFH cells. Therefore, in the 'broad' subjects, we hypothesize that the maintenance of GC TFH cells results in improved B cell help, affinity maturation and generation of B cell with BCRs of high enough affinity to withstand antigen variation **(Figure 4.1).** Cleary these antibodies need to target conserved epitopes to become highly cross-reactive. Therefore by combining immunogens which focus the antibody response to conserved epitopes with immunization strategies to optimize B cell help perhaps we will finally make progress in inducing CRNA by vaccination. In which case devising consistent and validated methods for evaluating GC TFH and pTFH cell vaccine responses will be critical for evaluating vaccine strategies moving forward.

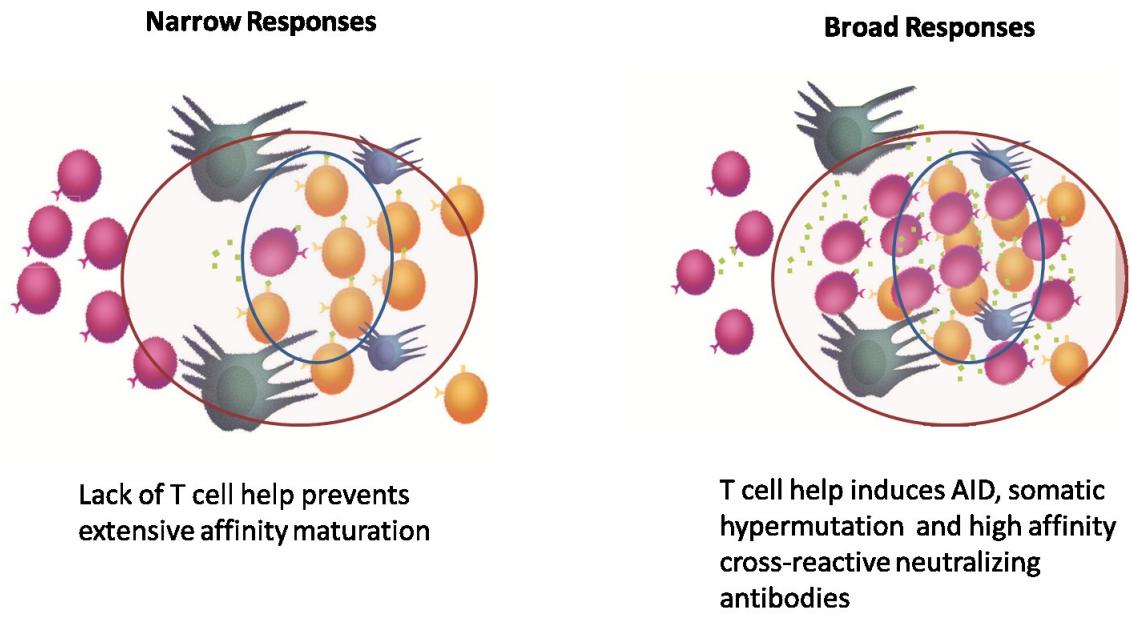


Figure 4.1 Model of development of CRNA during HIV-1 Infection. The red circles symbolize B cell follicles of a secondary lymphoid organ, and the blue circles represent a germinal center. In subjects with narrow responses, TFH cells are infected and depleted during early infection; B cells lack sufficient 'help' undergo less affinity maturation (left panel). In subjects with broad CRNA able TFH cells respond to CXCL13 and potentially secrete more CXCL13 to recruit more B and TFH cells, provide 'help' to B cells, support affinity maturation and development of CRNA (right panel).

During vaccination, TLR7/8-mediated induction of CXCL13 may be beneficial to generation of CRNA in uninfected individuals and should be thoroughly considered. In murine studies, TLR7 agonists have been used as vaccine adjuvants and demonstrated to be highly effective at promoting high-affinity antibody responses through mechanisms which depended on TLR7 signaling in DCs and on T cell help (Kasturi et al., 2011; Neighbours et al., 2012). In addition, viral infections of TLR7 deficient mice have also highlighted the significant contributions TLR7 activation makes to the generation of the antibody response and subsequent protection (Kasturi et al., 2011; Neighbours et al., 2012). Similarly, in influenza vaccine studies, type I IFN has been associated with development of antigen-specific neutralizing antibodies (Proietti et al., 2002; Stetson and Medzhitov, 2006). Type I IFN signaling in DCs has also been shown to be important for the development of LN-resident TFH cells (Cucak et al., 2009). Therefore, taken

together our results implicate induction of type I IFN as a potential mechanism driving the preferential preservation of TFH cells and concomitant increases in CXCL13. Our results suggest that the induction of CXCL13 and TFH cells by type I IFN may be of functional relevance in these models and TLR7/8 adjuvants should be thoroughly considered for modulating the antibody responses. Vaccines should also be evaluated for induction of CXCL13 and pTFH/TFH responses in the generation of high-affinity antigen-specific antibodies.

Concluding remarks

While these studies shed new light and understanding on the development of CRNA, they also raise important questions that merit investigation, such as the precise mechanisms that allow for the maintenance of pTFH/TFH during early infection, how to recapitulate these mechanisms by vaccination, and what the role of CXCL13 is in CRNA. Our results presented here identify multiple independent factors that correlated with CRNA including early frequency of pTFH cell and plasma concentrations of CXCL13, when other variables did not. Considering the dearth of understanding of development of CRNA, they may be especially informative in future vaccine design and evaluation. Understanding the mechanism(s) by which (p)TFH cells assist the development of broad neutralizing antibody responses during HIV-1 infection may contribute to the development of vaccination protocols that will elicit similar antibody responses by immunization.

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