Qualitative Characteristics of HIV-1-Specific CD4+ T Cells Responses Associated With Broadly Neutralizing Antibody Response

Qualitative characteristics of HIV-1-specific $CD4^+$ T cells responses associated with broadly neutralizing antibody response

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A Thesis Submitted to the Faculty of

The Harvard Medical School

in Partial Fulfillment of the Requirements

for the Degree of Master of Medical Sciences in Immunology

Harvard University

Boston, Massachusetts.

May, 2016

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Abstract

The underlying mechanism responsible for the development of broadly neutralizing antibodies (bNAbs) in natural infection is poorly understood. Current findings suggest that a sufficient help of $CD4^+$ T cells to B cells is required in the development of bNAbs. At the clinical level, it is unclear whether bNAbs generating individuals exhibit more robust HIV-1-specific $CD4^+$ T cells responses, thereby providing better help to B cells during infection. We hypothesized bNAbs response generating individuals to possess superior qualitative characteristics in their HIV-1-specific $CD4^+$ T cells responses compared to non-neutralizers. In this study, *in vitro* stimulation assay that allows the evaluation of the combined CD4-orchestrated cellular immune response to HIV-1 antigens was performed. $CD8^+$ depleted peripheral blood mononuclear cells (PBMCs) of chronically HIV-infected subjects with different capability of generating bNAbs response were stimulated with Gag peptide pools for 48 hours. Qualitative characteristics of HIV-1-specific $CD4^+$ T cells are analyzed based on 34 chemokines and cytokines secretion in the supernatant. HIV-1-specific $CD4^+$ T cells from broad neutralizers showed to have a unique capacity to stimulate production of the cardinal cytokine CXCL13 that has been previously associated with germinal center formation and development of broadly neutralizing antibodies against HIV. Linear discriminant analysis (LDA) and partial least square discriminant analysis (PLSDA) also showing

CXCL13 to be positively correlated with neutralization. Immunofluorescence staining of the lymph node section showed that CXCL13 was exclusively found in the follicle. Although CXCL13 is thought to be a natural ligand for CXCR5, not all cells that expressed CXCL13 have CXCR5 co-staining. It may suggest that CXCL13 is not exclusively expressed by $CXCR5^{hi}$ $CD4^{+}$ T cells in the germinal centers and other follicular cell subset may contribute.

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Acknowledgements

I would like to express my deepest gratitude for my thesis advisor Dr. Filippos Porichis who provided invaluable guidance and intellectually challenging discussion throughout the course of my Master's Thesis and laboratory project. His valued advice will remain with me in my future endeavors both inside and outside the laboratory.

I would also like to extend my deepest appreciation for Dr. Shiv Pillai, the Program Director of Master of Medical Sciences (MMSc) in Immunology, who provided endless support and guidance during the Master's program. His advice and kindness will be everlasting in my life.

I also want to thank Alexandra Massa, Ngoc Ly, Julio Silva, and Dr. Adrienne Yanez who provided technical support during my laboratory project. To Drs. Takashi Maehara and Hamid Mattoo, I also want to thank you for their technical support.

I would like to thank the MMSc in Immunology Drs. Shiv Pillai, Michael Carroll, Kevin Bonham and Selina Labriola for their endless support and passion in every step during the program.

This Master's Thesis would never be realized without the financial support from the Fulbright Scholarship, United States Department of States, Airlangga University School of Medicine, and the Indonesian Secretary of Education.

To my MMSc in Immunoloy classmates thank you for the wonderful friendships inside and outside of the classroom. They have provided me with an amazing support in finishing this Master's Thesis.

I express my gratitude to Drs. Agung Pranoto, Djoko Santoso, Kuntaman, and Ni Made Mertaniasih who introduced me to the research world and for their personal as well as professional support that made this master's study possible.

Finally, I want to thank my husband Maulana A. Empitu, my wonderful and tough son Maulana Bahagia Lillah, my cheerful and strong daughter Miratu Sophia Ananta, my parents, and my siblings Hendry and Derry Kadarisman. You have taught me that life always has a beautiful side, even if it is tough at times.

"This work was conducted with support from Students in the Master of Medical Sciences in Immunology program of Harvard Medical School. The content is solely the responsibility of the authors and does not necessarily represent the official views of Harvard University and its affiliated academic health care centers."

Chapter 1: Background

1.1 Current state of the Human Immunodeficiency Virus pandemic

Three years after the first case of acquired immunodeficiency syndrome (AIDS) case was discovered in 1981, Human Immunodeficiency Virus (HIV) was formally identified as the etiology of AIDS [1-3]. HIV infection became one of the major health problems worldwide. It has infected more than 78 million people and caused 39 million death since the beginning of the AIDS pandemic [4]. In 2014 alone, HIV infection caused 1.2 million deaths and 2 million newly infections from 37 million people who live with HIV infection [5]. Low and middle income countries are the most affected, especially in sub-Saharan Africa where 70% of new HIV infection occurs [5].

Between the two etiologies of AIDS, HIV-1 is the predominant HIV infecting global population, with most patients progressing to AIDS [6-8]. HIV-2 infection also contribute to the AIDS burden, but only 30% of people with HIV-2 infection progress to AIDS, indicating that HIV-2 is less pathogenic that HIV-1 [6-8].

AIDS is characterized by deterioration in the immune system, hallmarked by depletion of the $CD4^+$ T cell number. This perturbation in the immune system allows for numerous opportunistic infections and cancers leading to serious morbidity and mortality. This disease is transmitted through blood transfusion, vertical transmission (mother to child through delivery or breast feeding), and sexual intercourse.

Relatively low access to antiretroviral therapy (ART) is one of major contributors to the high rate of AIDS-related deaths worldwide. Only about 40% of HIV-infected

individuals have access to ART, and even less of those who respond positively to the therapy [5]. Additionally, ART needs to be taken life-long in order to maintain a viral suppression effect which greatly depend on the patients' adherence. Therefore, ensuring access and adherence to ART presents a logistical, financial, and behavioral challenges especially in developing countries where HIV infection is most prevalent [9].

Beside the public health related challenges in its application and distribution, the role of ART in the context of combating the AIDS epidemic mainly focused on secondary and tertiary prevention. Despite the advances of ART in decreasing mortality and morbidity amongst people infected with HIV, ART cannot provide an absolute cure. It does suppress HIV replication, but neither eradicate the virus from the human body nor preventing new infection.

However, based on successful experience of prior vaccines for preventing many other infectious diseases, a preventive vaccine against HIV-1 is thought to be the most effective approach to hamper new HIV infections which hopefully lead to the ending of the AIDS pandemic. A modeling study indicates that a preventive AIDS vaccine with 70% efficacy could significantly reduce the number of new infection by 44% in 10 years and by 65% in 25 years in low and middle income countries [10]. An effective HIV-1 vaccine will also be more cost effective compared to life-long ART. It is also beneficial for people who do not have access to ART or lack consistency in using ART. It could prevent tens of millions of infections and save millions of lives [10].

1.2 HIV-1 vaccine development

After more than 30 years of effort to develop an HIV-1 vaccine, there is still no HIV-1 vaccine that could reach these goals. The development of an effective vaccine has been extremely difficult. The process of HIV-1 vaccine development takes years to transition from basic science research into clinical trials. While a large number of preclinical and clinical studies have been performed, only 4 differ vaccine regimens have gone to clinical efficacy studies [11-15].

The first HIV-1 vaccine regiment that went on to efficacy trials was based on viral envelope protein gp120 (AIDSVAX B/E gp120) [16-18]. It was designed to induce type-specific antibody response. This approach worked for Hepatitis B vaccine [19-21]. However, this regimen could not elicit HIV-1 specific antibodies to prevent infection [11, 13].

The second HIV-1 vaccine efficacy study called STEP was conducted to test the efficacy of a regimen using a recombinant adenovirus type 5 as the vector that directed against group specific antigen (Gag), polymerase (Pol), and negative factor (Nef) proteins of HIV. This vaccine regimen was designed to induce $CD8^+$ T cells response to prevent disease progression. This efficacy trial resulted in a non-protective result [12]. Safety concerns were also raised during this trial when a subset of people who received the vaccine was found to be at higher risk of infection after vaccination [22]. Pre-existing immunity to adenovirus 5 increased the risk of gaining HIV-1 infection [22].

The next efficacy trial for an HIV-1 vaccine also used recombinant adenovirus type 5 as a delivery vehicle, but included multi-clade Env to generate antibodies in

addition to Gag and Pol for eliciting T cell-responses [23]. This regimen was also unsuccessful in preventing HIV-1 infection though there were no safety concerns related to increased risk of infection for the vaccinated subjects [14].

Promising results came from the RV144 vaccine efficacy trials. The RV144 used a canarypox vector (ALVAC) expressing HIV-1 clade B Gag, protease, and CRF01_AE Env gp120 as a prime and used both ALVAC and AIDSVAX B/E gp120s as boosts. These efficacy trials showed an estimated vaccine efficacy of 31% in preventing transmission [15].

Many approaches have been taken to develop an effective vaccine against HIV-1 infection. The partial success of the RV144 vaccine trial provided the first evidence that it might be possible to protect against HIV acquisition. Results from this vaccine trial highlight the need for an HIV-1 vaccine that is able to elicit both arms of the adaptive immune system: cell-mediated and humoral immunity. In humoral immunity, the objective is eliciting broadly neutralizing antibodies (bNAbs) response.

1.3 The adaptive immune response to HIV-1 infection

HIV-1 is a part of the genus lentivirus from the retroviridae family that infects cells of the human immune systems, specifically $CD4^+$ T cells. In the human body, HIV could be found in blood, semen, cervicovaginal fluid, cerebrospinal fluid, milk, urine, tears, ear secretion and saliva. The amount of virus in bodily fluids and the extension of contact with infected bodily fluids determine the transmission frequency of HIV-1. Transmission typically occurs through intimate sexual contact, contaminated blood, and mother to child transmission (during delivery or breast feeding) [24].

Transmission of HIV-1 requires successful interaction of the virus with receptors on a cell surface. HIV-1 has tropism to CD4 molecule [25]. After the virus attaches to the CD4 molecule, the next step is fusion between viral and host cell membrane. The HIV-1 conical capsid core, which contains the viral RNA genome, is released into the cytoplasm of the infected host cell. Then, the capsid core goes to the host's nucleus where reverse transcription of the viral RNA genome results in viral DNA. The viral DNA then integrates itself into the host genome. This directs the formation of progeny virions that are eventually released to infect other new cells [25].

1.3.1 Cell-mediated immune response against HIV-1

1.3.1.1 $CD4^+$ T cells immune response against HIV-1 infection

$CD4^+$ T cells play a pivotal role in the human immune system, orchestrating adaptive immune responses to form an effective response against pathogens while also suppressing non-essential immune responses [26-28]. The term "T helper cells" underscores their specialized role in orchestrating immune responses. They provide help to other cells in adaptive immunity. In HIV-1 infection, $CD4^+$ T cells help $CD8^+$ T cells in memory development. IL-21 produced by $CD4^+$ T cells helps maintain antiviral activity of $CD8^+$ T cells [29, 30]. $CD4^+$ T cells also provide help to B cells in the process of antibody development [28, 31].

$CD4^+$ T cells also have direct effector functions against HIV-1. Cytotoxic activity of $CD4^+$ T cells correlates with a delay in disease progression [32]. It has been suggested that $CD4^+$ T cells use similar killing mechanism as $CD8^+$ T cells and natural killer cells [32]. Cytotoxic $CD4^+$ T cells have also been suggested to play a potent role in controlling

viral replication in infected macrophages or dendritic cells while $CD8^+$ T cells are impaired [33]. In addition, $CD4^+$ T cells that produce IL-17 and IL-22 are essential for maintaining the integrity of mucosa. Therefore, these cells may also play indirect role in controlling viral replication through reducing immune activation [34].

1.3.1.2 $CD8^+$ T cells immune response against HIV-1 infection

The most important function of $CD8^+$ T cells is to recognize and kill infected cells. The killing mechanism predominantly used by $CD8^+$ T cells is through secretion of perforin and granzymes [35, 36]. Perforin is a pore-forming protein that is required for delivery of granzymes into the target cells while granzymes are serine proteases that cleave caspases to induce apoptosis [37-40]. These proteins are contained within lytic granules and are released early after $CD8^+$ T cells activation into immunological synapse formed between $CD8^+$ T cells and target cells. $CD8^+$ T cells can also mediate killing by the Fas-Fas ligand (FasL) pathway. However, cytolysis of HIV-1-infected cells appears to be primarily mediated by perforin and granzymes mechanism [36].

$CD8^+$ T cells also inhibit HIV-1 replication through chemotactic cytokines secretion. β-chemokines macrophage inflammatory protein-1α (MIP-1α), MIP-1β and regulated upon activation normal T-cell expressed and secreted (RANTES) are noncytotoxic factors secreted by $CD8^+$ T cells that directly inhibit HIV-1 replication by binding to CCR5 [33, 41]. This mechanism results in blocking access and induces internalization of the receptor [33, 41]. In HIV-1 infection, $CD8^+$ T cells also provide another noncytotoxic function mediated by $CD8^+$ T cell antiviral factor (CAF) [42]. CAF

functions by suppressing HIV-1 long terminal repeat (LTR)-mediated gene expression in $CD4^+$ T cells [43].

1.3.2 Humoral immune response to HIV-1 infection

Antibodies are able to prevent infection by binding viral epitopes that are important for replication [44]. This neutralization process is thought to be the pivotal immune response in eradication of HIV-1 infection. Whereas most individuals with HIV-1 infection develop neutralizing antibodies (NAbs) against HIV-1 several months after initial infection, the HIV-1 is always one or more steps in front of these NAbs. Consequently, these NAbs are only able to control viral replication in a limited manner [45]. Therefore, NAbs against HIV-1 have to overcome a high level of viral diversity.

About 15-20% of infected individuals develop broadly neutralizing antibodies (bNAbs) several years after infection [46]. These bNAbs are capable of blocking a wide range of HIV-1 stains and are characterized by a high degree of somatic hypermutation. This characteristics results from extensive affinity maturation and Ag-specific interaction of T follicular helper (Tfh) cells with B cells within germinal centers (GCs) [47-56]. The broad range of neutralization from these antibodies suggest that in some individuals, the host immune system is capable of overcoming the flexibility of HIV-1 evolution.

1.3.3 T-B interaction: Importance of CD4+ T cell help to B cells

The vast majority of neutralizing antibody responses to pathogens are dependent on $CD4^+$ T cell help. Development of bNAbs against HIV-1 requires functional germinal center formation and sufficient help of $CD4^+$ T cells to B cells [32, 57, 58]. Germinal centers are the sites of B cell selection and mutation [59]. Each round of B cell

proliferation and selection depends on survival, proliferation and differentiation signals provided by T follicular helper (Tfh) cells, a subset of $CD4^+$ T cells, in the form of cell surface co-stimulatory molecules and secreted factors [31].

Follicular B cells are activated through a T-cell-dependent mechanism. Starting with activation at the site of infection, dendritic cells migrate to the paracortex where they present a viral antigen as a class II MHC peptide complex to naïve $CD4^+$ T cells [60, 61]. In the lymphoid follicles, B cells are in part activated through the binding of their cognate antigen that result in MHC II antigen presentation. Activated B cell then enter para-cortex in chemotactic guided in order to find $CD4^+$ T cell that specific for the MHC II peptide complex displayed in its surface. B cells are further activated when creating primary focus formation after encountering the antigen specific $CD4^+$ T cell. The higher affinity B cell clones primarily become short-live plasma cell that create extra follicular formation [62]. A smaller portion of these cells become long live plasma cell that eventually reside in bone marrow or other secondary lymphoid tissues [63].

Other progeny of an activated B cell clones re-entry the follicle along with their cognate T-cell. Then, they interact with other cells including $CD4^+$ follicular T helper (Tfh) cell and dendritic cells. This interaction results in development of the germinal center formation. Isotype switching, somatic hypermutation, and affinity maturation processes taking place in the germinal center produce antibodies with higher affinity. These processes also lead to maturation of antigen-specific naïve B cells into memory B cells that persist in circulation [64, 65].

Most HIV bNAbs have a high number of mutations, indicating massive selection and affinity maturation in germinal centers before the development of sufficient broadly

neutralizing binding capacity [48, 49, 51, 53]. The evidence indicates that the majority of those mutations are functional products of the affinity maturation process, not bystander accumulation of irrelevant mutations [53].

1.3.4 Lack of knowledge in HIV-1 specific CD4+ T cell responses associated with the development of broadly neutralizing antibodies

$CD4^+$ T cells play a pivotal role in the human immune system, orchestrating the adaptive immune response to shape an effective response against pathogens while suppressing non-essential immune responses [26-28]. The potential importance of functional $CD4^+$ T cells responses in the development of HIV-1 vaccine gains limited attention because $CD4^+$ T cells are the primary targets of HIV-1. This raised the concern that they might fuel viral replication [66]. However, the large majority of HIV-1-specific $CD4^+$ T cells are not infected in vivo [66].

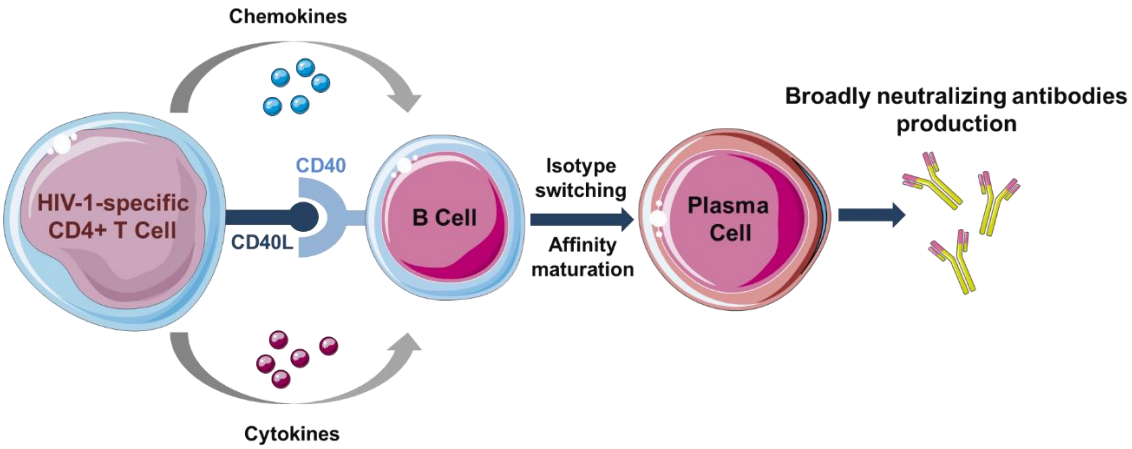


Figure 1. Schematic diagram of HIV-1-specific $CD4^+$ T cells help to B cells. Sufficient help of $CD4^+$ T cells to B cells is required for the development of bNAbs.

A fundamental problem in HIV-1 vaccine design is how to induce development of broadly neutralizing antibodies (bNAbs). Sufficient help of $CD4^+$ T cells to B cells is required for the development of bNAbs (Figure 1). There have been extensive efforts to characterize the blood bulk $CD4^+$ T cell subsets of unknown antigen specificity that might be associated with help to B cells [67-70]. Despite the critical role of HIV-1-specific $CD4^+$ T cell in the development and maintenance of the germinal center and humoral immune response [32, 57, 58], the quality of HIV-1-specific $CD4^+$ T cells associated with optimal help to B cells in natural HIV-1 infection are only partially understood. One important question is whether individuals with the capability of generating broadly neutralizing antibodies have more robust HIV-1-specific $CD4^+$ T cells responses to provide optimal help to B cells.

Due to the evidence that most HIV-1 bNAbs show high level of mutation, it is likely that outstanding $CD4^+$ T cell help to B cells must be elicited by an HIV-1 vaccine to meet the overall challenge of having optimal germinal centers for extensive selection events to generate HIV-1 bNAbs. We hypothesize that individuals who generate broadly neutralizing antibodies response have superior qualitative characteristics of HIV-1-specific $CD4^+$ T cells responses compared to non-neutralizers. Furthermore, this characteristics may lead to more optimal help to B cells in generating broadly neutralizing antibodies responses against HIV-1.

In this study, we performed *in vitro* stimulation assay that allows the evaluation of the combined CD4-orchestrated cellular immune response to HIV-1 antigens (Figure 2). $CD8^+$ depleted peripheral blood mononuclear cells (PBMCs) of chronically HIV-infected subjects were stimulated with Gag peptide pools for 48 hours. Subjects were categorized

based on their ability to generate broadly neutralizing antibody responses. Qualitative characteristics of HIV-1-specific $CD4^+$ T cells are analyzed based on chemokines and cytokines secretion in the supernatant. These soluble factors are both critical mediators of HIV-1-specific $CD4^+$ T cell functions and key regulators of this lymphocyte subset.

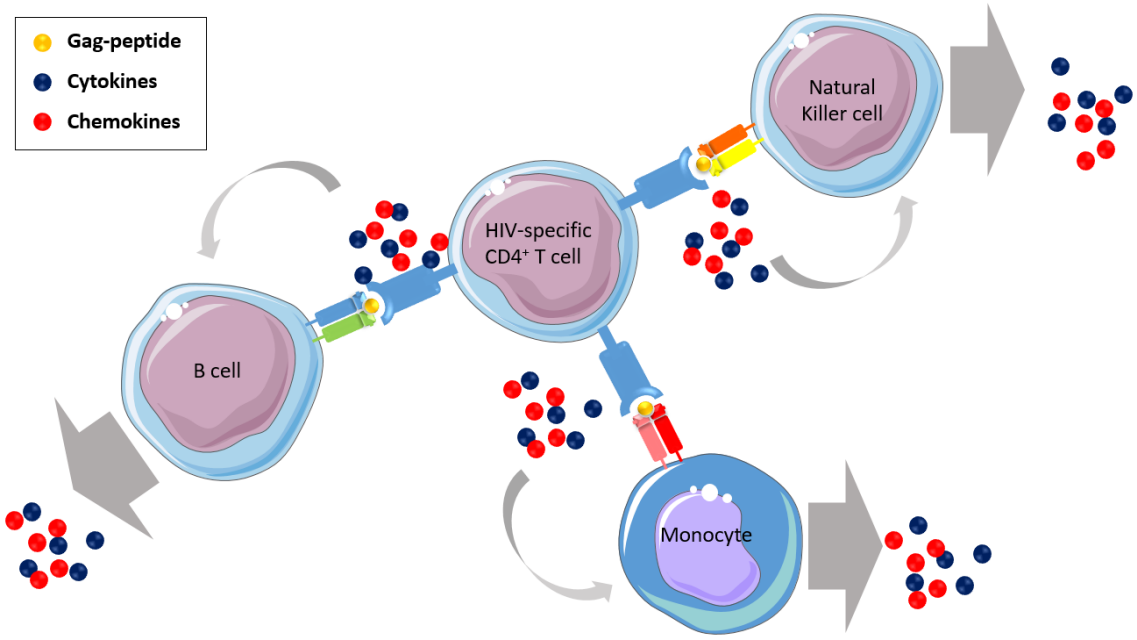


Figure 2. Schematic diagram of *in vitro* stimulation assay that allows the evaluation of the combined CD4-orchestrated cellular immune response to HIV-1 antigens. $CD8^+$ depleted peripheral blood mononuclear cells (PBMCs) of chronically HIV-infected subjects were stimulated with Gag peptide pools for 48 hours.

Understanding the qualitative characteristics of HIV-1-specific $CD4^+$ T cells responses associated with broadly neutralizing antibodies response will fill a major unmet need for rational design of vaccine strategies.

Chapter 2: Methods and Results

To characterize HIV-1-specific $CD4^+$ T cells responses associated with broadly neutralizing antibody responses, *in vitro* stimulation assay that allows the evaluation of the combined CD4-orchestrated cellular immune response to HIV-1 antigens was performed. This experimental design previously described in studies by Porichis *et al* [71-73].

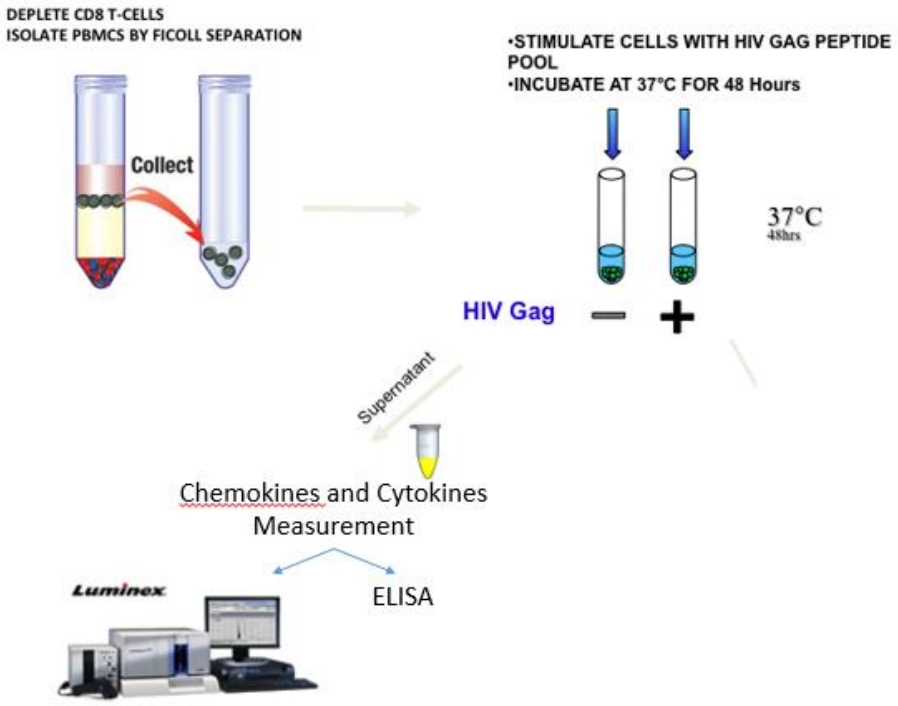


Figure 3. *In vitro* experimental design for measuring secreted chemokines and cytokines of HIV-1-specific $CD4^+$ T cells responses associated with broadly neutralizing antibodies response. CD8 T cells were depleted from PBMCs and that were isolated using Ficoll separation method. CD8-depleted PBMCs then stimulated with an HIV-1 Gag peptide pool and incubated at 37 °C, 5% CO_2. After 48 hours, supernatant was collected for chemokines and cytokines analysis using Luminex bead arrays and ELISA. A previously in studies by Porichis *et al* [71-73]

In this study, CD8-depleted PBMCs from 21 broad neutralizers and 21 non-neutralizers were stimulated in the presence of Gag peptide pools for 48 hours (Figure 3). After this period, Luminex bead arrays and enzyme linked immunosorbent assay (ELISA) were performed to measure a total of 34 secreted cytokines and chemokines in the supernatant in response to antigen recognition by the HIV-1-specific CD4+ T cells.

2.1 Materials and Methods

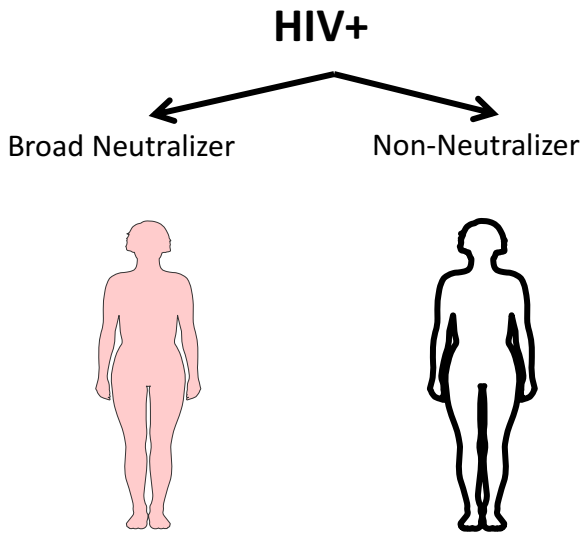


Figure 4. Subjects were categorized into broad neutralizers and non-neutralizers based on TZM.bl neutralization results. Individuals who could neutralize three or more tier 2/3 viruses were classified as broad neutralizers, while individuals who neutralized less than three tier 2/3 viruses were classified as non-neutralizers

2.1.1 Subjects

A total of 42 HIV-infected subjects were recruited from Massachusetts General Hospital. Based on TZM.bl neutralization assay result, 21 subjects were categorized as broad neutralizers and 21 were categorized as non-neutralizers (Figure 4). Fresh blood from each enrolled subject was obtained and processed. The peripheral blood

mononuclear cells (PBMCs) were isolated from blood and then frozen. The frozen PBMCs were processed and measured for chemokines and cytokines released using Luminex bead arrays and enzyme linked immunosorbent assay (ELISA).

2.1.2 Neutralization assay

A luciferase-based TZM.bl cell assay was utilized to measure neutralizing antibody titers, indicated by a decrease in luciferase reporter gene expression after single-round viral infection of TZM.bl cells. As described previously [74], 3-fold serial dilutions of heat-inactivated plasma samples (56°C for 1 h) were done in duplicate and incubated with HIV-1 Env pseudoviruses for 1 h at 37°C.

TZM.bl cells were subsequently added in growth medium containing DEAE-dextran at a final concentration of 11 ug/ml, and assay plates were incubated for 48 h at 37°C in 5% CO2. Bright-Glo luciferase reagent (Promega) and a Victor 3 luminometer (PerkinElmer) were used to measure Luciferase reporter gene expression. Neutralization titers or 50% inhibitory dose [ID50]) were defined as the serum dilution at which relative light units (RLU) were decreased by half, compared to the number of RLU in virus control wells after subtraction of background RLU counts in cell control wells.

The plasma from each subject was tested against a standardized panel of clade B tier 1 and tier 2/3 Env pseudoviruses. Neutralization was determined as positive if the ID50 titer against an HIV-1 isolate was 3-fold higher than that observed against a negative-control murine leukemia virus (MuLV).

In this study, individuals who could neutralizer three or more tier 2/3 viruses were classified as broad neutralizers, while individuals who neutralized less than three tier 2/3

viruses were classified as non-neutralizers. From 42 enrolled subjects, 21 subjects were categorized as broad neutralizers and 21 were categorized as non-neutralizers.

2.1.3 PBMC isolation

PBMCs were isolated from the blood using the Ficoll (Hystopaque) density-gradient centrifugation method. After centrifugation, harvested PBMCs were washed 3 times in Hanks Balance Salt Solution. PBMCs were then resuspended at a concentration of 10^7 cells per milliliter in complete RPMI 1640 medium containing 200 mM l-glutamine, 5000 IU penicillin, 5000 ug/ml streptomycin, 1M HEPES and supplemented with 10% fetal calf serum (FCS). Cell viability was evaluated using NucleoCounter® NC-200™. PBMCs were frozen down in 10% dimethyl sulfoxide (DMSO) in FCS at a concentration of 10^6 cells per milliliter.

2.1.4 CD8 Depletion

After thawing and resting the frozen PBMCs for 3 hours, $CD8^+$ T cells were depleted using Dynabeads CD8 Positive Isolation Kit (Life Technologies, cat# 113-33D). Using this magnetic cell separation method ensured separated cells were free of attached beads or antibody.

Before each experiment, the beads were washed once with PBS buffer containing 1% FBS to remove the sodium azide stabilizing agent from the bead buffer. The beads were then suspended in an equivalent volume of PBS buffer with 1% FBS. After adding the beads to the PBMCs (25 ul beads/10^7 PBMCs), the suspension was placed them on a rotator for 20 minutes at 4^{o}C. The suspension was then placed in the magnet for 5

minutes to separate bead-labeled $CD8^+$ T cells from the suspension. The remaining cells were then placed in a new tube and washed twice.

2.1.5 Stimulation and supernatant collection

CD8 depleted PBMCs were resuspended in R10 at concentration $4x10^6$ cells/milliliter. 500 microliters of supernatant was transferred to the tube. Each patient sample was subjected to 2 different conditions: no stimulation and Gag stimulation. Gag peptide pool comprised of Gag peptides 1-66 (1µg/ml/peptide) was used for Gag stimulation. 5µl of gag peptide pool was added to 500µl of sample supernatant to stimulate. Samples were then incubated for 48 hours at 37ºC in 5% CO_2.

After 48 hours of stimulation, samples were centrifuged at 1500 rpm for 10 minutes to collect the supernatants. 5uL of 0.5% PBS-Tween-20 were added to each 50 uL of supernatant for virus inactivation. 700uL of Qiazol was added to the remaining cell pellets and stored at -80^OC.

2.1.6 Chemokines and cytokines secretion measurement

Table 1. Chemokines and cytokines that were measured

CXCL13	IL-1α	IL-7	IL-15	MIP-1α
Fractalkine	IL-1β	IL-8	IL-17α	MIP-1β
GM-CSF	IL-1rα	IL-9	IL-21	MIP-3α
Granzyme A	IL-2	IL-10	IP-10	Perforin
Granzyme B	IL-4	IL-12p40	MCP-1	sCD40L
IFN-α2	IL-5	IL-12p70	MCP-3	TNFα
IFN-ɣ	IL-6	IL-13	MDC	

2.1.6.1 Luminex

MILLIPLEX® MAP, a Luminex bead-based array, was used to measure the concentration of cytokines and chemokines. Pre-coated magnetic beads (EMD Millipore, catalog# HCYTOMAG) were used to measure concentration of several secreted cytokines and chemokines (Table 2)

Table 2. Measured chemokines and cytokines using MILLIPLEX® MAP HCYTOMAG

Fractalkine (22.7)	IL-1β (0.8)	IL-12p40 (7.4)	MCP-1 (1.9)
GM-CSF (7.5)	IL-1RA (8.3)	IL-12p70 (0.6)	MCP-3 (3.8)
IFN-α2 (2.9)	IL-7 (1.4)	IL-15 (1.2)	MDC (3.6)
IFN-ɣ (0.8)	IL-8 (0.4)	IP-10 (1.1)	sCD40L (5.1)
IL-1α (9.4)	IL-9 (1.2)		

Values in the bracket indicated the minimum detectable concentrations (pg/ml).

Luminex kit from catalog# HCD8MAG was used to measure the concentration of other chemokines and cytokines (Table 3).

Table 3. Measured chemokines and cytokines using MILLIPLEX® MAP HCD8MAG

Granzyme A (8.9)	IL-6 (0.1)	MIP-1α (0.3)	Perforin (3.8)
Granzyme B (0.6)	IL-10 (1.1)	MIP-1β (2.4)	TNFα (0.2)

Values in the bracket indicated the minimum detectable concentrations (pg/ml).

For the other remaining chemokines and cytokines, Luminex kit from catalog# HSCYTOMAG was used (Table 4).

Table 4. Measured chemokines and cytokines using MILLIPLEX® MAP HSCYTOMAG

IL-2 (0.19)	IL-13 (0.23)	IL-21 (0.14)	MIP-3α (0.83)
IL-4 (1.12)	IL-17α (0.33)		

Values in the bracket indicated the minimum detectable concentrations (pg/ml).

The method for 96 well plates described by the manufacturer was followed. Samples were added along with standard solutions and controls as described in manufacturer's protocol for catalog # HCYTOMAG, HCD8MAG, HSCYTOMAG (EMD Millipore). Beads were combined added to each well for an 18 hours incubation at 4°C in the dark. After incubation, the plate was washed twice, detection antibody added, incubated at RT in darkness for 60 min, before streptavidin–phycoerythrin addition and further incubated for 30 min. After washing, the plates were run on a Luminex 3D with xPONENT software for acquisition and BioPlex for data analysis. The median fluorescent intensity was analyzed using a 5-parameter logistic curve-fitting to calculate the concentrations of the cytokines in the samples.

2.1.6.2 ELISA

The Human CXCL13 Quantikine ELISA Kit (R&D Systems, catalog# DX130) was used to measure the concentration of CXCL13. The minimum detectable concentration of CXCL13 of this kit is 0.43 pg/ml. Manufacturer's protocol was followed. Samples were added along with prepared standards and controls and incubated

for 2 hours at room temperature. The plate was washed, human BLC/BCA-1 conjugate was added to each well and incubated for 2 hours at room temperature. After washing, substrate solution was added to the plate and incubated for 30 minutes at room temperature. After the addition of stop solution, optical density was measured on a microplate reader set at 450 nm.

Results were calculated by taking the average of duplicate readings for each standard, control, and sample and then subtracting the average zero standard optical density (O.D.). Standard curves were generated using Microsoft Excel 2010 and used to calculate the concentration of CXCL13 in pg/ml.

2.1.7 Immunofluorescence analysis

Paraffin-embedded sections of lymph node from HIV negative subjects were prepared. After baking the slides in 60^{O}C for 1 h, slides were de-paraffinized and rehydrated. Antigen retrieval was then performed using antigen retrieval buffer (Abcam, catalog# ab93684). Blocking was performed using peroxidase and alkaline phosphatase blocking agent (Dako, catalog# S2003) followed by Bloxall endogenous peroxidase and alkaline phosphatase blocking solution (Vector Laboratories, catalog# SP-6000). Slides were incubated with human CXCR5 antibody (cat# MAB-190, R&D Systems) for 2 hours followed by washing with PBS-Tween, incubating with TSA polymer HRP anti-mouse (Life Technologies, catalog# 87-9163) for 15 min, washing with PBS-Tween, and incubating with Cy5 (Perkin Elmer, catalog# NEL794001KT) for 15 min. Slides were then washed again with PBS-Tween. Before performing second antigen staining, antigen retrieval and Bloxall blocking were performed. Then, slides were incubated with human

CXCL13 Antibody (R&D Systems, catalog # AF801,) for overnight in 4^{O}C followed by washing with PBS-Tween, incubating with TSA polymer HRP anti-goat (Life Technologies, catalog# 87-9363) for 15 min, washing with PBS-Tween, and incubating with Cy3 (Perkin Elmer, catalog# NEL794001KT) for 15 min. After that, slides were washing again with PBS-Tween followed by counterstaining for DAPI with 3,3′-diaminobenzidine (Vector Laboratories, catalog# D1306). Stained slides were mounted then analyzed using a TissueFAXS Whole Slide Scanning System fluorescence microscope. Quantification of CXCL13 and CXCR5 was performed using TissueQuest 4.0.

2.1.8 Data analysis

Normality distribution of the data was tested using D'Agostino-Pearson omnibus normality test. To see the differences between broad neutralizers and non-neutralizers in no antigen stimulation, data that passed normality test would be tested using parametric test and data that did not have normal distribution would be tested using non parametric test. Univariate analyses were using GraphPad Prism 6.0. Multivariate analyses were carried out with JMP Pro 12 and XLSTAT.

2.2 Results

To investigate qualitative characteristics of HIV-1-specific $CD4^+$ T cell responses associated with broad neutralizing antibodies (bNAbs) response, we evaluated a cohort of 42 subjects with chronic HIV-1 infection for circulating *ex vivo* Gag-specific $CD4^+$ T cell responses in Luminex and ELISA using $CD8^+$ T cells-depleted PBMCs incubated for 48 hours. These responses were evaluated using 34 different chemokines and cytokines. A TZM.bl neutralization assay was used to determine the neutralization breadth of subjects. Individuals who neutralized three or more tier 2/3 viruses were classified as broad neutralizers, while individuals who neutralized two or less tier 2/3 viruses were classified as non-neutralizers. In this study, 21 subjects were classified as broad neutralizers and 21 subjects were categorized as non-neutralizers. There were no significant differences in viral loads and duration of infection between groups (Fig. 5a and 5b).

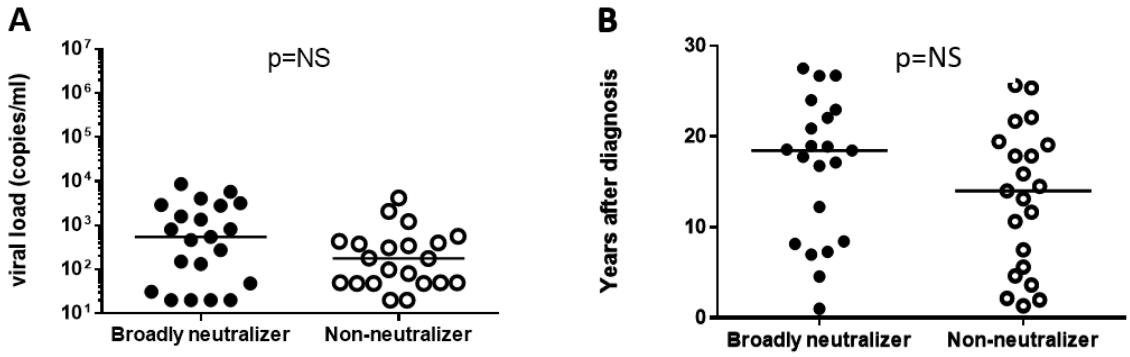


Figure 5. Clinical characteristics of broad neutralizer and non-neutralizer subjects. No significant differences were observed between broad neutralizers and non-neutralizers in HIV-1 viral loads (A) and duration of infection (B). Scatter plots show distribution of data for broad neutralizers and non-neutralizers for graphs in panel A and B, with lines denoting the median values. Statistical significance was tested using the Mann-Whitney test.

Table 5. Cytokines and chemokines response after Gag stimulation

Upregulated			Not Upregulated
CXCL13 (p=0.0095)	IL-4 (p=0.0166)	IL-21 (p=0.0001)	IL-2
GM-CSF (p=<0.0001)	IL-6 (p=0.0257)	IP-10 (p=<0.0001)	IL-5
Granzyme A (p=<0.0001)	IL-7 (p=0.023)	MCP-3 (p=0.0002)	IL-10
Granzyme B (p=<0.0001)	IL-8 (p=0.0098)	MIP-1a (p=0.0002)	IL-17a
IFNa2 (p=0.0473)	IL-9 (p=0.0012)	MIP-1b (p=0.0133)	MCP-1
IFNg (p=<0.0001)	IL-12p40 (p=0.0306)	MIP-3a (p=0.0063)	MDC
IL-1a (p=<0.0001)	IL-12p70 (p=0.0069)	Perforin (p=<0.0001)	sCD40L
IL-1b (p=<0.0001)	IL-13 (p=0.0223)	TNFa (p=<0.0001)	
IL-1ra (p=<0.0001)	IL-15 (p=<0.0001)		

Wilcoxon matched pairs, one tailed, t test was used to assess statistical significance. P value >0.05 was considered significant.

To characterize qualitative characteristics of HIV-1-specific CD4 T cell response associated with broadly neutralizing antibody responses, we first examined secretion levels following Gag stimulation compared to no stimulation condition. Using Wilcoxon matched pairs, one tailed, t test, we found that not all cytokines and chemokines were upregulated with Gag-peptide pool stimulation (Table 5).

Gag-specific $CD4^+$ T cells response results in higher level of CXCL13 in Broad neutralizers

Then, we characterized chemokines and cytokines level between broad neutralizers and non-neutralizers. Amongst all cytokines and chemokines that were measured, only CXCL13 showed a statistically significant higher concentration in response to Gag stimulation in broad neutralizers compared to non-neutralizers (Figure 6).

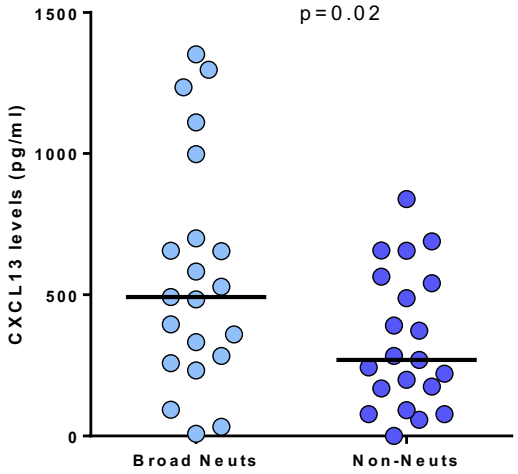


Figure 6. Gag-specific CD4$^+$ T cells response results in higher level of CXCL13 in broad neutralizers. Significance was tested using a Mann-Whitney, one-tailed test. P value <0.05 was considered significance. Median values shown (Broad neutralizer: 492, Non-neutralizers: 269).

Significantly higher response in CXCL13 secretion in broad neutralizers after Gag-peptide stimulation compared to unstimulated condition

In order to further characterize HIV-1-specific CD4$^+$ T cells responses after Gag peptide pool stimulation, we investigated the characteristics within each group. Wilcoxon matched pairs, one tailed, t test was performed for this analysis. In broad neutralizers we did see significantly enhanced CXCL13 concentration after Gag stimulation as the result of HIV-1-specific CD4$^+$ T cells responses (Figure 7a). However, this result was not seen in non-neutralizers. The response of HIV-1-specific CD4$^+$ T cells in non-neutralizers after Gag stimulation did not cause elevation of CXCL13 concentration (Figure 7b).

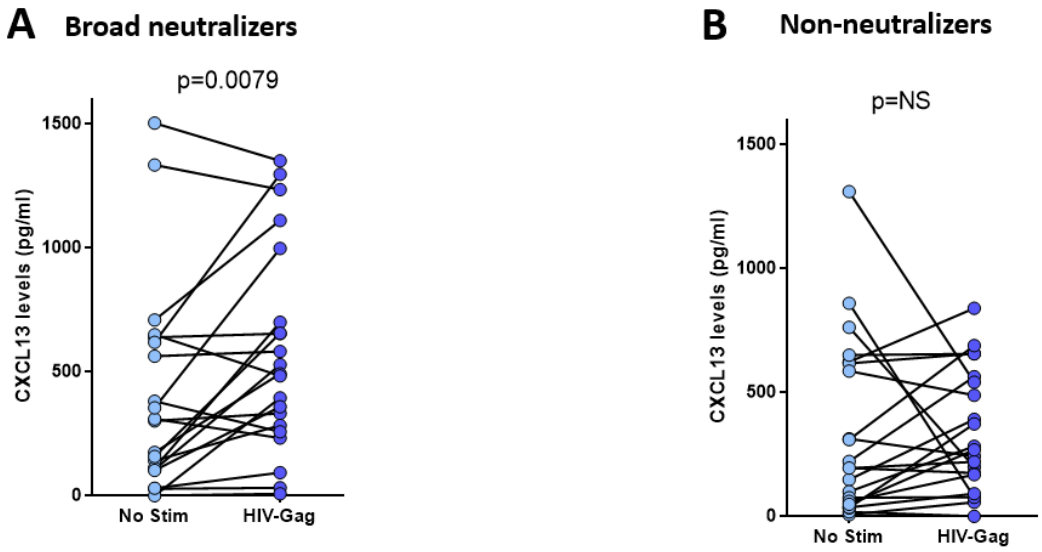
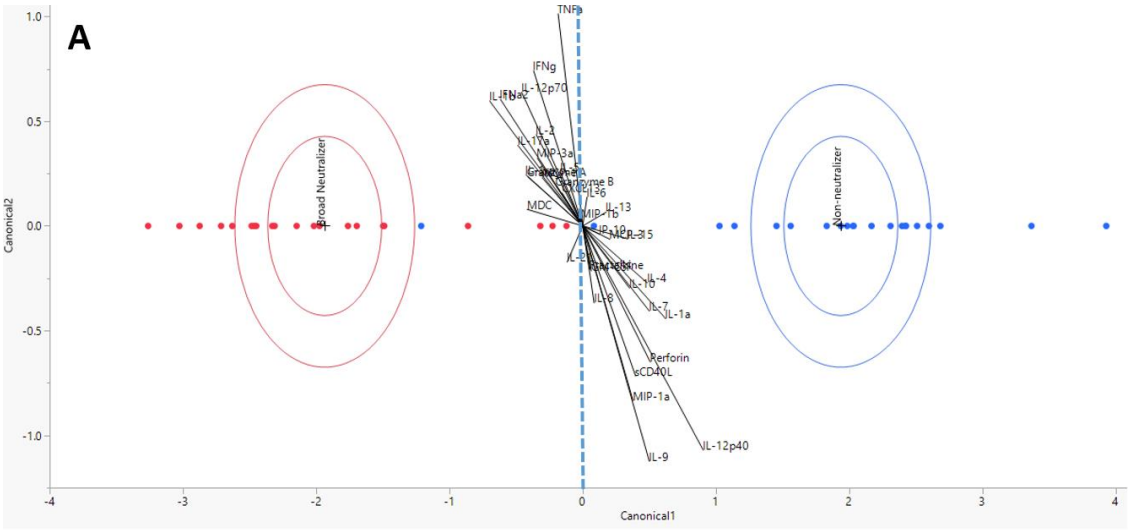


Figure 7. Significantly higher response in CXCL13 secretion in broad neutralizers after Gag-peptide stimulation compare to unstimulated condition. After Gag-peptide stimulation, broad neutralizers provided a statistically significant higher levels of CXCL13 (Figure 7a). No elevation in CXCL13 secretion was detected in non-neutralizers after Gag-peptide stimulation (Figure 7b). A Wilcoxon matched pairs, one tailed, t test was used to assess statistical significance. P value >0.05 was considered significant.

Identifying networks of cytokines and chemokines that are associated with neutralization

Since chemokines and cytokines work together in network, we performed multivariate analysis to identify the relationship in our measured chemokines and cytokines. Linear discriminant analysis and partial least square discriminant analysis (PLSDA) were performed to identify network of cytokines and chemokines that are associated with neutralization.

Linear discriminant analysis



B

Score Summaries

Source	Count	Number Misclassified	Percent Misclassified	Entropy RSquare
Training	42	3	7.14286	0.78043

Training

Actual Neutralization Status	Predicted Neutralizer	Predicted Non-neutralizer
Neutralizer	20	1
Non-neutralizer	2	19

Figure 8. Linear discriminant analysis modeling. This discrimination analysis method could distinguish broad neutralizers and non-neutralizers (Figure 8a) with more than 92% accuracy (Figure 8b). Analysis was performed using JMP Pro 12.

Linear discriminant analysis (LDA) showed that several cytokines and chemokines such as CXCL13, Granzyme A, Granzyme B, IFNα2, IFNɣ, IL-1β, IL-1RA, IL-2, IL-5, IL-12p70, IL-17α, MDC, and MIP-3α are positively associated with neutralization (Figure 8a). This linear discriminant analysis could distinguish broad neutralizers and non-neutralizers (Figure 8a). Discriminant analysis model was tested by removing one sample. In the test there were 3 misclassified samples: 1 predicted non-neutralizer that was a neutralizer and 2 predicted neutralizers that were actually non-

neutralizers. This model gave 92.8 % accuracy with more than 0.78 correlation (Figure 8b).

Partial least square discriminant analysis (PLSDA)

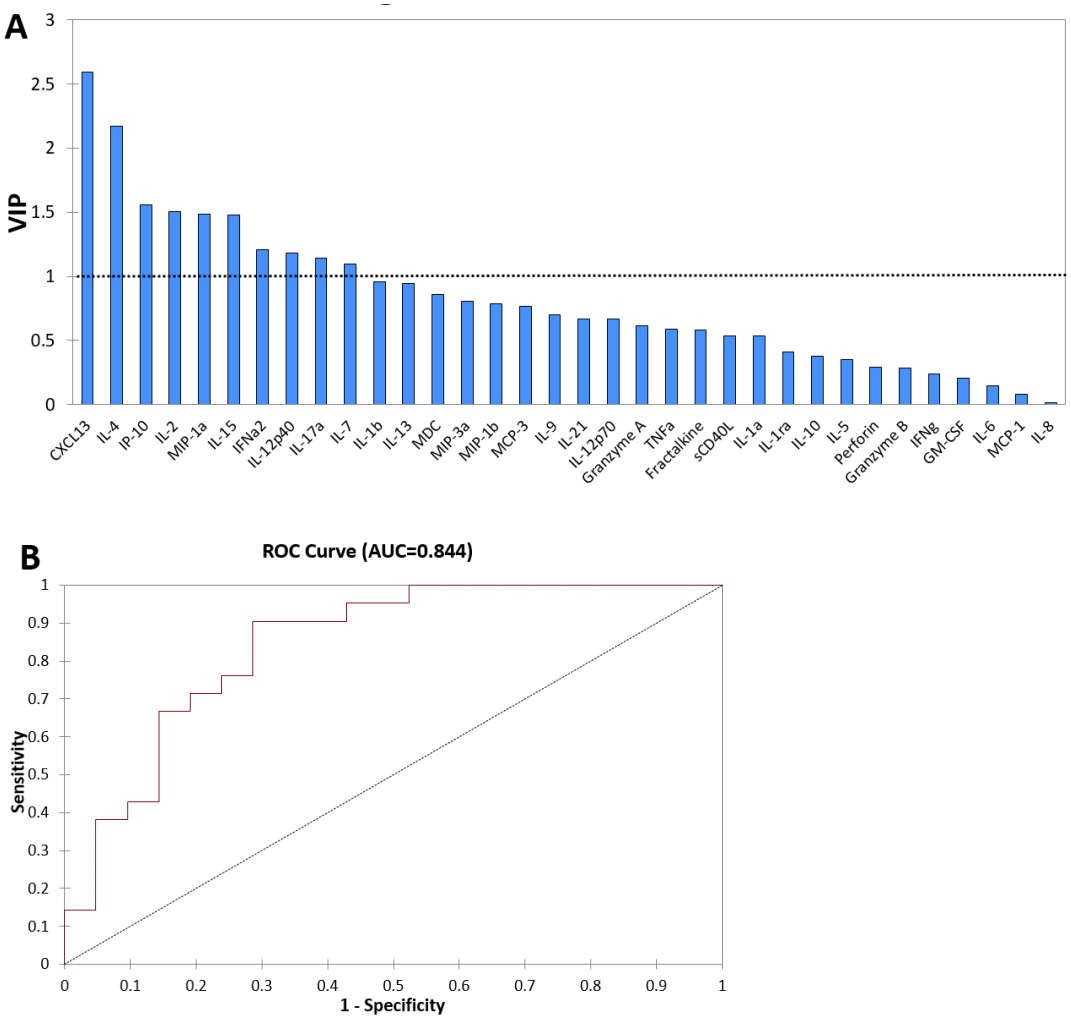


Figure 9. Partial least square discriminant analysis (PLSDA) modeling. In PLSDA model, CXCL13 appeared as the most important variable that distinguishes between broad neutralizers and non-neutralizers (Figure 9a). This PLSDA model had 84.4% accuracy (Figure 9b). Analysis was performed using XLSTAT.

Partial least square discriminant analysis (PLSDA) showed that CXCL13, IL-4, IP-10, IL-2, MIP-1α, IL-15, IFNα2, IL-12p40, IL-17α, and IL-7 had high variable in projection (VIP) score (Figure 9a). A VIP score close to or greater than 1 is considered

important in the PLSDA model. In our PLSDA model, CXCL13 appeared as the factors that had the highest variable in projection (VIP) score (Figure 9a). It showed that CXCL13 was the highest contribution factor to the given model. This PLSDA model had more than 84% accuracy (Figure 9b).

Immunofluorescence analysis

Due to both univariate and multivariate analyses showing CXCL13 to be positively correlated with HIV-1-specific $CD4^+$ T cells response that are associated with neutralization, we wanted to further characterized CXCL13 in lymph node of human body. Immunofluorescence-based microscopy assay was performed for this purpose.

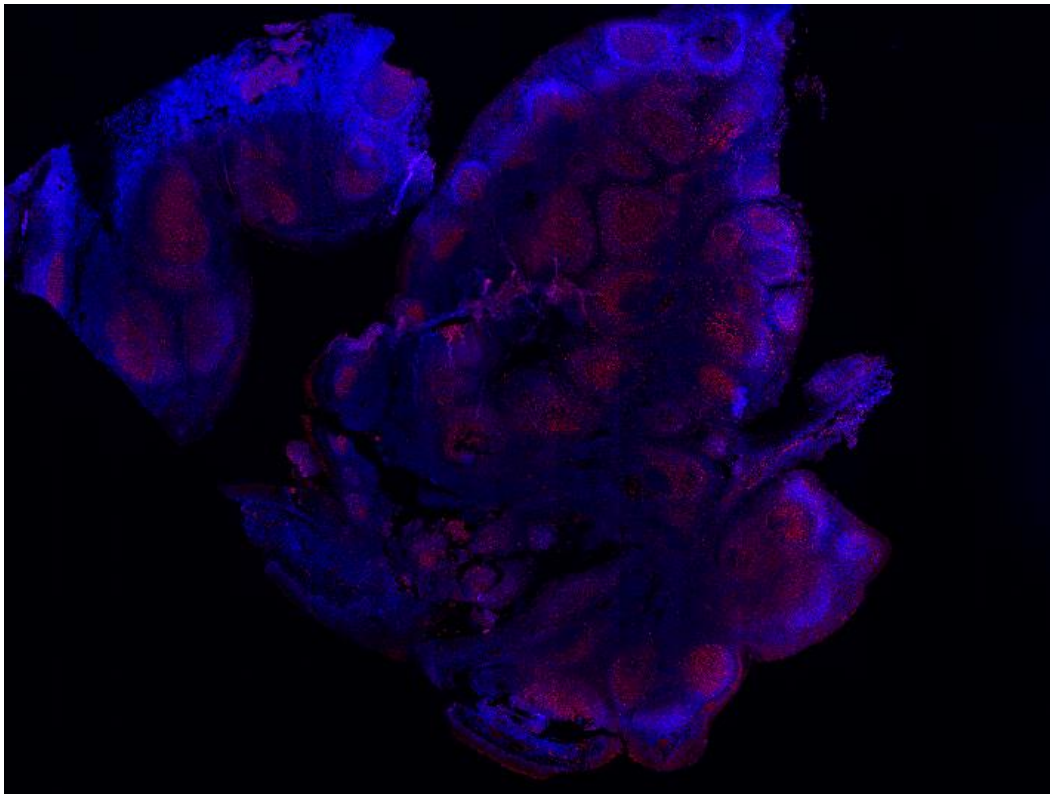


Figure 10. Expression of CXCL13 in the lymph node of an HIV negative individual. Immunofluorescence staining of the lymph node with anti-CXCL13, and 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI) of HIV negative individual. CXCL13 (red) and DAPI (blue) staining is shown

Immunofluorescence staining of CXCL13 was performed in lymph node section of HIV negative individual (Figure 10). There, we saw CXCL13 was exclusively found in the follicle of the lymph node.

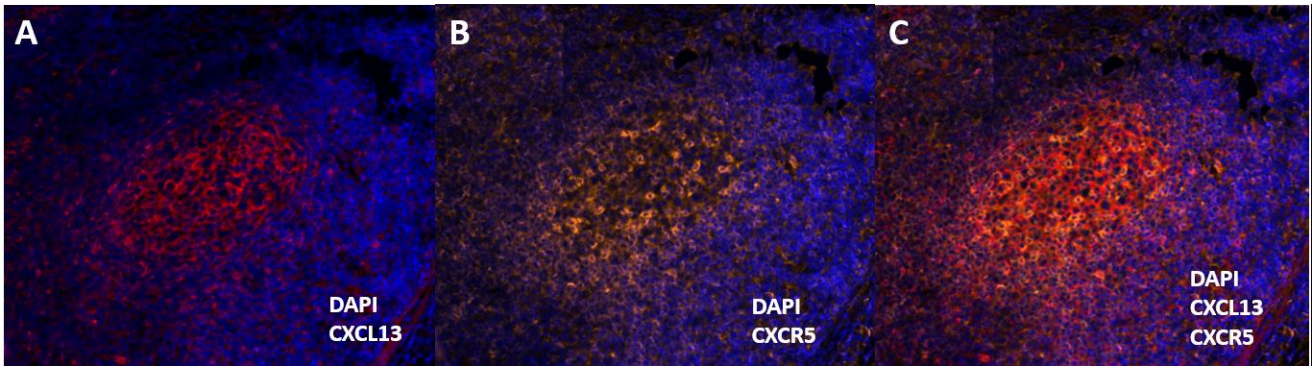


Figure 11. Expression of CXCL13 and CXCR5 in the germinal center formation in a lymph node of an HIV negative individual. Immunofluorescence staining of the lymph node with anti-CXCL13, anti-CXCR5, and 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI). CXCL13 (red) and DAPI (blue)(Figure 11a); CXCR5 (yellow) and DAPI (yellow) (Figure 11b); CXCL13 (red), CXCR5 (yellow) and DAPI (blue) staining is shown (Figure 11c).

Then, immunofluorescence staining for CXCL13 and its ligand, CXCR5, was performed using lymph node sections of an HIV negative individual (Figure 11). In the germinal center formation, some of the cells expressed both CXCL13 and CXCR5 (Figure 11c).

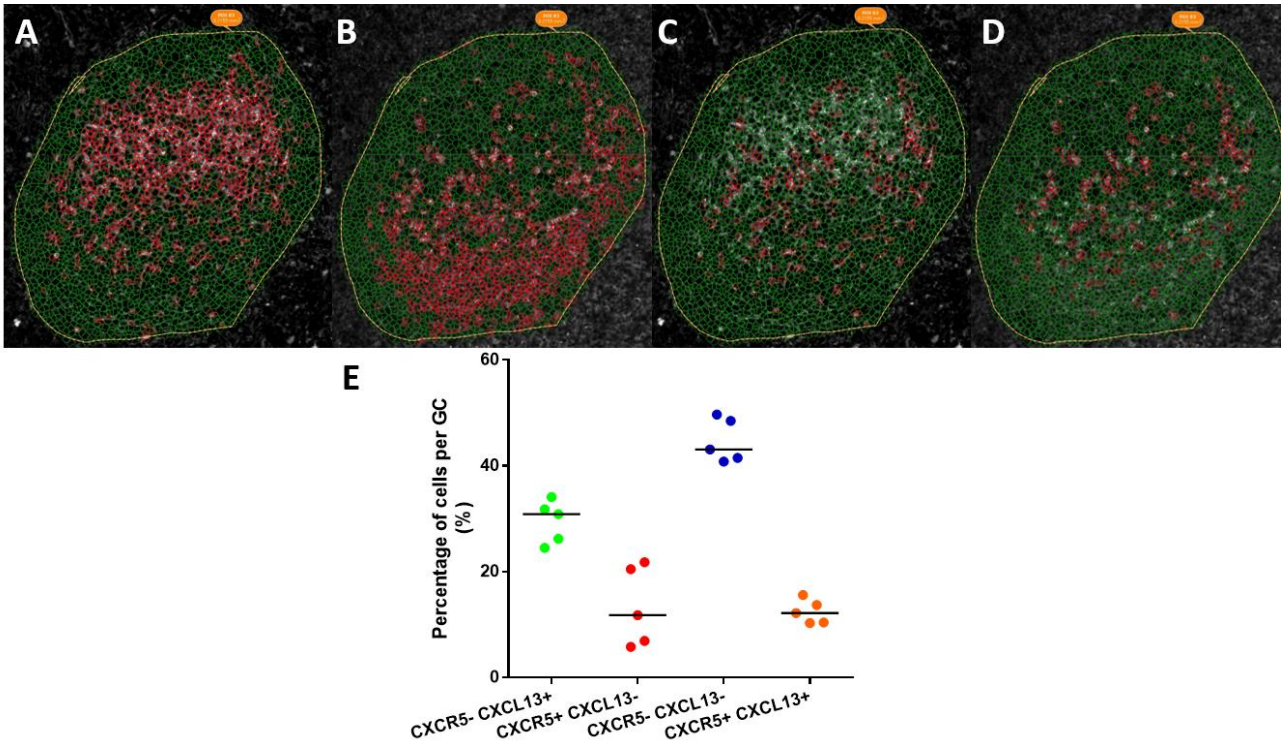


Figure 12. Quantification of the expression of CXCL13 and CXCR5 in the germinal center in a lymph node of an HIV negative individual. Representative germinal center in a lymph node of an HIV negative individual: $CXCL13^+$ cells (red) (Figure 12a), $CXCR5^+$ cells (red) (Figure 12b), $CXCR5^+$ $CXCL13^+$ cells (red) amongst $CXCL13^+$ cells (white) (Figure 12c), $CXCR5^+$ $CXCL13^+$ cells (red) amongst $CXCL5^+$ cells (white) (Figure 12d). Quantitative comparison of cells expressed $CXCR5^-$ $CXCL13^+$, $CXCR5^+$ $CXCL13^-$, $CXCR5^-$ $CXCL13^-$ and $CXCR5^+$ $CXCL13^+$. Median values shown.

Then, we quantified the number of cells expressed $CXCR5^-$ $CXCL13^+$, $CXCR5^+$ $CXCL13^-$, $CXCR5^-$ $CXCL13^-$ and $CXCR5^+$ $CXCL13^+$ (Figure 12). Results showed that not all cells that expressed CXCL13 have CXCR5 co-staining, and vice versa (Figure 12e). It may suggest that CXCL13 is not exclusively expressed by $CXCR5^{hi}$ $CD4^+$ T cells in the germinal centers, other follicular cell subset may contribute.

Chapter 3: Discussion and Perspective

3.1 Discussion

The underlying mechanism responsible for the development of broadly neutralizing antibodies (bNAbs) in natural infection is poorly understood. At the clinical level, it is unclear whether bNAbs generating individuals exhibit more robust HIV-1-specific $CD4^+$ T cells responses, thereby providing better help to B cells during infection. We hypothesized bNAbs response generating individuals to possess superior qualitative characteristics in their HIV-1-specific $CD4^+$ T cells responses compared to non-neutralizers. Furthermore, these superior characteristics would lend themselves to optimized help to B cells in generating bNAbs against HIV-1. Our findings suggest that HIV-1-specific $CD4^+$ T cells from broad neutralizers have a unique capacity to stimulate production of chemotactic cytokines CXCL13.

Higher viral loads and longer infection periods may result in an increased like hood for developing bNAbs [75-78]. It is important to account for these variables in the study design to eliminate potential confounds. In our study, there were no significant differences in viral loads and duration of infection between broad neutralizers and non-neutralizers (Figure 5a and 5b).

After 48 hours of stimulation with Gag-peptide pool, not all chemokines and cytokines being measured were up regulated (Table 5). It was predicted that some of the chemokines and cytokines would not be up regulated since they might act as anti-inflammatory cytokines that may negatively correlated with neutralization. However, inflammatory cytokine such as IL-2 was not up regulated. It is possible that IL-2 was

consumed by cells during the incubation period causing measurements in the supernatant to underestimate the cytokine levels produced [72].

Amongst the 34 measured cytokines and chemokines, only CXCL13 showed a statistically significant higher concentration in response to Gag stimulation in broad neutralizers compared to non-neutralizers (Figure 6). In broad neutralizers, we showed a significantly higher concentration of CXCL13 after Gag stimulation as the result of HIV-1-specific $CD4^+$ T cells responses (Figure 7a). Conversely, the response of HIV-1-specific $CD4^+$ T cells in non-neutralizers following Gag stimulation did not produce elevated level of CXCL13 concentration (Figure 7b). This findings suggested that HIV-1-specific $CD4^+$ T cells from broad neutralizers have a unique capacity to stimulate production of CXCL13.

Since chemokines and cytokines work together in network, we performed multivariate analysis to identify the relationship in our measured chemokines and cytokines. Linear discriminant analysis showed that several cytokines and chemokines such as CXCL13, Granzyme A, Granzyme B, IFNα2, IFNɣ, IL-1β, IL-1RA, IL-2, IL-5, IL-12p70, IL-17α, MDC, and MIP-3α are positively associated with neutralization (Figure 8a). Another multivariate analysis method, partial least square discriminant analysis (PLSDA), showed that CXCL13, IL-4, IP-10, IL-2, MIP-1α, IL-15, IFNα2, IL-12p40, IL-17α, and IL-7 were factors with high variable in projection (VIP) score with CXCL13 appeared as the factors that had the highest VIP score (Figure 9a). These cytokines and chemokines were not belong to a single cell subset. Some of them are belong to T helper 1 (Th1), Th2, Th17 or cells other than $CD4^+$ T cells. However, in these two different multivariate analysis method, CXCL13 was consistently appeared as a

factor that play a role in the network of cytokines and chemokines that are associated with neutralization.

Both univariate and multivariate analyses showing CXCL13 to be positively correlated with HIV-1-specific $CD4^+$ T cells response that are associated with neutralization. CXCL13 is a ligand for CXCR5. In mice, this chemokine has a significant role in organizing B cell follicle and germinal centers [79-82]. CXCL13 is expressed in follicular dendritic cells and T folicullar helper (Tfh) cells in the B-cell follicles [83-85]. This chemokine is crucial for secondary lymphoid structure development [81, 82]. CXCL13 facilitates migration of B cells and Tfh cells to B cell follicles and germinal centers [86, 87]. In humans, perturbations in CXCL13 secretion has been related with pathogenesis of various chronic inflammatory condition, including several infections and autoimmune disorders associated with lymphoid genesis dysregulation and humoral responses [88-91].

Immunofluorescence staining of the lymph node section showed that CXCL13 was exclusively found in the follicle (Figure 10). In the germinal center formation, some of the cells expressed both CXCL13 and CXCR5 (Figure 11c).However, our results showed that not all cells that expressed CXCL13 have CXCR5 co-staining, and vice versa (Figure 12e). It may suggest that CXCL13 is not exclusively expressed by $CXCR5^{hi}$ $CD4^+$ T cells in the germinal centers, other follicular cell subset may contribute.

3.2 Limitation and Future Direction

Using an *in vitro* stimulation assay where cytokines and chemokines are measured in the supernatant, we showed that HIV-1-specific $CD4^+$ T cells from broad neutralizers have a unique capacity to stimulate production of the cardinal cytokine CXCL13 that has been previously associated with germinal center formation and development of broadly neutralizing antibodies against HIV [92]. Our immunofluorescence-based microscopy assay showed that in the lymph node, CXCL13 is exclusively found in the follicle of the lymph node and not exclusively expressed by $CXCR5^{hi}$ $CD4^+$ T cells such as Tfh cells and B cells. Conversely, a study performed by Crotty *et al* found that CXCL13 expression is restricted to germinal center Tfh cells. This study also showed that CXCL13 protein was not produced in the other cell types present in tonsil, PBMCs, spleen and lymph node.

Further study need to be conducted to determine the source of CXCL13 and further clarify these findings. CXCL13 should be further characterized in matched lymph nodes and blood of HIV positive and HIV negative individuals using different approach that could provide more accurate result. Immunofluorescence staining alone or in combination with in situ hybridization may produce more precise findings. Particular attention should be given to follicular dendritic cell (fDC), follicular reticular cell (frc), T follicular helper cell (Tfh), T follicular regulatory cell (Tfr), B cell, and monocytes since they may relate to germinal center formation and development of broadly neutralizing antibody.

Future experiments, such as transcriptional analysis, should be performed to uncover other potential biomarkers implicated in broadly neutralizing antibodies

development. In addition, the predictive models employed in this study should be optimized further and validated in order to increase their accuracy and correlation.

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