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Enhancing T-Cell Responses to Vaccination of HIV-1 infected Subjects on Antiretroviral Therapy

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Abstract
With the advancement in anti-retroviral therapy (ART) regimens there has been a significant improvement in the quality of life and survival of those individuals infected with HIV-1. Even with the benefits to CD4+ cell counts, decrease in viremia and inflammatory biomarkers, HIV-1 infected individuals continue to exhibit functional issues in their T-cell immune responses to recall antigens and vaccines. Additionally, researchers believe that T-cell mediated responses will be important to elicit in a therapeutic vaccination setting. These T-cell functionality issues can leave individuals infected with HIV-1 at risk from opportunistic infections and co-morbidities. Furthermore, a therapeutic HIV-1 vaccine is needed that can elicit responses to help infected subjects better control HIV infection so as to potentially reduce the need for long-term therapy. However, basic research on HIV is still needed to solidify potential immune correlates against HIV and other pathogens affecting HIV-1 infected subjects. Likewise, investigation of therapeutic targets that can aid in enhancing T-cell immune responses in these individuals is of importance.

In this thesis, we examined whether a therapeutic HIV-1 DNA vaccine delivered with in vivo electroporation to HIV-1 infected subjects on ART could elicit potent cellular immune responses previously suggested to be important in the control of HIV. This vaccine strategy demonstrated an enhancement in cell-mediated IFN-γ production and cytotoxic immune responses to HIV-1. However, until a vaccine or therapy for HIV-1 is developed, these individuals also continue to be at risk for other opportunistic infections, such as influenza infection. Supported by previous studies that focus on influenza vaccination, we found that a standard dose of the H1N1 vaccine (15μg; Novartis) did not elicit sero-protection in all individuals. Importantly, the ability of these individuals to respond to vaccination was associated with the frequency of naÃ¯ve CD4+ T-cells prior to vaccination, thereby reinforcing the importance of CD4+ T-cell help and the need for better CD4+ T-cell reconstitution. In addition, HIV-1 infected subjects, despite ART, have an altered cytokine/chemokine environment. Thereby it is important to explore whether targeting the cytokine milieu can lead to improvements in responses to vaccination in these individuals. We specifically found that the pro-inflammatory chemokine IP-10 was elevated in the sera of those infected with HIV-1 while on ART. Additionally, elevated levels of IP-10 were associated with decreased cellular immune responses, which could be improved by neutralizing IP-10 prior to antigen stimulation. Therefore, the studies herein support the need for better understanding of the basic science of HIV-1 infection to uncover and comprehend what potential immune correlates are needed for therapeutic treatment of these individuals.

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Dedication

Dedicated to my parents:

Celia Ramirez Macias

And

Ramiro Ramirez

Amazing parents whose drive to see their children succeed, inspires me and keeps pushing me forward

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ABSTRACT

ENHANCING T-CELL RESPONSES TO VACCINATION OF HIV-1 INFECTED SUBJECTS ON ANTIRETROVIRAL THERAPY

Lorenzo Ramirez

Jean D. Boyer

With the advancement in anti-retroviral therapy (ART) regimens there has been a significant improvement in the quality of life and survival of those individuals infected with HIV-1. Even with the benefits to CD4+ cell counts, decrease in viremia and inflammatory biomarkers, HIV-1 infected individuals continue to exhibit functional issues in their T-cell immune responses to recall antigens and vaccines. Additionally, researchers believe that T-cell mediated responses will be important to elicit in a therapeutic vaccination setting. These T-cell functionality issues can leave individuals infected with HIV-1 at risk from opportunistic infections and co-morbidities. Furthermore, a therapeutic HIV-1 vaccine is needed that can elicit responses to help infected subjects better control HIV infection so as to potentially reduce the need for long-term therapy. However, basic research on HIV is still needed to solidify potential immune correlates against HIV and other pathogens affecting HIV-1 infected subjects. Likewise, investigation of therapeutic targets that can aid in enhancing T-cell immune responses in these individuals is of importance.

In this thesis, we examined whether a therapeutic HIV-1 DNA vaccine delivered with *in vivo* electroporation to HIV-1 infected subjects on ART could elicit potent cellular immune responses previously suggested to be important in the control of HIV. This vaccine strategy demonstrated an enhancement in cell-mediated IFN-γ production and cytotoxic immune responses to HIV-1. However, until a vaccine or therapy for HIV-1 is

developed, these individuals also continue to be at risk for other opportunistic infections, such as influenza infection. Supported by previous studies that focus on influenza vaccination, we found that a standard dose of the H1N1 vaccine (15μg; Novartis) did not elicit sero-protection in all individuals. Importantly, the ability of these individuals to respond to vaccination was associated with the frequency of naïve CD4+ T-cells prior to vaccination, thereby reinforcing the importance of CD4+ T-cell help and the need for better CD4+ T-cell reconstitution. In addition, HIV-1 infected subjects, despite ART, have an altered cytokine/chemokine environment. Thereby it is important to explore whether targeting the cytokine milieu can lead to improvements in responses to vaccination in these individuals. We specifically found that the pro-inflammatory chemokine IP-10 was elevated in the sera of those infected with HIV-1 while on ART. Additionally, elevated levels of IP-10 were associated with decreased cellular immune responses, which could be improved by neutralizing IP-10 prior to antigen stimulation. Therefore, the studies herein support the need for better understanding of the basic science of HIV-1 infection to uncover and comprehend what potential immune correlates are needed for therapeutic treatment of these individuals.

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INTRODUCTION

1.1 HIV-1 Infection

Identified in the early 1980's, despite the years of extensive research, HIV-1 infection continues to contribute to significant morbidity and mortality around the world[1,2]. During the acute phase of infection there is increased viral replication, immune activation, depletion of CD4+ cells, dissemination of the virus into lymphoid tissues, and elevation of inflammatory biomarkers[3, 4, 5]. The acute phase is followed by a phase of chronic infection with continuous immune activation and viral replication, which further contributes to the loss of CD4+ T-cells and the eventual progression to AIDS, where opportunistic infections place these individuals at risk for complications and death[6, 7, 8].

Several studies examine the role of markers of inflammation and immune activation suggesting a predictive role for disease progression. Such studies show several prognostic markers during untreated HIV-1 infection that are predictive of rapid disease progression. These markers include immune activation[9, 10], inflammatory cytokines/chemokines, in particular IP-10,[4, 11, 12] viral loads, and CD4+ cell counts[13]. Furthermore, chronic HIV-1 infection leads to altered T-cell characteristics, such as an accumulation of terminally differentiated T-cells and dysfunction[10, 14].

1.2 HIV-1 Infection and Anti-Retroviral Therapy

Since the approval of zidovudine (AZT) in 1987[15], significant enhancements in anti-retroviral therapy (ART) have led to an improvement in the quality of life and survival of those living with HIV-1 infection[16, 17]. Even more, improvements to drug regimens have led to better adherence and reduction in side-effects that were seen with older regimens; these enhancements in turn have helped in reducing transmission[18, 19, 20].

In addition to better survival of these individuals, use of ART also helps lead to the rebound of CD4+ cell counts, which are shown to increase for up to seven years in

these individuals[21, 22, 23]. However, these increases in CD4+ cell counts are shown to be influenced by pre-ART CD4 counts[22], suggesting that the timing of ART initiation can influence immune reconstitution. Nonetheless, ART treatment is also shown to lead to decreases in viremia to levels below detection within months of starting therapy[24, 25]. Finally, ART also leads to a decline in immune activation and inflammatory markers[26, 27].

Despite these advancements, it is demonstrated that although there is a decrease in viremia, a viral reservoir is still present[24, 28, 29]. This fact may be due to the inability of ART drugs to reach all tissues, classified as sanctuaries that could harbor viral reservoirs[29, 30]. Additionally, Hunt *et al.*[31] demonstrate that despite ART, these individuals continue to have elevated activated CD4+ and CD8+ T-cells compared to healthy HIV-negative individuals. Also, even with ART, these individuals are shown to have an imbalanced cytokine and chemokine environment[26, 27, 32, 33, 34].

Observations of residual immune activation and inflammation have led to interest in early initiation of ART. A study of long-term ART initiated during early HIV-1 infection suggested that there is potential for better immune system preservation and a functional cure[35]. A functional cure is defined as the ability to control infection without the need for medication. For example, while not successful in the long-term, very early ART initiation in an infant showed the potential for a functional cure[36]. Therefore, the timing of ART initiation could aid in preserving the immune system, and may also help avoid damage that may persist in individuals who initiate ART later.

1.3 HIV-1 Infection and Cellular Immune Responses

Adaptive cell mediated immunity is directed by T-lymphocytes[37]. Upon infection or vaccination naïve CD4+ and CD8+ T-cells become activated and undergo differentiation after interacting with a specific antigen that is displayed in the context of

self-major histocompatibility (MHC) complex molecules[38]. CD4+ T-cells differentiate into T-helper subsets. Th1 and Th2 subsets dominate most immune reactions, whose functions depend on the cytokines they secrete[37]. Specifically, development of these helper subsets is dependent on the type of stimuli present at the initiation of immune responses. The presence of the cytokines IL-12 and IFN-γ and transcription factors STAT-1, STAT-4, and T-bet is shown to induce Th1 subsets important for responses to pathogens that infect cells or activate macrophages[37]. On the other hand the cytokine IL-4 and transcription factors GATA-3 and STAT-6 are the major inducers of Th2 subsets, important in responses against helminthes and allergens[37]. CD4+ Th1 cells are also involved in activating cells such as macrophages, which aid in the production of additional immunological mediators and improves T-cell activation[37]. As well, CD4+ helper T-cells, particularly follicular helper T-cells are involved in promoting humoral immunity[37]. Specifically, upon activation, T-cells migrate to B-cell follicles where they help promote B-cell responses, such as clonal expansion, antibody production and isotype switching, through cytokines or CD154 dependent mechanisms[39]. Finally, there also exist subsets of CD4+ T-cells involved in regulation of immune responses, these cells are know as regulatory T-cells[37].

While naïve CD8+ T-cells with antigen stimulation undergo differentiation into cytotoxic T-lymphocytes (CTLs)[37]. CD8+ T-cells respond by producing cytokines and chemokines, such as IL-2, IFN-γ, TNF-α, and MIP-1α/β, as well as the release of cytotoxins, such as perforin and granzyme, from cytolytic granules[40]. CD8+ cytotoxic T-lymphocytes can directly kill cells infected with intracellular pathogens[37]. CD8+ CTLs are known to mediate target cell killing, as discussed above through degranulation and the production and secretion of cytotoxins such as perforin and granzyme B, which help permeabilze cells and activate apoptotic pathways. In addition to cytotoxin-mediated

killing, CD8+ CTLs are also able to use contact-dependent mechanisms through their expression of Fas ligand, which binds the Fas receptor found on the surface of many cell types[37]. During acute HIV-1 infection a strong CTL response is shown to contribute to the decline in viremia observed[41].

Individuals who are able to control HIV-1 infection in the absence of therapy, exhibit strong cellular immune responses that are shown to be important in control. Considering this, eliciting these responses in individuals receiving ART, may be necessary in targeting HIV-1. Similarly, exploring cellular mechanisms of viral control can help researchers understand why those with progressive disease fail to control HIV-1 infection[42]. The suggestion that CD8+ T-cells play an important role in controlling HIV-1 infection come from studies involving CD8+ T-cell depletion and SIV infected rhesus macaques, which demonstrate that the presence of a potent CD8+ response is associated with lower viral replication and slower disease progression[43]. In addition, the occurrence of viral escape mutations suggests that there is pressure from CD8+ T-cells[44, 45]. Additionally, it is proposed that early ART leads to increases in CD4+ helper subsets important in maintaining CTLs[42, 43, 45]. So examining how to enhance the CD4+ helper response is of interest as well. In particular it has been suggested that CD4+ T-cells, specifically of central memory phenotype, that produce IL-2 are able to help maintain proliferative T-cell responses and effector CD8+ T-cell responses[46]. As well, CD4 help may be involved in regulating T-cell differentiation and memory formation, as studies during HIV-1 infection suggest changes in T-bet expression in the absence of CD4 help[47]. These findings and others have suggested better HIV control in the presence of higher numbers of HIV-specific CD27- CD8+ T-cells[48]. So if differentiation is affected, through abnormal expression of T-bet, this could impact T-cell mediated responses against HIV.

In regards to cellular correlates of protection, Betts *et al.*[40, 49] suggest that poly-functional T-cells, which include proliferative capacity, secretion of IL-2, IFN-γ, TNF-α, and MIP-1α/β, ability to degranulate and produce cytotoxins, may be important in the control of HIV-1 infection[50]. However, while IFN-γ is a measure of an active anti-viral immune response, it does not directly inhibit HIV-1 replication or kill cells[50, 51]. Therefore, surrogate markers of killing, such as degranulation and production of perforin and granzyme B are theorized to be crucial. Specifically, non-progressors compared to progressors are shown to have better proliferative capacity of T-cells, which is associated with perforin expression[45]. Furthermore, use of more direct measures of killing, demonstrate that non-progressors are also better at eliminating autologous HIV-infected CD4+ T-cells *in vitro* compared to progressors[52]. Though in regards to HIV-1 infected subjects receiving ART, Migueles *et al.*[53] indicate that despite ART, these individuals, compared to long-term non-progressors, have a lower proliferative and cytotoxic capacity[53, 54]. While this may be due in part to a decay in CD8+ CTLs with long-term ART[50], these findings suggest even more that enhancing these responses in HIV-1 infected individuals on ART will be necessary to target HIV-1.

1.4 Prophylactic vs. Therapeutic HIV-1 Vaccines

Many viral vaccines are based on either live-attenuated of whole inactivated viruses[55]. However, for HIV those vaccine strategies have been deemed unsafe due to the risk of integration of HIV proviral DNA into the host genome[55]. Therefore additional vaccine modalities such as recombinant and DNA-based vaccines have been of interest. Regardless, there has yet to be an effective prophylactic or therapeutic vaccine for HIV. The goal of a prophylactic vaccine against HIV would hope for the prevention of HIV infection, if possible, or the reduction of viral set points and/or better control once

infected[55, 56]. As well, a prophylactic vaccine will need to be effective at all portal of HIV entry and must have broad and durable immunity. On the other hand a therapeutic vaccine for HIV would aim to treat those individuals who are already infected. Potential goals with a therapeutic vaccine are to help enhance the immune responses against HIV, reduce secondary transmission, as well as possibly helping in controlling infection, aiding in targeting the viral reservoir, and/or eliminate infection (sterilizing cure)[57]. In addition, in ART-treated individuals, it would be a goal for a therapeutic vaccine that could potentially help reduce or eliminate the need for prolonged anti-retroviral therapy (functional cure).

The preventative HIV vaccine field has undergone different iterations of vaccines, focusing on the induction of neutralizing antibodies, CTL responses, or a combination of different immune responses[55]. With years of different HIV vaccine designs, three major trials have elucidated the need to further understand the basic immunology of HIV-1 infection and possible immune correlates of protection. The STEP and Phambili trials aimed at investigating the efficacy of cell-mediated immunity as a prophylactic vaccine[58]. However, both studies were terminated early due to analysis suggesting that vaccination was associated with an increase in HIV acquisition[55]. The researchers concluded that the cell-mediated immunity induced by this vaccine did not prevent infection or reduce viral set-points. This was suggested to be due to pre-existing immunity to the adenoviral 5 vector used as well as circumcision status[55]. A third study, known as the RV-144 trial was suggested to show moderate efficacy in individuals who had received the vaccine compared to placebo. After analysis of the study it was suggested that antibodies against the V1V2 loops[55] may be playing a role. Nonetheless, the vaccine did not aid in better control of HIV replication or loss of CD4+ T-cell subsets[56]. The findings in these trials suggest that better understanding of the basic science of HIV infection as well as

additional investigation of possible correlates of protection are necessary. While studies of non-progressors have revealed some potential correlates for HIV-1 control that does not mean that those responses would be effective at preventing HIV-1 acquisition.

In further regards to therapeutic vaccines against chronic infections, like HIV, it is important to understand what immune correlates are needed during continuous viral replication. This could mean that the antigens targeted prophylactically could differ from therapeutic targets[57]. Likewise, during non-chronic infections there is an important role of immune memory, which can help dramatically during a second round of infection[**57**]. However, it is known that HIV-1 infection can impact normal immune cell differentiation[3, 57]. This effect on differentiation could affect the ability of memory cell formation, even after the antigen is significantly reduced, as is with ART. Therefore, a therapeutic vaccine may need to stimulate effector cells and possibly enhance not only their function, but expansion and survival. Even more, understanding the basic science of HIV infection, in particular in subjects on ART, could help elucidate potential adjuvant/therapeutic strategies to enhance immune responses. Lastly, since CD4+ T-cells play a role in helping both arms of the immune system, including maintaining CD8+ CTLs, exploring how to improve immune reconstitution with ART regimens could help if then combined with a therapeutic vaccine that would enhance the anti-HIV response.

1.5 HIV-1 Infection and Non-HIV Infections

Prior to effective antiretroviral therapy HIV-infected individuals were at heightened risk for opportunistic infections due to their immune-compromised status[59]. Specifically, CD4 counts are shown to contribute to the severity and risk for different opportunistic infections, however, ART aids in reconstituting these cells[22, 60]. While issues associated with unknown HIV-status, and lack of drug accessibility continue to

contribute to morbidity and mortality from opportunistic infections in those infected with HIV, some individuals despite ART and CD4+ cell reconstitution are not able to attain necessary responses to non-HIV vaccination to protect them completely.

The Centers for Disease Control and Prevention (CDC)[61] establish recommended treatment and vaccination guidelines for individuals living with HIV[59]. The CDC details that opportunistic infections can still occur in individuals on ART, but maintain that ART should not be interrupted to administer treatment for the opportunistic infection. In regards to preventative vaccines, the CDC for 2014 recommends that HIV-infected individuals with CD4 counts above 200 cells/μl consider receiving vaccines against influenza, whooping cough and tetanus (Tdap), pneumococcal disease, hepatitis B virus, human papilloma virus, measles, mumps and rubella (MMR if born after 1957, but not received the vaccine), chickenpox (if born after 1980 and not received the vaccine)[61, 62]. However, not all vaccines are safe to administer to individuals depending on the individual's immune-compromised severity.

Current studies suggest that standard influenza vaccines are only moderately effective in those infected with HIV and that even with ART, individuals may still be at risk of morbidities due to influenza infection[63, 64, 65]. In order to achieve sero-protection individuals infected with HIV often require higher or multiple doses of the influenza vaccine[66, 67]. Similarly, while the conjugate pneumococcal vaccine is shown to protect HIV-infected infants, pneumococcal infections remain elevated in those infected with HIV despite effective ART[68, 69]. It is thereby suggested that herd immunity may be most beneficial for protecting these individuals. Of particular concern to HIV-1 infected subjects is HCV co-infection due to shared routes of acquisition. Studies reveal mixed findings regarding the potential benefit of ART on HCV infection. Specifically, Klein *et al.*[70] and Kottilil *et al.*[71] show that ART contributes no significant benefit to HCV control.

However, effective treatment of HCV in those co-infected with HIV may differ. For example in those infected with HIV, treatment with peg-interferon and ribavirin was more effective than interferon and ribavirin[72]. These findings suggest that despite ART, benefit to other co-infections may not be present and so these individuals may still be at increased risk and may need to receive alternative treatment combinations.

Finally, in a study by Lange *et al.*[73], they demonstrated that even with the re-establishment of normal CD4 cell counts, the pre-ART environment is more predictive of whether HIV-infected individuals would respond to vaccination against tetanus toxoid, diphtheria-toxoid, and keyhole limpet hemocyanin. These findings suggest that despite immune reconstitution individuals may still be at risk of disease if they are unable to respond adequately to vaccines. Furthermore in HIV-infected children, even with ART and increases in CD4 counts and B-cells, antibodies generated to MMR and varicella zoster vaccines decline over time, potentially leaving these children at risk despite vaccination and can potentially require additional immunizations to maintain protection[74]. Similar findings were found in children who received Tdap[75]. Even more, while the varicella zoster vaccine is safe in HIV-1 infected children, even after two immunizations only 60% of children who received the vaccine developed antibodies as compared to the HIV-uninfected counterparts[76].

1.6 HIV-1 Infection and Co-morbidities

Antiretroviral therapy has increased the survival and quality of life of those living with HIV-1 infection, but has turned HIV-1 infection into a chronic disease[17]. It is estimated that by 2015, 50% of HIV-infected individuals will be over 50 years of age[77]. In addition, as these individuals live longer, their risk of other co-morbidities, which include cardiovascular, renal, pulmonary, neurologic, gastrointestinal, and bone diseases, increase[78]. With the development of better ART regimens the contribution from toxicities

due to ART is being minimized[79]. Therefore the contribution to these co-morbidities from ART, HIV-1 infection, and aging needs to be explored. In fact, the elderly, who often receive medications other than ART, are at risk for side-effects due to drug-drug interactions[77]. Furthermore, compared to younger HIV-infected individuals, older individuals may have less immune reconstitution[80] so a balance to deal with HIV-1 infection and other co-morbidities needs to be investigated especially since these co-morbities could possibly affect cellular immune responses.

i. T-cell Function

With HIV-1 infection thymic involution is seen to occur. This thymic involution is also shown to occur with age, which in turn affects these individuals' generation of naïve T-cells[81]. Without the generation of new cells, the accumulation of terminally differentiated cells increases, leaving those infected with HIV and the elderly at risk for other infections/co-morbidities, such as influenza virus infection[82, 83]. Even more, Rickabaugh *et al.*[84] demonstrate that despite ART, the changes in naïve CD4+ T-cell subsets in those infected with HIV appear to be accelerated compared to age-matched healthy controls. Similarly, the damage that occurred during untreated HIV-1 infection can contribute to replicative senescence or exhaustion of T-cells due to their chronic stimulation[14, 85]. Therefore, considerations to the initiation of ART are important to evaluate, but as is discussed below, ART itself may contribute to issues seen in HIV-1 infected individuals as they age.

ii. Neurocognitive Disease

Even with ART, HIV-1 infected individuals' brain volumetric measures are decreased compared to healthy individuals[86]. Of particular concern in regards to individuals on ART, is whether ART drugs are able to enter tissues such as the brain

where possible viral sanctuaries may exist[14, 87]. The presence of neural viral sanctuaries could contribute to ongoing damage to the central nervous system. Aside from this, the changes seen in metabolic function in those treated with ART can contribute to complications in neurocognitive function[85]. In contrast, older HIV-infected individuals with already present neurocognitive impairment may have issues adhering to their ART medication, which can have trickledown effects to their immune function, responsiveness to vaccination, and response against opportunistic infections[88].

iii. Cardiovascular Disease

Cardiovascular disease is a leading cause of death in the United States[89]. Several studies have found an increased risk for cardiovascular disease in those infected with HIV when compared to age-matched HIV-uninfected individuals[89]. It is proposed, since age impacts immune reconstitution, inability to reconstitute CD4 cell counts may contribute to cardiovascular issues[90]. Yet while ART may be beneficial in improving CD4 counts, certain ART drugs, such as protease inhibitors, contribute to alterations in lipids, which can exacerbate issues in fat and insulin metabolism and in turn promote cardiovascular disease[91]. However, the benefits associated with ART are suggested to outweigh these issues and therefore alternative therapeutic targets are being investigated.

iv. Cancer

Development of ART has led to a decrease in HIV-associated cancers like Kaposi's sarcoma[92]. On the other hand, cancers not associated with HIV/AIDS, including cancers of the lungs, liver, skin, anus, and others, are on the rise, particularly in men[93]. Reasons behind this increased risk are theorized to be due to the HIV virus itself affecting normal cell-cycle regulation, stimulation of potential oncogenes, and

enhancement of angiogenesis[93]. By the same token, those infected with HIV are at risk for co-infection with cancer-causing viruses such as HPV, HCV, and EBV[93, 94, 95]. When treating these individuals in the ART era, understanding the risks of chemotherapy while on ART is of importance since cancer treatment could contribute to further complications and side-effects[93].

v. Other Co-morbidities

As individuals age, there is an increase in frailty, reduced mobility, and activity. These changes are suggested to be accelerated in those infected with HIV and is seen to occur despite treatment with ART[96]. Additionally, toxicities from ART or non-HIV drugs are shown to stimulate inflammatory pathways associated with frailty[97].

1.7 HIV-1 Infection and the Cytokine/Chemokine Environment

With effective antiretroviral therapy, researchers demonstrate that viremia can fall below the limits of detection, and immune activation and inflammation is reduced. Regardless of these benefits, even with long-term ART, the levels of immune activation remain elevated compared to healthy uninfected individuals[31, 98, 99]. This chronic immune activation can then contribute to further dysregulation of cytokine and chemokine production and augment the risk of potential co-morbidities[3, 100]. Furthermore, a study by Almeida *et al.*[101] demonstrated that despite receiving ART for a year, PMBCs from HIV-infected individuals continue to exhibit abnormal cytokine production. These findings are supported by other studies showing that despite ART, abnormal cytokine, such as IL-6, and chemokine levels, such as CCL2 and CXCL10, fail to normalize and may contribute to disease progression[33, 102, 103].

Since cytokines and chemokines are important in the control of immune activation and inflammation, cell function, and mobility, investigating their altered

regulation may further reveal relevant therapeutic targets. Such investigation of targets like the CCR5 receptor and its ligands has led to the development of anti-HIV therapeutic agents[104]. Conversely, certain cytokines thought to be important in the maintenance of T-cells and which are normally anti-viral in function are shown to actually further contribute to viral replication. For example, IL-7, a cytokine important in thymocyte proliferation and survival, is shown to lead to an increase in HIV viral replication, and in those individuals on ART, IL-7 is demonstrated to reactivate latent proviral DNA[105, 106].

Understanding what contributes to the observed chronic activation is of interest in order to better target these issues and so as to improve immune function in these HIV-infected individuals. Studies suggest several potential sources for the chronic activation including residual low-level replication, microbial translocation, co-infection with other pathogens such as HCV, depletion of immuno-regulatory cell subsets, as well as defects that are a byproduct of HIV-infection prior to ART initiation[99, 107, 108]. Further, understanding the effect that the dysregulated cytokine and chemokine environment plays in affecting immune function and reconstitution can have implications for correcting these issues. For instance, HIV-1 infected subjects with high levels of the pro-inflammatory chemokine IP-10 are more likely to have immunological treatment failure following HAART[109]. As mentioned above investigation of CCR5 and its chemokine ligands has led to development of therapeutics that have aided in understanding how to better inhibit HIV replication[110]. Now with ART, HIV-1 replication is decreased significantly, however investigating other possible associations HIV-1 infection post-ART has with the chemokine system can reveal additional targets to improve immune function in these individuals.

i. IP-10/CXCL10

Interferon-γ-inducible protein-10kDa (IP-10), also referred to as CXCL10 or small-inducible cytokine B10, is a 10kDa chemokine part of the CXC family of chemokines[9]. IP-10 is produced by a wide-range of cell types that include monocytes, innate immune cells such as neutrophils and eosinophils, lymphocytes, epithelia cells, endothelial cells, stromal cells, hepatocytes, astrocytes, and keratinocytes[111, 112]. The IP-10 protein can be induced by a host of factors, which in addition to IFN-γ, can be induced by Type-I interferons IFN-α/β, weakly by TNF-α (unless it synergizes with IFNs), IL-12, stimulation of toll-like receptors, RIG-I like receptors, and RNA helicases[112, 113, 114, 115, 116]. Expression of the IP-10 protein requires binding of a STAT1-STAT2 heterodimer to the IP-10 promoter[117]. The promoter itself for IP-10/CXCL10 contains a functional IRSE and NF-κB1 element [118].

Along with MIG/CXCL9 and I-TAC/CXCL11, IP-10 binds the CXCR3 receptor, a seven trans-membrane G-protein coupled receptor, with intermediate affinity compared to MIG and I-TAC[9, 119]. The CXCR3 receptor can be expressed on NK cells, NKT cells, plasmacytoid dendritic cells, certain B-cell subsets, macrophages, and activated T-lymphocytes[9, 120]. Expression of CXCR3 on immune cells is then shown to allow entry of these cells into sites of inflammation and restricted sites, such as the brain[121, 122]. In humans, the CXCR3 receptor is coupled to a $G\alpha_i$ protein and has 3 isoforms, CXCR3-A, CXCR3-B, and CXCR3-Alt. The CXCR-B isoform can also bind CXCL4 and acts inhibitory in nature compared to the CXCR3-A isoform[123].

For binding of IP-10, MIG, and I-TAC to the CXCR3 receptor, the sulfated N-terminus of the receptor is necessary. For IP-10 the proximal16 amino acid residues of the N-terminus of the receptor are also required[120]. In regards to internalization of the CXCR3 receptor, this also requires the carboxyl terminal and beta-arrestin-1 domains[124].

Along with IP-10's binding to these regions for receptor internalization, binding also initiates signaling pathways involved in chemotaxis and calcium mobilization[124, 125]. Moreover, internalization of the receptor and calcium mobilization initiate signaling cascades involving Akt and kinases such as p38[126]. Additionally, IP-10 is shown to play a role in regulating apoptosis, angiostasis, cell growth, and proliferation[9]. Furthermore, the IP-10/CXCR3 signaling pathway is shown to exert signals that may disrupt the immunological synapse, thereby potentially affecting normal TCR signaling[127].

The IP-10 protein can exist both in its "long" agonist form and as a shorter competitive antagonist form[128]. In addition, the antagonist form can still bind the CXCR3 receptor, but blocks signaling[129]. The antagonist form results from a two amino acid N-terminal truncation due to processing by the amino-peptidase CD26 (dipeptidyl peptidase IV, DPPIV)[130]. CD26 is a 110kDa protein expressed on the surface of mature thymocytes, activated T and B-cells, NK cells, macrophages, endothelial, and various tissue epithelial cells[131, 132]. CD26's expression on cells increases 5-10 fold following activation of cells and can also exist in an active soluble form. CD26 exerts its function through its extracellular domain, which can cleave dipeptides from the N-terminus of proteins, such as IL-8 and IP-10, and in turn leads to different isoform production or protein degradation[130, 131].

ii. IP-10 and HIV-1 Infection

IP-10 is found to be elevated not only during untreated HIV-1 infection, but despite ART, remains elevated compared to healthy HIV-uninfected controls[133, 134, 135]. CXCL10 is also shown to stimulate HIV viral replication and blocking IP-10's interaction with CXCR3 reduces this replication[136]. Furthermore, with this elevation, IP-10 is shown to be predictive of not only viral loads in HIV-1 infected individuals, but also predictive of rapid disease progression[12, 134]. On a similar note, HIV controllers with elevated IP-10

levels are shown to have higher immune activation and lower CD4+ cell counts[137]. Conversely, Lajoie *et al.*[138] demonstrated that HIV-exposed sero-negative sex-workers have significantly lower levels of IP-10 in their mucosa compared to their HIV-negative and HIV-positive counterparts. These findings suggest a protective role of low IP-10 levels. In addition to canonical induction of IP-10, HIV-1 infection can induce high levels of IP-10 through stimulation of TLR7/9 dependent pathways[114]. IP-10 has also been found to be elevated in the cerebrospinal fluid of HIV-1 infected subjects exhibiting viral replication in the CNS[139]. Furthermore, HIV-1 proteins are shown to take advantage of the pleiotropic effects of IP-10 and exacerbate disease. Specifically, HIV's Tat helps induce IP-10 by antigen-presenting cells thereby recruiting more targets[140, 141]. While gp120 and Nef are shown to induce IP-10 in astrocytes contributing to toxicity and in turn neuronal cell death[142, 143]. Besides its effects during untreated HIV-1 infection, IP-10 is also associated with immunological treatment failure during HAART[109].

iii. IP-10 and HCV Infection

The CDC reports that 25% of individuals living with HIV are co-infected with hepatitis c virus and in those HIV-infected individuals who are injection drug users, 80% are co-infected with HCV[144]. Furthermore, co-infection with HIV is shown to accelerate HCV disease progression[145]. Thereby understanding HCV infection can help inform HIV-1 infection. As mentioned above, IP-10 is elevated in HIV-1 infection and when examining subjects mono-infected with HCV, the levels of IP-10 are not only elevated, but also correlate with markers of liver damage and inflammation. Moreover, the levels of IP-10 during co-infection when compared to mono-infected individuals are shown to be even more elevated[145]. These findings suggest that exploring IP-10 in these patient populations can have potential benefits for both HIV and HCV infection.

Increased levels of IP-10 in HCV infection are also associated with increased HCV viral loads, fibrosis of the liver, and are predictive of responsiveness to therapy[146]. The effect of IP-10 on responsiveness to HCV therapy has exhibited similar results in subjects co-infected with HIV[147]. Finding it contradictory that a chemokine involved in the recruitment of immune cells to sites of infection serves as a negative prognostic marker led to a more in depth investigation of IP-10 during HCV infection. Casrouge *et al.*[130] demonstrated that IP-10's antagonist form through potential processing by CD26 is elevated in HCV infected individuals, suggesting that IP-10 antagonism may be present when IP-10 is elevated in these individuals. Another study by Soderholm *et al.*[148] indicate that individuals with lower concentrations of soluble CD26 (sCD26) have better treatment outcomes compared to individuals with elevated sCD26. Additionally, lower sCD26 levels are associated with higher HCV-specific CD8+ T-cells in the blood[148]. These findings would suggest that lower sCD26 could be associated with less IP-10 antagonism and better T-cell functionality.

iv. IP-10 and Other Diseases

Similarly in subjects with hepatitis B virus infection, Hou *et al.*[149] have shown that IP-10 correlates not only with HBV viral loads, but also with increased levels of the exhaustion marker PD-1 on T-cells, suggesting a potential impact of elevated IP-10 levels on functionality of T-cells. Additionally, in individuals with HIV-1 infection, elevated levels of IP-10 can potentially affect the ability of these subjects to clear H1N1 virus infections[150].

Aside from viral infections, IP-10 is also elevated in a number of other diseases. Specifically, IP-10 is suggested to play a role in the immuno-pathogenesis of rheumatoid arthritis[151]. Likewise, a study by Yellin *et al.*[152] further demonstrated the impact of IP-10 in rheumatoid arthritis, showing that blocking of IP-10 led to clinical efficacy in patients

that were part of a phase-II clinical trial. Again, IP-10 is elevated in both Type-1 and -2 diabetes[153, 154, 155], chronic spinal cord inflammation[156], and airway diseases, such as asthma[157]. Findings on elevated levels of IP-10 have targeted it as a potential biomarker for not only HCV infection[158], but also tuberculosis[159].

Additionally, while IP-10 is beneficial in certain cancers, such as breast cancer[152**160**], this may not always be the case. Rainczuk *et al.*[161] found evidence of the antagonistic form of IP-10 in serous epithelial tumors, which correlated with less T-lymphocyte infiltration and potentially negative prognosis for patients having higher levels of the IP-10 antagonistic form.

1.8 Introduction to Aims of Thesis

Years of extensive research in the hopes of identifying and developing an effective vaccine against HIV that could lead to a functional cure and/or better targeting of the viral reservoir has yet to be accomplished. With amazing progress in the field of antiretroviral therapies, HIV-1 has gone from being a death sentence to a manageable chronic disease that allows individuals to live a better and longer life. Aside from potential side-effects of long-term ART use, the HIV-1 infected population continues to age and it has become apparent that damage caused by the HIV virus has left individuals with compromised immune systems, particularly the preferential depletion of CD4+ helper subsets. Therefore, this thesis investigates hypotheses aimed at understanding 1) whether a therapeutic DNA-based vaccine strategy can elicit potent cell-mediated immune responses suggested to be important in HIV-1 control; 2) the role that pre-vaccination cell-mediated factors may play in vaccine responses to non-HIV infections, specifically influenza; And 3) the impact that the imbalanced cytokine/chemokine milieu may have on T-cell responses in HIV-1 infected individuals

on stable therapy. These hypotheses can lend support for research involved in better understanding the timing of ART initiation and preservation of important cell-subsets and its effect on cell-mediated responses to vaccines. As well, the research contained herein can elucidate what responses may still need to be improved further as well as identify possible therapeutic targets to enhance cell-mediated immunity.

i. Improving cell-mediated immune correlates of protection against HIV-1

Studies by Betts *et al.*[40, 49] and Migueles *et al.*[162] have expanded our understanding of potential cell-mediated immune correlates of protection against HIV-1 infection. These studies have examined how long-term non-progressors and controllers who in the absence of antiretroviral therapy are able to control HIV-1 infection and increase their time to disease progression.

Specifically, these studies demonstrate the significance of multi-functional T-cell responses, proliferative capacity, and ability of CD8+ CTLs to kill virally infected cells. Thereby in the development of a therapeutic vaccine against HIV-1, eliciting these responses will be a significant task. Therefore, the first study examined in this thesis explores the use of a DNA based vaccine, not only as a safe strategy to administer to HIV-1 infected individuals, but also a good method to elicit cell-mediated immune responses against HIV-1 antigens. An additional aim is to examine potential factors that may dampen vaccine responses against HIV-1.

ii. Understanding non-responsiveness to vaccines against potential opportunistic infection. As has been discussed by various researchers, HIV-1 infected individuals despite effective antiretroviral therapy continue to show issues in T-cell regeneration and function[3, 14]. Additionally, findings as to the benefit of ART for the protection from opportunistic infections are varied. This means that HIV-1 infected individuals may still

be at risk for complications from opportunistic infections, one such being influenza infection. The need to vaccinate the population against influenza viruses yearly and the risk of the rise of new pandemic strains leaves immuno-compromised individuals in jeopardy. Therefore, in order to better understand why certain individuals respond properly to non-HIV vaccines while others do not, we sought to examine responses to H1N1 vaccination. We hypothesized that chronic immune activation that persists in individuals on ART may play a role in responses to H1N1 vaccination and in turn the ability to achieve sero-protection.

iii. Exploring residual issues affecting immunogenicity in the presence of stable antiretroviral therapy. With stable ART, it is demonstrated that viremia declines, inflammation and immune activation also decline, and there is immune reconstitution of certain cell subsets, specifically CD4+ cells[10, 21, 25]. Therefore, in the absence of high levels of HIV viral replication and immune activation the question arises as to why there remain problems in T-cell functionality. In addition to potential complications that may come about from possible low-level HIV viral replication, other co-pathogens, and microbial translocation, the mediators of some of these issues, the cytokines and chemokines, may be potential therapeutic targets that can aid in boosting immunogenicity and sero-protection against vaccines. Therefore, we hypothesize that exploring the altered cytokine/chemokine environment that is present in HIV-1 infected individuals can offer us ways in which to boost cell-mediated immune responses.

iv. Investigating IP-10 as a potential therapeutic target in improving vaccine immunogenicity. IP-10/CXCL10 is up-regulated in several diseases and is a negative prognostic marker for many of them including HCV[130] and HIV-1 infection[12]. Interestingly, IP-10 remains elevated in HIV-1 infected individuals on stable ART. So using research

from the HCV field regarding the role of IP-10 in responsiveness to therapy can help inform the field of HIV in better understanding IP-10's role in chronic HIV infection. Furthermore, the ovarian cancer field has further expanded a potential role that IP-10's antagonistic form may play in successfully treating patients[161]. Hence, in the third study of this thesis we hypothesize that elevated levels of IP-10 present in HIV-1 infected individuals on ART can impact T-cell function and in turn responsiveness to vaccination. Additionally, targeting of IP-10 and/or its receptor, CXCR3 could aid in improving cellular immune responses.

v. Overarching aims of this thesis. The aims of this thesis contained herein focus on improving T-cell mediated immune responses shown to be important in the control of HIV-1 infection during acute infection and in long-term non-progressors. Additionally, this thesis aims to understand what factors play a role in non-responsiveness to vaccination and what factors present after the initiation of ART contribute to non-responsiveness. Finally, examining the impact from the dysregulated cytokine/chemokine milieu, specifically elevated IP-10 levels, on T-cell function, can aid our understanding in improving vaccine strategies both for therapeutic HIV-1 vaccination and non-HIV vaccination, specifically influenza.

CHAPTER 2:

Therapeutic Immunization of Synthetic Consensus HIV env, gag, and pol DNA in HIV Infected Individuals Induces Potent Cellular Immune Responses and Synthesis of Granzyme B, Perforin.

"Even if HIV prevention efforts were optimally implemented to achieve a new infection rate of near zero, recidivism could threaten this success."

–Dr. Anthony Fauci (*N. Engl. J. Med.* 2014; NIAID)

2.1 Abstract

In this phase-I clinical trial we examined the safety and immunogenicity of immunization of HIV-1 infected individuals on stable antiretroviral therapy with therapeutic vaccination. Twelve HIV-1 infected subjects on stable ART received four doses of the PENNVAX®-B vaccine (encoding synthetic consensus HIV env, gag, and pol) delivered with *in vivo* electroporation. The vaccine was safe and well tolerated. Investigating the immunogenicity elicited by the vaccine demonstrated the production of IFN-γ by both CD4+ and CD8+ T-cells. Additionally, immunization with this vaccine also induced CD8+ T-cells to degranulate and produce perforin and granzyme B in response to stimulation with HIV antigens. This study demonstrates the capability of a therapeutic DNA vaccine against HIV-1 to induce potent responses suggested to be important in the control of HIV-1 infection.

The study in this chapter was conducted in collaboration with others, including Drs. Morrow, Tebas, and Weiner. Our lab performed ELISpot and flow cytometric immunological assessments.

Citation: Morrow, MP, Tebas, P, Yan, J, **Ramirez, LA,** Slager, A, Kraynyak, K, Diehl, M, Shah, D, Khan, A, Lee, J, Boyer, J, Kim, JJ, Sardesai, NY, Weiner, DB, Bagarazzi, ML. Synthetic Consensus HIV-1 DNA induces potent cellular immune responses and synthesis of granzyme B, perforin in HIV infected individuals. *In Review.*

2.2 Introduction

i. Development of a Therapeutic Vaccine Against HIV-1

With several years of arduous research, the question remains as to whether a cure against HIV is possible. Over twenty years of research has led to significant enhancements in antiretroviral drug regimens, which can succeed at inhibiting HIV-1 replication. Similarly, providing condoms, male circumcision, pre-exposure prophylaxis drugs in addition to ART has reduced the risk of HIV transmission and acquisition[163]. However, these drugs and resources have and continue to have obstacles, including issues with adherence, accessibility, and side-effects/toxicities[164]. Furthermore, while this has indeed improved the lifestyle and survival of people living with HIV[17], the long-term need to have these individuals on drugs for the rest of their lives is not only costly to those being treated, but also to the institutions that need to keep developing, improving, and delivering these drugs. Therefore, the need to generate a functional cure, or a therapeutic treatment that could reduce the need for long-term therapy is necessary[164]. Additionally, as Katlama *et al.*[165] explain, the development of a therapeutic vaccine that could assist in boosting immune responses against HIV can help better target the viral reservoir. Specifically, if a therapeutic vaccine can boost cell mediated immune responses, combine that vaccine with an agent that elicits the virus from the latent reservoirs, together this strategy could help eliminate and target the virus[164, 166].

However, the HIV vaccine field has gone back in forth in regards to T-cell based vaccines and those that attempt to elicit broadly neutralizing antibodies. Nonetheless, the field of HIV vaccines has seen both setbacks as with Merck's STEP trial[167] and slight successes as with the RV144 trial; it is still suggested that in order to better treat HIV

infection both arms of the immune system will likely play significant roles[168]. Nonetheless it is possible that responses necessary in a prophylactic setting may not be effective in a therapeutic setting. In regards to T-cell vaccines, Hansen *et al.*[169] demonstrated that a T-cell based vaccine against SIV, while not protective from HIV acquisition, could elicit SIV-specific CD8+ T-cell responses which are associated with the control of infection. In addition, research on therapeutic vaccines has shown that several vaccine modalities including viral vector-based (e.g. Modified vaccinia Ankara vector-based), dendritic cell-based (e.g. DCV2/MANON07-ORVACS), subunit-based (e.g. Vacc-4x), and DNA-based (e.g. DermaVir) vaccines can elicit immunogenic T-cell responses[264]. Therefore, a therapeutic HIV vaccine that could help infected individuals control infection, possibly with reduced need for ART, or help target latently infected cells through the boosting of T-cell mediated responses is of investigation in this study.

Moreover, understanding of potential cell-mediated immune correlates of protection have come from examining those individuals who are able to maintain control of HIV in the absence of therapy and have limited their disease progression. Studies, by Migueles *et al.*[52, 53], Hersperger[54] and Betts *et al.*[49] have demonstrated the role of not only a poly-functional T-cell response, but also the importance of CTL cytotoxic capacity and killing of infected cells in the control of HIV-1 infection. Recenlty, Ndhlovu *et al.*[166**170**] also demonstrated that HIV controllers maintain a broad HIV-specific CD8+ T-cell memory response against HIV gag, which may play a role in long-term viremic control. Additionally, while broadly neutralizing antibodies may be important to elicit as well, B cells also need assistance from functional T-cells in order to help initiate and maintain B cell responses.

As mentioned above, a variety of vaccine modalities have been investigate for the use as therapeutic vaccines, these strategies include DNA based vaccines, dendritic

cell-based vaccines, and nonreplicative viral vectors, such as canarypox-based vaccines[264]. The study in this chapter implemented the use of a DNA vaccine combined electroporation for enhanced transfection of cells. Compared to other vaccine modalities, such as live vaccines or recombinant viral vectors, DNA vaccines, which use plasmid constructs, offer a safer strategy to target HIV-1[171]. With improved design and delivery of DNA vaccines, their immunogenicity has and continues to improve in generating both humoral and cell-mediated immune responses[171]. Furthermore, the DNA PENNVAX®-B vaccine delivered with electroporation was previously shown to be safe and elicit CD4+ and CD8+ T-cell responses in HIV sero-negative individuals (HVTN protocol 080 NCT00991354). Thus, in this study we investigate and characterize T-cell responses, including IFN-γ production and cytotoxic capacity, in response to vaccination with a therapeutic DNA vaccine against HIV env, pol, and gag antigens.

2.3 Results

i. Study design

The study described in this chapter was an open label, phase I clinical trial conducted at one center in the United States (NCT01082692). The study protocol was approved by an Institutional Review Board and adhered to the guidelines of Good Clinical Practice and the Declaration of Helsinki. Written informed consent was obtained prior to study enrollment. For inclusion into the study, adult HIV-1 infected male and female subjects had to be between 18 and 55 years of age, currently receiving a highly active antiretroviral therapy (HAART), needed to have undetectable plasma viral loads (<75 copies/ml), CD4+ lymphocyte counts ≥400 cells/μl, and nadir CD4+ lymphocyte counts >200 cells/μl. Counts were documented twice on different occasions within 60 days of enrollment into the study. Female subjects could not be pregnant or nursing, and

needed a negative serum pregnancy test within 30 days of entry into the study, and finally must have a negative urine pregnancy test on the day of the first therapeutic vaccine dose. Individuals were excluded if they had any past or present AIDS-defining illness, malignancy needing chemotherapy, autoimmune disease, or had received any other immunomodulatory therapy within 4 weeks of study entry. A timeline depicting screening for eligibility up to and including analysis can be seen in **figure 2.1**.

The aims of this study were to examine the safety and immunogenicity of a therapeutic HIV-1 DNA vaccine delivered with electroporation in HIV-1 infected individuals on stable antiretroviral therapy. The PENNVAX®-B vaccine is a cocktail of 3 expression plasmids that contain the genes that encode synthetic HIV-1 Clade B env, gag, and pol. Subjects (n=12) received four doses of the PENNVAX®-B vaccine delivered intramuscularly followed immediately by electroporation with the CELLECTRA® 2000 Adaptive Constant current device. Each dose contained 3mg of the expression plasmids in equal proportions. Doses were administered as shown in **figure 2.2**. Blood was also collected at the time-points depicted in **figure 2.2** as well as safety assessments on time-points where the vaccine was administered. Demographics and characteristics of the study population are depicted in **Table 2.1**. The average age of the subjects was 42.6 years and ranged between 31-55 years. Eleven of the twelve subjects were male, and 58% identified as black and 92% as non-Hispanic or Latino.

All in all immunization with the PENNVAX®-B vaccine delivered with electroporation was well-tolerated and no severe, life-threatening, or adverse events occurred during the course of this study.

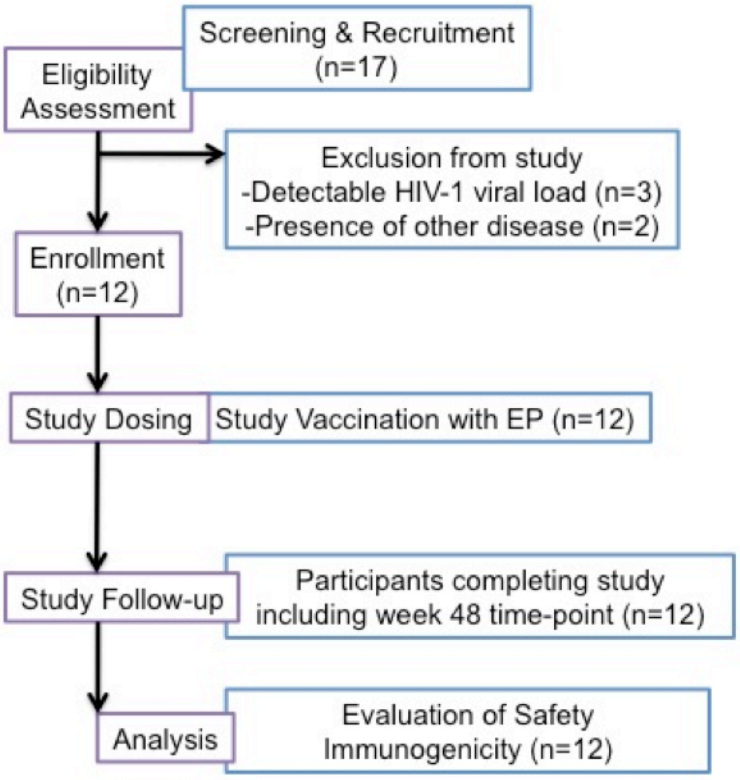


Figure 2.1. Schematic of eligibility, determination, enrollment, study conduct, and analysis.

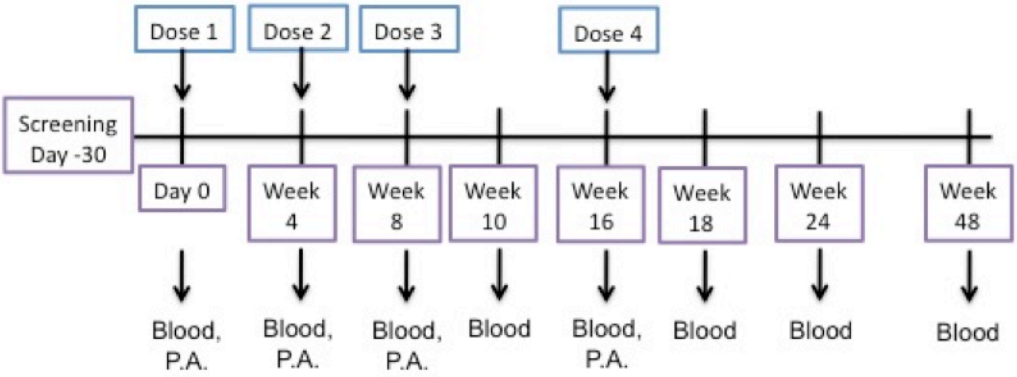


Figure 2.2. Study schedule and immunization time-line for participants of the study. The schematic depicts time-points of immunization, pain assessment (P.A.), and collection of blood for immunologic and virologic assays.

Table 2.1. Demographic and immunologic characteristics of study participants.

Characteristic	Subjects (n=12)
Age, y	
Mean	42.6
Range	31-55
Sex, n (%)	
Male	11 (92)
Female	1 (8)
Race, n (%)	
White	5 (42)
Black or African American	7 (58)
Ethnicity, n (%)	
Hispanic or Latino	1 (8)
Non-Hispanic or Latino	11 (92)
Body Mass Index, kg/m^2	
Mean	27.7
Range	21-40.4
CD4+ T-cell count at screening, cells/μl	
Mean	733
Range	461-993
Nadir CD4+ T-cell count*, cells/μl	
Mean	505
Range	203-877
HIV-1 viral load at screening, copies/ml	
Modes	20, 48
Range	20-63

*n=11, data not available for one subject

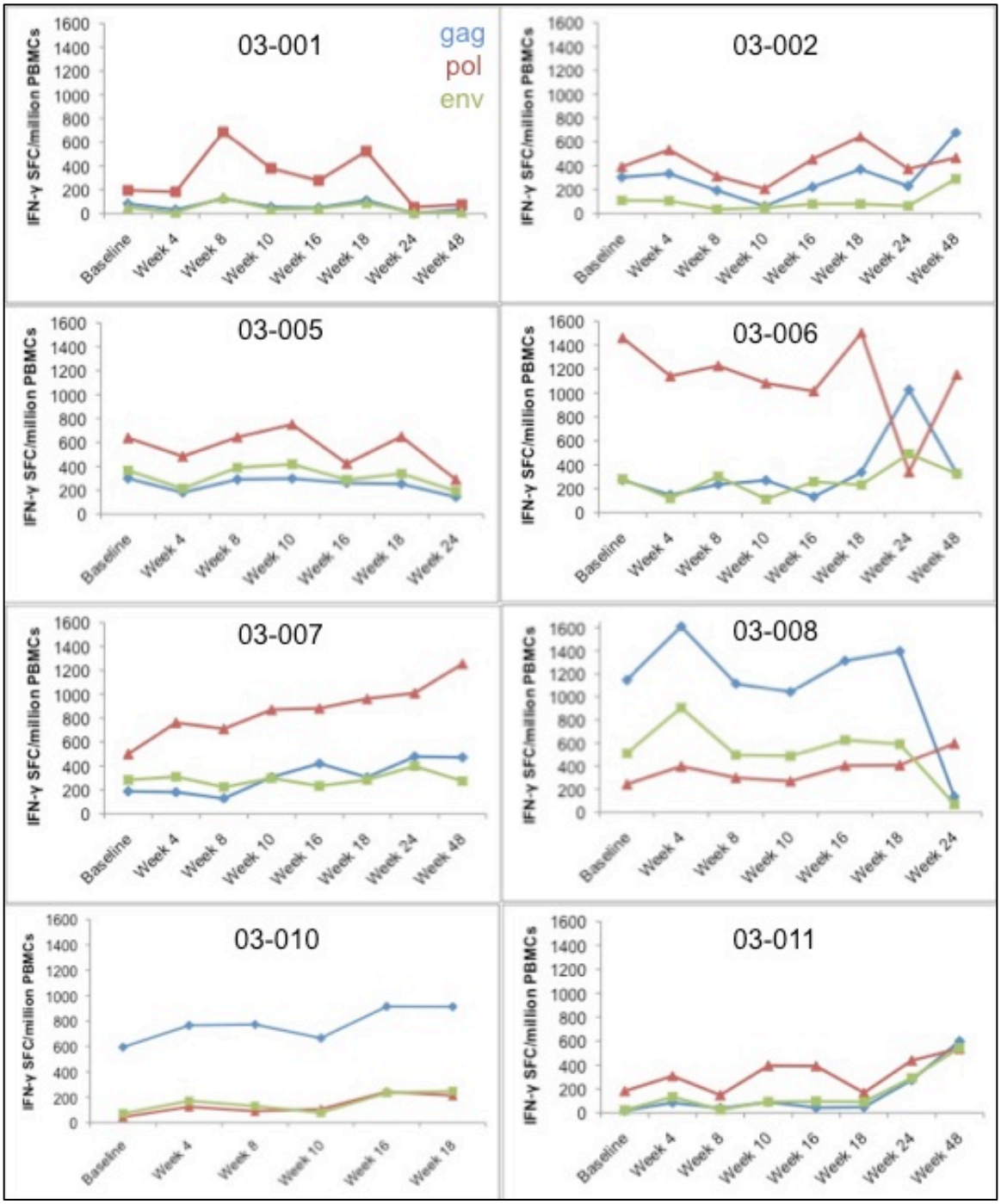
ii. IFN-γ Induction in PBMCs by Vaccination with PENNVAX®-B

IFN-γ is an important measure of innate and adaptive anti-viral responses[261]. Using a standard IFN-γ ELISpot assay we measured cellular immune responses elicited by immunization with PENNVAX®-B. We specifically examined IFN-γ production in response to the antigens included in the vaccine, that is env, gag, and pol, across the immunization time-line. Each of the individual 12 subjects' IFN-γ responses to env, gag, and pol at each time-point can be seen in **figure 2.3**. Time-points for each subject's peak responses are as follows: Subject 03-001 (gag: wk 18; pol: wk 8; env: wk 8), subject 03-002 (gag: wk 48; pol: wk 18; env: wk 48), subject 03-005 (gag: wk 10; pol: wk 10; env: wk 10), subject 03-006 (gag: wk 24; pol: wk 8; env: wk 24), subject 03-007 (gag: wk 24; pol: wk 48; env: wk 24), subject 03-008 (gag: wk 4; pol: wk 24; env: wk 4), subject 03-010 (gag: wk 16; pol: wk 16; env: wk 18), subject 03-011 (gag: wk 48; pol: wk 48; env: wk 48), subject 03-013 (gag: wk 48; pol: wk 48; env: wk 48), subject 03-015 (gag: wk 48; pol: wk 48; env: wk 48), subject 03-016 (gag: wk 10; pol: wk 10; env: wk 18), subject 03-017 (gag: wk 16; pol: wk 16; env: wk 16).

When examining day 0, peak, and memory (week 48) vaccine responses, we found IFN-γ responses peak at 692.6±436.4, 614±344.5, and 341.0±243.8 SFC/10^6 PBMCs for gag, pol, and env respectively. Comparing day 0 responses to peak responses demonstrated a significant increase in response to vaccination (Day 0-gag: 364.2±310.2 SFC/10^6 PBMCs, p=0.0010; Day 0-pol: 372.0±384.0 SFC/10^6 PBMCs, p=0.0068; Day 0-env: 175.1±155.1 SFC/10^6 PBMCs, p=0.0010; **figure 2.4**). When looking at the subjects individually, we also see that vaccination led to IFN-γ responses great than 1000 SFC in some of the subjects (**figure 2.3**) Additionally, while there was an increase in IFN-γ production during memory responses (Week 48), these responses were not significantly elevated compared to Day 0 (gag: 364.2±310.2 vs. 412.0±398.8

SFC/10^6 PBMCs; pol: 372.0±384.0 vs. 454.5±454.2 SFC/10^6 PBMCs; env: 175.1±155.1 vs. 199.4±195.9 SFC/10^6 PBMCs; **figure 2.4**). In addition, two subjects did not have enough sample available to examine memory responses (Week 48). Furthermore, to determine if there was an increase in ELISpot responses to non-vaccine antigens, we examined subjects' IFN-γ ELISpot response to HIV Nef antigen, which was not part of the vaccine. However, vaccination did not lead to a significant increase in IFN-γ response to Nef (p=0.6569; **figure 2.5**).

We next sought to determine which individuals exhibited responses that would classify them as responders to vaccination. Since subjects' responses prior to the first vaccine dose were examined multiple times, a pre-vaccine ELISpot response for each antigen (gag, pol, env) was determined for each subject. Using this pre-vaccine response we required subjects' IFN-γ ELISpot responses to be at least 2 standard deviations above the pre-vaccine response in order to be classified as a responder to the particular HIV antigen (gag, pol, or env). Following these criteria, all of the twelve subjects were shown to be responders to at least one of the vaccine antigens, with the majority of the subjects (11/12) being responders to pol. Nonetheless, 8/12 subjects were positive responders to gag and half showed a response to env (**Table 2.2**). Each individual's response to each antigen is depicted in **Table 2.3**. Moreover, nine of the twelve subjects showed positive responses to more than 1 of the antigens, and 4 subjects exhibited a response to all three. Additionally, eight of the twelve subjects demonstrated positive responses at more than one time-point during the immunization time-line.


03-001
gag
pol
env
03-002
03-005
03-006
03-007
03-008
03-010
03-011
IFN-γ SFC/million PBMCs
1600
1400
1200
1000
800
600
400
200
0
Baseline
Week 4
Week 8
Week 10
Week 16
Week 18
Week 24
Week 48

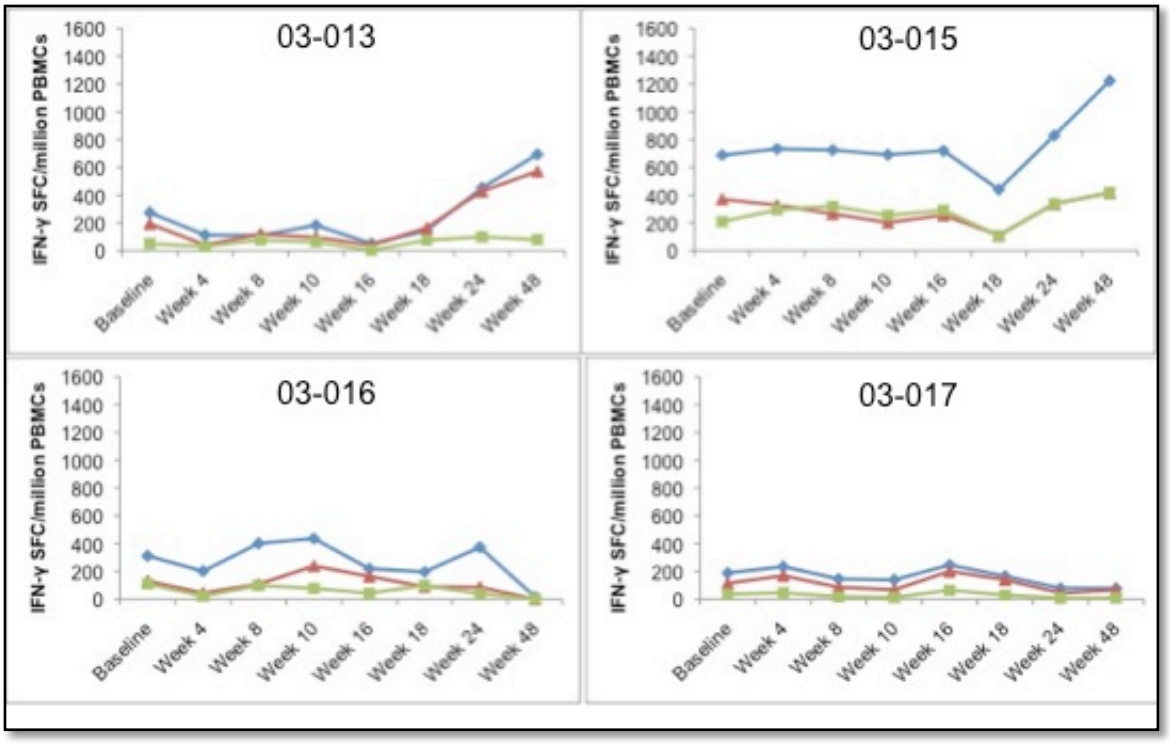


Figure 2.3. IFN-γ ELISpot responses to gag, pol, and env across the study time-line for each individual study participant. Note that subject 03-010 did not have sample available for week 24 and 48; and subject 03-008 did not have sample available for week 48. Graphs depict IFN-γ SFC/10^6 PBMCs. Blue color indicated response to gag; red color represents response to pol; and green color represents response to env.

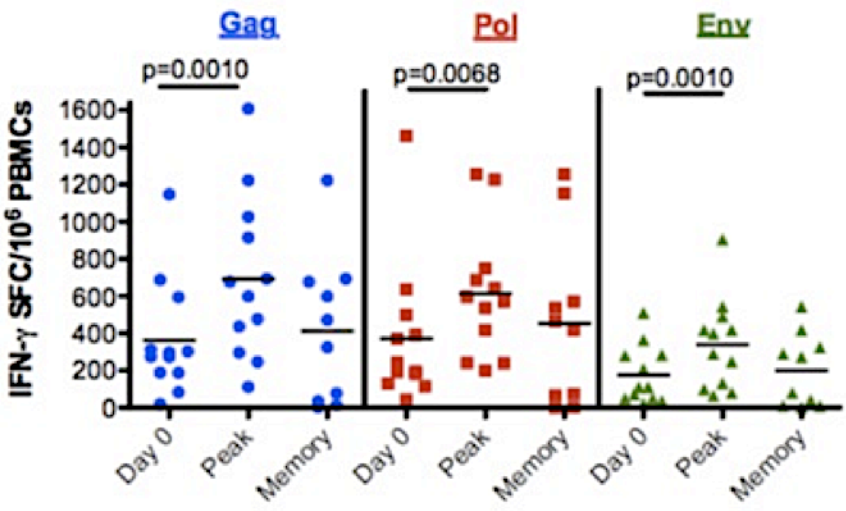


Figure 2.4. IFN-γ ELISpot responses to individual gag, pol, and env HIV-1 antigens part of the PENNVAX-B® vaccine on day 0, the peak of response, and memory (8 months after the final dose of PENNVAX-B®). Day 0 responses were significantly lower than peak responses (gag: p=0.001; pol: p=0.0068; env: p=0.001). Graph depicts IFN-γ SFC/10^6 PBMCs.

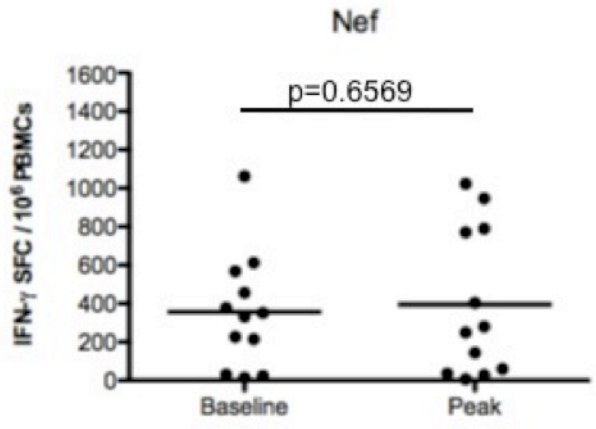


Figure 2.5. IFN-γ ELISpot responses to HIV-1's Nef antigen (not part of PENNVAX-B®). This served as a control for natural variation in responses to HIV antigens. ELISpot responses to Nef did not differ significantly between baseline and peak response for the individuals in this study. Graph depicts IFN-γ SFC/10^6 PBMCs.

Table 2.2. IFN-γ ELISpot response to PENNVAX-B® HIV-1 antigens. Responders to gag, pol, and env by subject.

Patient ID	Gag	Pol	Env
03-001	-	+	-
03-002	+	+	+
03-005	-	+	-
03-006	+	-	+
03-007	+	+	-
03-008	-	+	-
03-010	-	+	+
03-011	+	+	+
03-013	+	+	-
03-015	+	+	+
03-016	+	+	-
03-017	+	+	+

Table 2.3. IFN-γ ELISpot, PENNVAX-B® response summary.

# of Responders to at least 1 antigen	12/12
# of Responders to Gag	8/12
# of Responders to Pol	11/12
# of Responders to Env	6/12
# of Responders to more than 1 antigen	9/12
# of Responders to all 3 antigens	4/12
# of responders at more than 1 time-point	8/12

iii. Cytotoxic capacity of CD8+ T-cells

We next wanted to determine if vaccination with PENNVAX®-B induced CD8+ T-cell responses associated with CTL functions. Specifically, while IFN-γ production is suggestive of an anti-viral response[5], functions associated with the identifying and killing of infected target cells are suggested, by studies of HIV controllers, to be important for control of HIV-1 infection[53]. We therefore examined the ability of CD8+ T-cells to degranulate, through expression of CD107a, and produce perforin and granzyme B. However, one subject (03-010) did not have enough sample to analyze this response. We used the gating strategy depicted in **figure 2.6** to identify CD8+ T-cells that could degranulate and produce the cytotoxins perforin and granzyme B. Furthermore, the lower cut off of the assay is 0.05%. Hence, we examined the impact of vaccination with PENNVAX®-B compared to baseline CD8+/CD107a+/Perforin+/Granzyme B frequencies.

We found that 7 of the 11 subjects examined demonstrated CD8+ cytotoxic responses that were above the lower cut off of the assay. In addition, 6 of those 7 subjects demonstrated responses that exceeded baseline frequencies and of which were as high as 0.49% (**figure 2.7**). While on the other hand 4 of the 11 subjects examined showed weak CD8+ CTL responses or responses that did not exceed baseline frequencies, with one subject not showing a response at all. This demonstrated that PENNVAX®-B can potentially enhance CD8+ CTL responses associated with control of HIV-1 infection, however, not all subjects exhibited a boost in response.

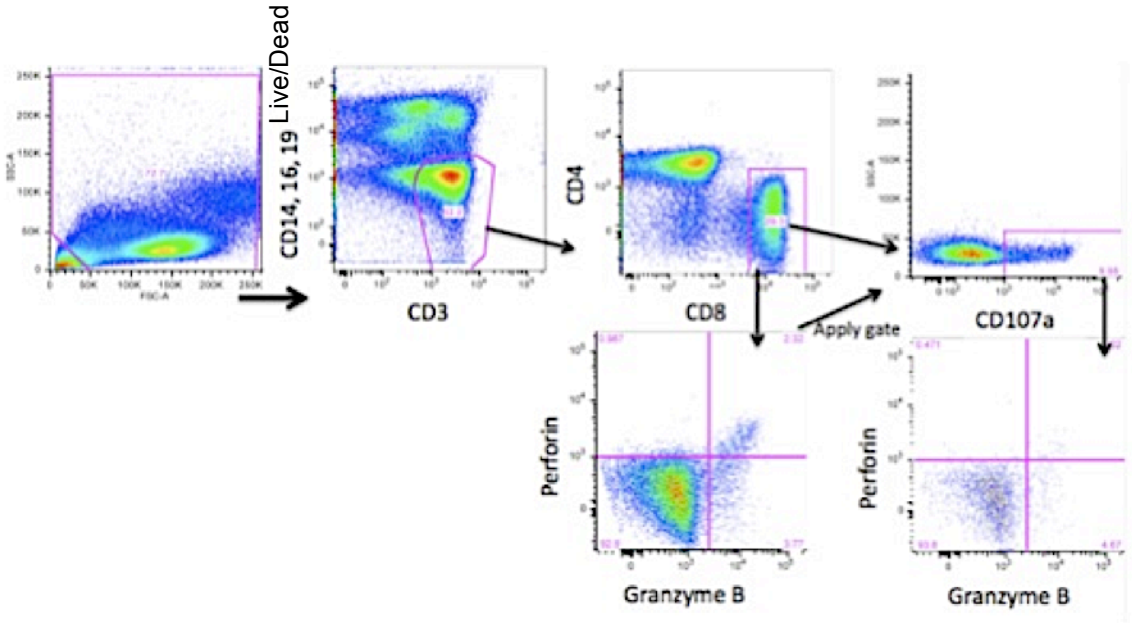


Figure 2.6. Multi-parameter flow cytometry panel used for gating the CD8+ CD107a+ Perforin+ Granzyme B+ population. We gate the live population followed by our CD3+ population, then for our CD8+ T-cell subset. On this subset we gate for both our CD107a+ and Perforin+ Granzyme B+ populations. We then apply the Perforin+ Granzyme B+ gate to the CD107a+ population to maintain consistency in our gating.

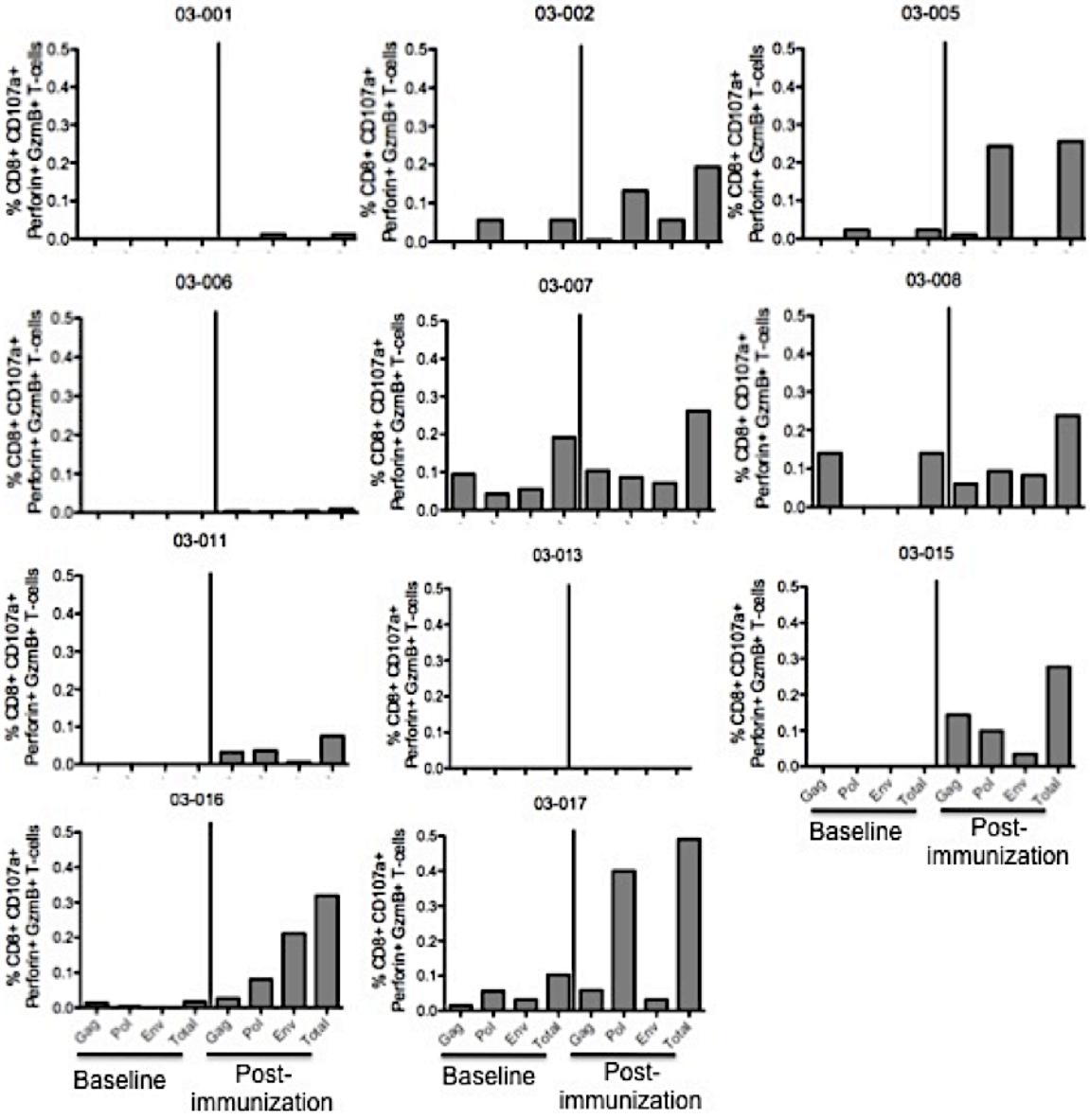


Figure 2.7. Frequency of CD8+ T-cells expressing the marker of degranulation CD107a and cytoxins perforin and granzyme B split up by HIV antigen (gag, pol, env), and total HIV for each study participant pre-immunization and post-immunization. Time-points for Post immunization responses for each subject are as follows: 03-001 (gag: wk 18; pol wk 24; env: no response), 03-002 (gag: wk 18; pol wk 18; env: wk 18), 03-005 (gag: wk 4; pol wk 10; env: no response), 03-006 (gag: wk 18; pol wk 10; env: wk 4), 03-007 (gag: wk 10; pol wk 10; env: wk 10), 03-008 (gag: wk 18; pol wk 10; env: wk 10), 03-011 (gag: wk 18; pol wk 18; env: wk 10), 03-013 (gag, pol, env: no response), 03-015 (gag: wk 8; pol wk 24; env: wk 24), 03-016 (gag: wk 8; pol wk 24; env: wk 48), 03-017 (gag: wk 16; pol wk 10; env: wk 24).

2.4 Discussion

In this study we examined the safety and immunogenicity of PENNVAX®-B delivered with electroporation in HIV-1 infected individuals who are on stable ART. This vaccine was shown to be safe and induced T-cell responses in HIV-uninfected individuals, thus we sought to examine if this vaccine could also aid in enhancing cellular immune responses in those infected with HIV. For those infected with HIV-1 and on stable ART, it would be the goal of a therapeutic vaccine or immuno-therapy, to enhance or modify the immune response to aid in controlling the virus with little or no need for ART, target the viral reservoir, or eliminate HIV-1 infection[164, 165]. Additionally, as research continues to explore how to better target the viral reservoir, researchers, like Archin *et al.*[166] have demonstrated that when they administered the HDAC inhibitor drug, vorinostat, to latently infected HIV-positive individuals, the levels of HIV RNA in resting CD4+ T cells increase. However, this also suggests the need for HIV-1 infected individuals to have the capability of targeting these infected cells, either through ART or in combination with a therapeutic vaccine/immuno-therapy that boosts immune functions associated with the identification and killing of virally infected cells[164, 166]. Therefore, without understanding how to eliminate these cells, it could leave individuals in a worse situation than the one they are in while on ART, such as further depletion of cell subsets necessary for avoiding complications from opportunistic infections. Nevertheless, the potential for a cure exists, as has been shown by the Berlin patient[172].

Nonetheless, while previous DNA vaccine strategies have suffered from poor immunogenicity and cell-mediated immune responses[173], continued research has demonstrated that DNA vaccine potency has significantly improved[174]. Importantly, this study shows that a DNA vaccine strategy can be immunogenic and elicit potent cellular

responses important against HIV-1 infection. Furthermore, this study supports the use of electroporation in delivering DNA-based immuno-therapies.

The results presented in this study suggest it is possible to enhance cellular immune responses in HIV-1 infected individuals on ART and reveals additional potential therapeutic targets. Specifically, this study demonstrated that IFN-γ production could be enhanced against more than one HIV-1 antigen and in all the individuals in the trial. However, the majority of responders responded to pol followed by gag, and env. Knowing this, enhancing responses to these antigens may be necessary in order to induce a broader cell-mediated response against HIV, which is suggested to be important[170, 175]. Moreover, the responses elicited were demonstrated to be specifically due to vaccination, since responses to antigens not present in the vaccine, Nef, were not boosted. In addition, the IFN-γ responses induced by this vaccine were potent, over 1000 SFCs, in several individuals. Likewise, this vaccine demonstrated that it is possible to use a therapeutic vaccine to elicit IFN-γ responses that were indicative of a long-lasting memory response. Thereby, suggesting that therapeutic vaccine could be used to help maintain long-term control of HIV-1 infection.

With chronic HIV-1 infection there is a gradual loss in CD8+ T-cells, partly due to the loss of CD4+ helper subsets[176, 177]. In spite of successful ART, these responses are not reconstituted leaving these individuals with the inability to respond properly to HIV-1 infection[45, 53]. Boosting these CTL responses in HIV-1 infected individuals on ART with a therapeutic vaccine or immuno-therapy could lead to reduced need for ART and better cell-mediated control of HIV-1 infection. Here, we demonstrate that vaccination with PENNVAX®-B could elicit CD8+ CTL responses, specifically the ability to degranulate and produce perforin and granzyme B, against HIV-1 antigens. Therefore, this would suggest that a therapeutic vaccine against HIV-1 can potentially enhance CD8+ CTL

cytotoxic capacity, which could lead to better control of infection and delay disease progression. Even more, if combined with an anti-latency agent, the ability to kill virally infected cells could be boosted. Although several subjects demonstrated an enhancement in cytotoxic capacity, not all individuals showed such a response, and one individual none at all. On that account, in addition to improving vaccination strategies, the need to identify additional therapeutic targets that could further enhance CD8+ CTL responses is of interest.

As well, as was suggested by the RV144 Trial, eliciting a potent antibody response may need to be combined with a cell-mediated therapy to better target HIV-1[168]. Additionally, further examining how to improve CD4+ T-cell helper responses will be beneficial to both humoral and CTL-mediated responses[178]. Nonetheless, this study demonstrates the need for an immuno-therapy against HIV and expands our understanding of eliciting potent cell-mediated immune responses in HIV-1 infected individuals on stable ART.

Limitations of Study

This study demonstrates that a therapeutic vaccine against HIV-1 can be used to elicit T-cell mediated immune responses. However, while all the individuals in this study demonstrated a positive response via IFN-γ as compared to baseline under the set definition of response, not all subjects' exhibited strong responses. For example subjects 03-016 and 03-017 had IFN-γ responses below 500 SFC/10^6 PBMCs. While statistically they are shown to be positive responses, this study cannot address whether responses of that low magnitude would be "helpful" responses to HIV-1. In addition, it may be necessary to place stricter definitions on a responder in order to ensure a better analysis of the impact of vaccination. In addition, other subjects such as 03-006 show higher IFN-

γ responses, but that subject's cytotoxic response is almost non-existent. Further analysis would be necessary to determine why cytotoxic capacity is not enhanced, such as the possible effect of T-cell exhaustion. As well, while this study does examine both the IFN-γ and CD8+ cytotoxic responses, this study may underestimate the overall impact on T-cell mediated responses since additional possible functions, such as IL-2 and TNF-α, were not measured in this study. Even more, the analysis of IFN-γ responses using ELISpot assays limit our findings as to what cells were producing that IFN-γ. While the goal of this study was not efficacy, studies like this sometimes have explored the impact of vaccination through the use of structured treatment interruptions of ART to see if control of infection was improved. Studies like those are necessary as we continue to investigate what immune correlates are important in a therapeutic setting. Additionally, studies like this would benefit from research investigating the timing of immunization, specifically, how long after ART/what level of immune reconstitution might be necessary to elicit responses important in a therapeutic setting. Finally, this study limits our ability to determine if the responses generated by this vaccine are an enhancement or boosting of already present responses or eliciting *de novo* ones.

Chapter 3:

Seroprotection of HIV-Infected Subjects After Influenza A(H1N1) Vaccination is Directly Associated with Baseline Frequency of Naïve T Cells.

"At the onset of the 2009 influenza season, there was a lot of speculation about whether this pandemic would rival the extent of the 1918 pandemic. Fortunately, that was not the case, but the lessons learned can be applied to preparations for future pandemics."
–Phillip LaRussa (*Semin. Respir. Care Med.* 2011; Columbia University)

3.1 Abstract

Individuals infected with HIV-1 are at risk for developing complications from influenza infection. Regardless of antiretroviral therapy use, these individuals often have blunted vaccine responses. We sought to better understand what factors may impact sero-protection to influenza A(H1N1). HIV-1 infected subjects on stable ART received a 15μg dose of the monovalent, unadjuvanted, inactivated, split virus H1N1 vaccine (Novartis). Prior to this study, subjects' antibody titers were evaluated before receipt of the vaccine and at 3 weeks post-immunization. Subjects were then determined to have achieved sero-protection (Responders) if by week 3 they had HAI titers that were ≥ 1:40 and had a ≥four-fold increase in their antibody titers from baseline. When assessing the role of immune activation and cellular phenotypes on sero-protection, we found that while the levels of immune activation did not differ between Responders and Non-responders, Responders had a higher frequency of naïve T-cell populations and lower frequency of terminally differentiated T-cell populations. We also assessed the cytokine and chemokine profiles of these individuals and compared the serum cytokine/chemokine levels to that of healthy HIV-negative controls and between Responders and Non-responders. Finally, we assessed the role that age plays in these factors associated with responsiveness and found that age was negatively associated with the frequency of naïve CD4+ T-cells. Therefore, this study suggests that preservation of naïve T-cell populations, through early ART initiation, could impact vaccine responses against influenza and other pathogens, especially as this population ages.

The study in this chapter was conducted in collaboration with others, including Drs. Tebas, and Frank.

Citation: **Ramirez, LA**, Daniel, A, Frank, I, Tebas, P, Boyer, JD. Seroprotection of HIV-Infected Subjects After Influenza A(H1N1) Vaccination is Directly Associated with Baseline Frequency of Naïve T Cells. *J. Infect. Dis.* 2014

3.2 Introduction

In 2009 the novel pandemic H1N1 influenza spread world-wide leading to widespread infection and death[179]. In addition to the severity of this pandemic, seasonal influenza can lead to serious illness in young children, the elderly, those with chronic infections, and immune-compromised individuals, which include those infected with HIV[180, 181, 182, 183, 184, 185]. Furthermore, the influenza vaccine is safe in those infected with HIV[186, 187, 188]. With ART, the rates of influenza-associated complications are reduced, but the rates remain elevated as is seen in other high-risk populations[189].

In spite of immune reconstitution that should be enough to prevent the development of opportunistic infections, HIV-1 infected individuals on ART are shown to have poor antibody and memory B-cell responses[190]. Additionally, poor responses are associated with CD4+ T-cell counts and HIV RNA levels[191, 192, 193, 194]. Even more, it is demonstrated that HIV-1 infected individuals often require higher or multiple doses of the influenza vaccine to achieve sero-protection[195], as similarly seen in other high-risk groups[196]. An early study in HIV-1 infected individuals demonstrated that a 15μg dose of the inactivated influenza vaccine induced weaker antibody responses in HIV-infected individuals compared to healthy controls, suggesting the need for alternative strategies to improve influenza immunization[197]. Recent studies have demonstrated that administering higher doses (60μg)[198, 199], multiple doses and/or adjuvanted doses[182, 200, 201, 202] can improve sero-protection, but not all individuals respond regardless.

Examining the study preceding the one in this chapter, demonstrated that HIV-1 individuals on ART do not all achieve sero-protection following H1N1 influenza vaccination[203]. Furthermore, despite ART, the durability of antibody responses following influenza vaccination is not as durable compared to HIV-uninfected controls[204]. Therefore the purpose of this study was to further understand what HIV-1 associated

dysregulation may affect the ability of these individuals to respond to influenza vaccination. While we do not examine the direct impact of HIV-associated dysregulation on B-cells, this study reinforces the impact on CD4+ T-cells can have impact on humoral responses and thus response to influenza vaccination.

HIV-1 infection leads to an imbalanced cytokine/chemokine, environment, and induces changes in apoptosis, exhaustion, and senescence[10, 205]. The altered cytokine/chemokine environment was shown to impact the severity of pandemic H1N1 influenza infection[206]. Thus, a dysregulated cytokine/chemokine milieu could also place HIV-1 infected individuals at risk, but understanding the role this environment plays can reveal possible targets to improve protection in these subjects. Also, regardless of ART, HIV-1 infected individuals maintain elevated levels of T-cell activation[31]. These levels of immune activation are associated with disease progression and are suggested to contribute to immune dysfunction[14]. Hence, it is possible that elevated levels of immune activation could contribute to blunted responses to vaccines. In addition, while the development and use of ART has improved the survival of individuals living with HIV-1[17], as this population ages their immune system ages as well, thereby contributing to defects of the immune system, a concept called “inflammaging.” This concept suggests that the immune systems of those infected with HIV-1 exhibit changes reminiscent of the elderly[207]. During physiological aging, there is progressive thymic involution, and in turn deceases in naïve T-cell numbers and reduced T-cell function[207, 208]. These changes are also seen in those infected with HIV and result in an accumulation of terminally differentiated immune cells, which can impact antigen responsiveness[3]. Regardless, studies have demonstrated the importance of vaccinating the elderly against influenza to reduce complications and co-morbidities[209, 210]. And as the CDC[211] suggests, giving the

influenza vaccine to those infected with HIV-1 is also necessary to protect these individuals[212, 213].

As a result, we hypothesize that in order to better improve protection in HIV-1 infected individuals, it is necessary to understand what factors, such as immune activation, cytokine/chemokine dysregulation, cellular phenotypes, and aging, can contribute to lack of sero-protection and help identify prospective therapeutic targets to aid in designing better vaccination strategies against influenza and other pathogens.

3.3 Results

i. Study Design

This research is a follow-up to the study previously presented by Tebas *et al.*[203]. The goal of that study was to examine the safety and immunogenicity of the recommended H1N1 vaccine (Novartis, Basel, Switzerland). HIV-1 infected subjects that were over the age of 18 and had an indication to receive the H1N1 vaccine were included in the study. Subjects were excluded if they had a known allergy to eggs or other components of the vaccines, had previous severe reactions to prior immunization to seasonal flu, or had known cases of H1N1 influenza during Spring 2009. Additionally, if subjects had received any licensed live vaccines 4 weeks prior to study entry, or inactivated vaccines prior to entry into the study. Participants were also excluded if they were currently receiving any other experimental treatments, systemic chemotherapy, steroids, immune-modulators, or had a history of Guillain-Barre syndrome.

A total of 120 participants were included in the primary study by Tebas *et al.*[203] and provided informed consent. For this follow-up study, the goal was to examine pre-vaccination baseline characteristics that can serve as predictors of their vaccine response. Forty-six subjects had enough frozen peripheral blood mononuclear cell

samples available for analysis in this study. These 46 subjects had baseline hemagglutination inhibition (HAI) titers of <1:40 and were on ART. Subjects' median age was 48 years, with a range of 26-77 years of age; 69.6% were male, 30.4% were female; 63% identified as Black/African-American, 10.9% were Hispanic/Latino, 23.9% were white, and 2.1% identified as Asian/Pacific Islander. These subjects had an average CD4+ T-cell count of 542 ± 306.8 cells/μl, an average nadir CD4+ T-cell count of 193 ± 187.2 cells/μl, and HIV RNA loads were <400 copies/mL in 90% of the subjects, and below the limit of detection in 85% of the subjects (**Table 3.1**).

Participants received a single 15μg intra-muscular dose of the monovalent unadjuvanted**,** inactivated, split virus H1N1 vaccine. As depicted in **figure 3.1**, each participant had blood collected and baseline studies performed prior to immunization. Following 21-28 days after vaccination, blood was collected and serological responses to the vaccine were evaluated by Tebas *et al.*[203]. They examined antibody titers of the 120 subjects using an HAI assay at Bioqual, Inc., as described previously by Kendal *et al.*[214]. Subjects were classified as sero-protected and Responders to the vaccine if at week 3 post-immunization their HAI titers were ≥1:40 and increased at least 4-fold higher than baseline, otherwise subjects not meeting these requirements were considered Non-responders. For the purpose of this study 27 subjects fit into the classification of Responder and 19 into the Non-responder classification.

Table 3.1. Demographic and immunologic characteristics of study participants.

Characteristic	Subjects (n=46)
Age, y	
Median	48
Range	26-77
Sex, n (%)	
Male	32 (69.6)
Female	14 (30.4)
Race, (%)	
White	23.9
Black or African American	63
Ethnicity, (%)	
Hispanic or Latino	10.9
Non-Hispanic or Latino	89.1
CD4+ T-cell count at screening, cells/μl	
Mean	544
Range	71-1396
Nadir CD4+ T-cell count, cells/μl	
Mean	193
Range	1-831
HIV-1 viral load at screening,%	
<400 copies/ml	90%
Below Limit of quantification	85%

Figure 3.1. Study and immunization timeline. Diagram depicts time-points of immunization, blood collection, and HAI assays.

ii. T-cell Activation

We hypothesized that the baseline levels of immune activation would affect the ability of subjects to achieve Responder status, since immune activation is a negative predictor of HIV-1 disease progression and contributes to cell turnover [9, 10]. In order to evaluate the levels of immune activation we used multi-parameter flow cytometry to examine the expression of the activation markers CD38 and HLA-DR on the surface of T-cells prior to vaccination. The diagram in **figure 3.2** depicts the gating strategy used to examine expression of the activation markers.

Using this strategy we compared the expression of CD38 and HLA-DR on CD4+ and CD8+ T-cells between Responders and Non-responders. We found that baseline expression levels of CD38+ HLA-DR+ CD4+ did not differ between Responders and Non-responders (1.56%±1.63% [n=27] vs. 1.95%±2.87% [n=19]; p=0.688; **figure 3.3**). Likewise, the expression levels of CD38+ HLA-DR+ CD8+ T-cells also did not differ between Responders and Non-responders (1.91%±1.79% [n=27] vs. 2.3%±3.63% [n=19]; p=0.8409; **figure 3.3**). This then suggests that baseline levels of activation do not play a significant role on the response to the H1N1 influenza vaccine.

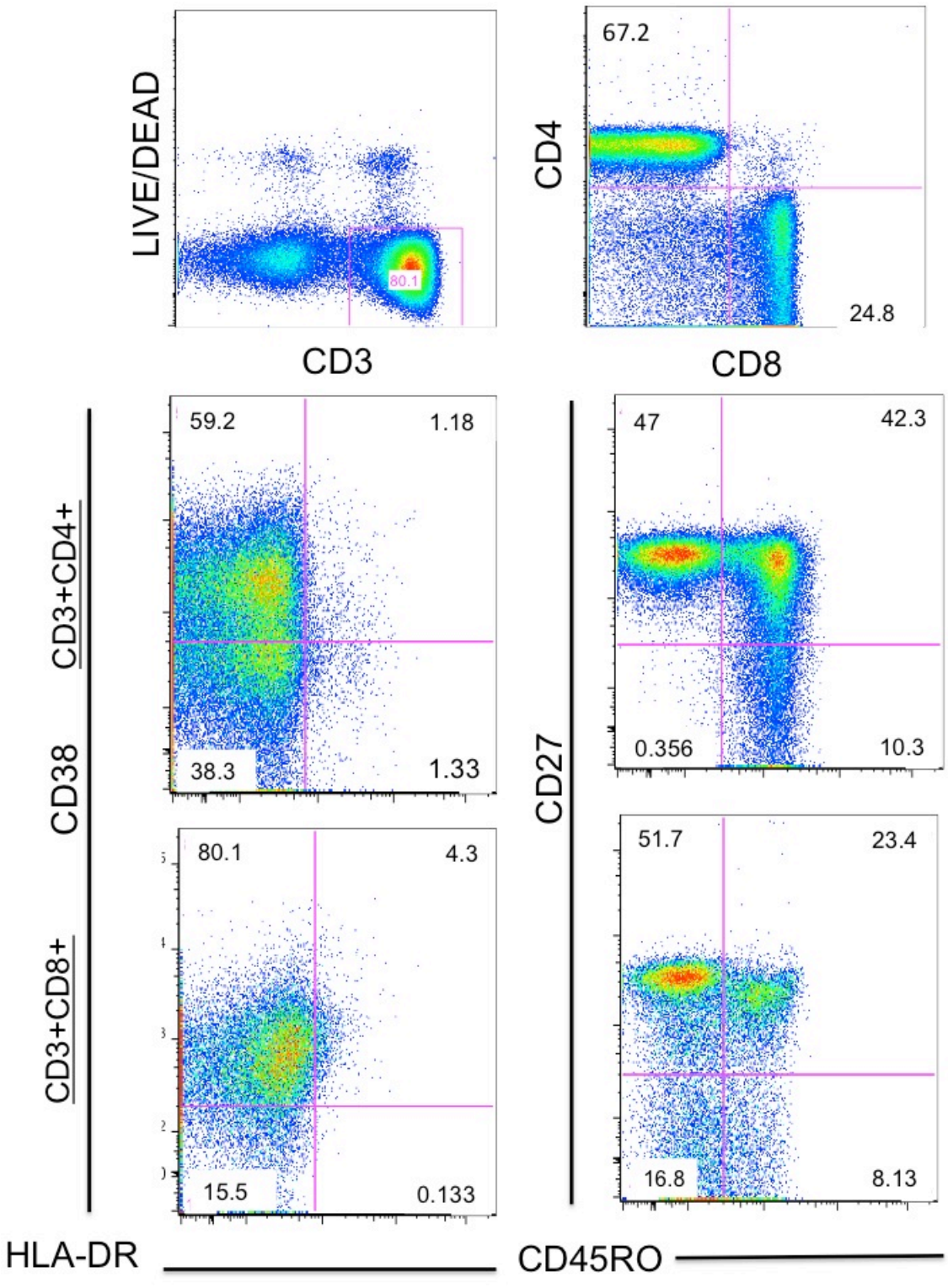


Figure 3.2. Example layout of gating strategy. First, gating for the live CD3+ T-cell population, we then gate for our CD4+ and CD8+ subsets. The CD4+ and CD8+ T-cell subsets are then gated for their expression of the activation markers CD38 and HLA-DR and for their memory phenotype populations (CD27, CD45RO).

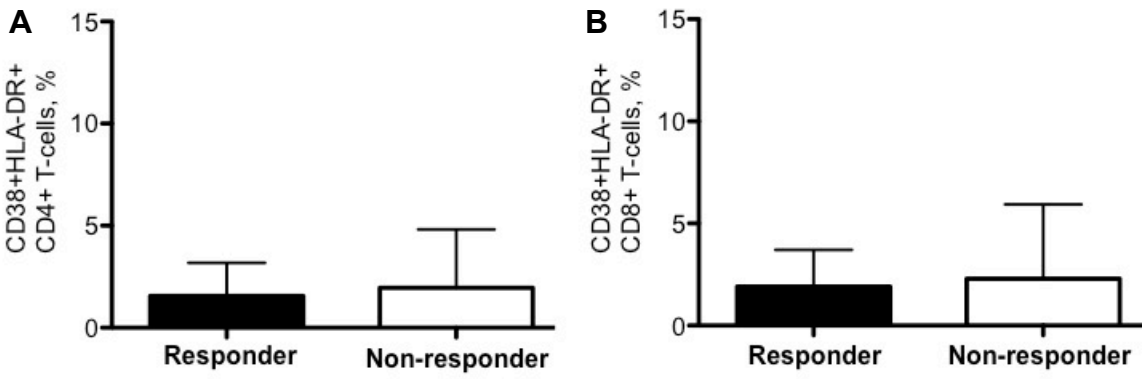


Figure 3.3. Mean percentages of activated (CD38+ HLA-DR+) **A)** CD4+ T-cells (1.56%±1.63% [n=27] vs. 1.95%±2.87% [n=19]; p=0.7) and **B)** CD8+ T-cells (1.91%±1.79% [n=27] vs. 2.3%±3.63% [n=19]; p=0.8) among Responders and Non-Responders.

iii. T-cell Differentiation Phenotypes

Research on HIV-1 infection has shown that chronically infected individuals exhibit an accumulation of terminally differentiated T-cells and have reduced regenerative potential[10, 14]. We wanted to examine whether the presence/lack of certain T-cell differentiation subsets could impact sero-protection to H1N1 in HIV-infected individuals. Using the gating strategy in **figure 3.2** we examined the expression of CD27 and CD45RO on the surface of CD4+ and CD8+ T-cells. We classified CD27+CD45RO- T-cells as naïve, CD27+CD45RO+ T-cells as central memory, CD27-CD45RO+ T-cells as effector memory, and CD27-CD45RO- T-cells as terminally differentiated effectors.

We found that Responders and Non-responders differed in their T-cell differentiation phenotype profiles. Specifically, Responders had a significantly higher baseline percentage of naïve (CD27+ CD45RO-) CD4+ (50.2%±23.1% [n=27] vs. 33.5%±23.1% [n=19]; p=0.02; **figure 3.4A**) and CD8+ T-cells (41.5%±17.9% [n=27] vs. 23.4%±17.4% [n=19]; p=0.001; **figure 3.4C**) as compared to the lower frequency observed in Non-responders. Additionally, we found that subjects classified as Non-responders had an increased baseline percentage of terminally differentiated (CD27-CD45RO-) CD4+ (3.2%±3.7% [n=27] vs. 11.3%±14.4% [n=19]; p=0.007; **figure 3.4B**) and CD8+ T-cells (26.1%±12.3% [n=27] vs. 40.2%±20.1% [n=19]; p=0.005; **figure 3.4D**) compared to a lower frequency observed in Responders. These findings suggest the possible importance of conserving the regenerative potential of T-cells in HIV-1 infected individuals.

When further examining potential predictors of response, which included viral load, baseline CD4+ T-cell counts, CD4+ T-cell nadirs, CD4+ and CD8+ naïve and terminally differentiated T-cells. We found that the CD4+ T-cell nadirs of the subjects were positively associated with the pre-immunization baseline CD4+ naïve (CD27+

CD45RO-) T-cells (n=46; R^2=0.141; p=0.01; **figure 3.5**). Thereby further illustrating the importance of preserving this T-cell subset.

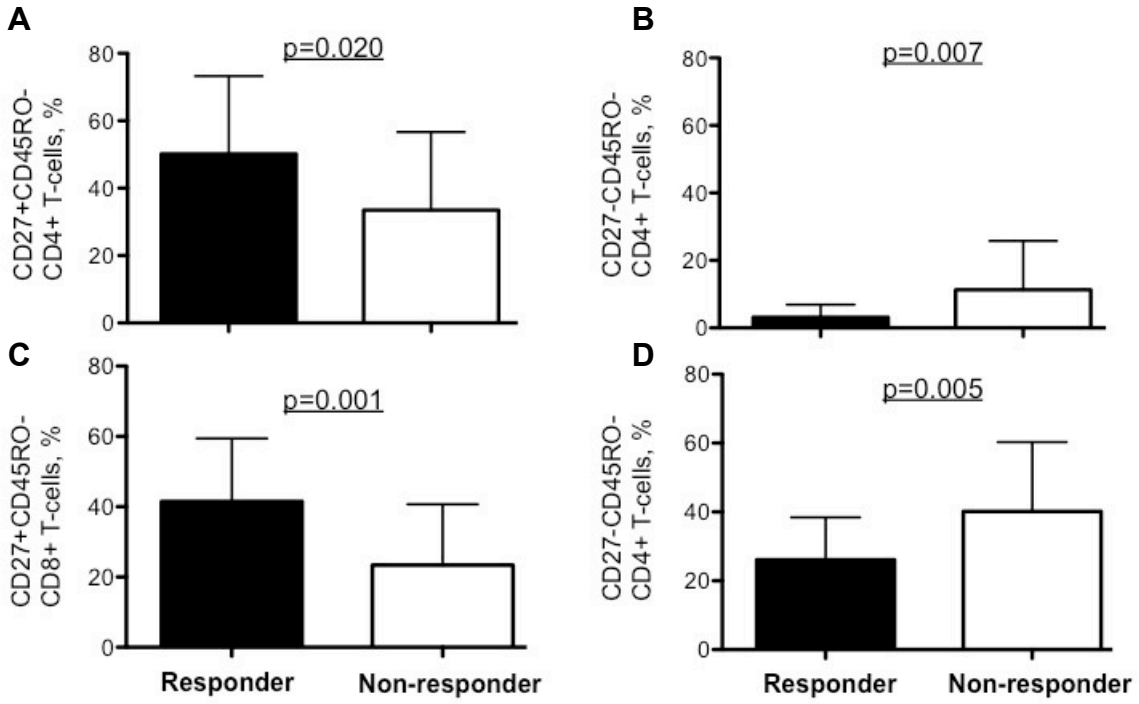


Figure 3.4. PBMCs were stained for markers of differentiation (CD27, CD45RO) and analyzed via flow cytometry. Mean baseline frequencies of **A)** naïve (CD27+ CD45RO-) CD4+ T-cells (50.2%±23.1% [n=27] vs. 33.5%±23.1% [n=19]; p=0.02), **B)** terminally differentiated (CD27-CD45RO-) CD4+ T-cells (3.2%±3.7% [n=27] vs. 11.3%±14.4% [n=19]; p=0.007), **C)** naïve (CD27+CD45RO-) CD8+ T-cells (41.5%±17.9% [n=27] vs. 23.4%±17.4% [n=19]; p=0.001), and **D)** terminally differentiated (CD27-CD45RO-) CD8+ T-cells (26.1%±12.3% [n=27] vs. 40.2%±20.1% [n=19]; p=0.005) among Responders and Non-Responders.

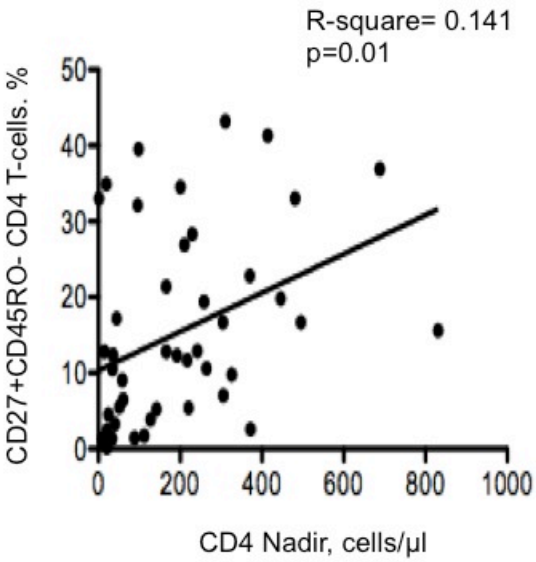


Figure 3.5. Multivariate regression analysis examining linear regression between the baseline frequency of naïve (CD27+ CD45RO-) CD4+ T-cells and the nadir CD4+ T-cell count (n=46; R^2=0.141; p=0.01).

iv. Cytokine and Chemokine Profiles

As discussed before, HIV-1 infected subjects exhibit an imbalanced cytokine and chemokine environment as compared to their healthy HIV-uninfected counterparts[206][205]. We thus examined the cytokine and chemokine profiles of the subjects in this study and compared them to the profiles of healthy HIV-negative subjects using a Luminex assay.

When comparing the cytokine/chemokine profiles of these subjects to that of healthy controls we found that the HIV-1 infected subjects had significantly lower serum levels of Th1 type cytokines that included IFN-γ (203.4±369.4 pg/ml [n=46] vs. 434.2±410.2 pg/ml [n=10]; p=0.0159), IFN-α2 (167.1±562.4 pg/ml [n=46] vs. 352.5±727.3 pg/ml [n=10]; p=0.0166), TNF-β (52.01±119.6 pg/ml [n=46] vs. 241.4±281.5 pg/ml [n=10]; p=0.0218), IL-10 (7.56±10.52 pg/ml [n=46] vs. 76.22±117.3 pg/ml [n=10]; p=0.0019); Th2 type cytokines: IL-5 (28.9±108.3 pg/ml [n=46] vs. 52.0±118.9 pg/ml [n=10]; p=0.0142); and others such as VEGF (760.8±874.2 pg/ml [n=46] vs. 1576±1267 pg/ml [n=10]; p=0.0197) and IL-1β (5.8±17.1 pg/ml [n=46] vs. 11.4±9.5 pg/ml [n=10]; p=0.0028). On the other hand, the HIV-1 infected subjects also demonstrated elevated serum levels of the chemokines MIP-1β (146.5±216.6 pg/ml [n=46] vs. 36.7±29.4 pg/ml [n=10]; p=0.036) and IP-10 (531.0±364.1 pg/ml [n=46] vs. 205.3±62.2 pg/ml [n=10]; p=0.0105), as well as elevated serum levels of IL-1α (1046±1451 pg/ml [n=46] vs. 23.1±23.1 pg/ml [n=10]; p=0.0009; **Table 3.2**).

Furthermore, in order to examine if the dysregulated cytokine/chemokine environment plays a potential role in sero-protection after H1N1 vaccination, we compared the serum cytokine and chemokine levels between Responders and Non-responders. We found that individuals who achieved sero-protection, otherwise known as Responders, had higher serum levels of IFN-α2 (272.6±741.4 pg/ml [n=27] vs.

30.03±57.0 pg/ml [n=19]; p=0.0493), IL-10 (10.7±11.9 pg/ml [n=27] vs. 2.3±3.9 pg/ml [n=19]; p=0.0009), and IL-6 (90.9±162.2 pg/ml [n=27] vs. 6.7±8.5 pg/ml [n=19]; p=0.0244) compared to lower serum levels observed in Non-responders (**Table 3.3**). These findings suggest that the pre-vaccination cytokine and chemokine environment could impact responsiveness to vaccination, and thus sero-protection.

Lastly, we examined whether pre-vaccination baseline serum cytokine/chemokine levels were related to factors that were associated with sero-protection above. Among the cytokine and chemokines that were altered in the HIV-1 infected participants in this study, we found a trending negative association with higher serum levels of IP-10 and the baseline percentage of naïve (CD27+ CD45RO-) CD8+ T-cells. Suggesting a role for this chemokine, IP-10, on the preservation of important CD8+ T-cell subsets (**figure 3.6**).

Table 3.2. Cytokine and Chemokine profiles of HIV-1 infected individuals in this study and of healthy HIV-negative controls measured using a Luminex assay.

	Immunological Marker	Healthy Control (conc. pg/ml)	HIV+ on ART (conc. pg/ml)	p-value
Primarily Th1	IL-12p40	210.5±351.1	102±144.6	0.3112
	IL-12p70	425.4±654.6	143±414.7	0.0878
	IFN-γ	434.2±410.2	203.4±369.4	**0.0159**
	TNF-α	10.96±7.172	22.28±38.15	0.4101
	TNF-β	241.4±281.5	52.01±119.6	**0.0218**
	IL-10	76.22±117.3	7.563±10.52	**0.0019**
Primarily Th2	IL-4	289.4±325.5	115.7±104.7	0.0771
	IL-5	52±118.9	28.88±108.3	**0.0142**
	IL-6	11.6±5.89	61.12±131.9	0.9731
	IL-13	278.5±295.2	176.8±298.4	0.1243
Growth Factors	IL-2	65.32±123.9	18.15±44.36	0.1238
	IL-3	14.17±27.21	25.73±61.87	0.6515
	IL-7	34.57±79.28	27.47±67.37	0.5852
	IL-15	44.25±94.63	7.310±5.342	0.1269
	G-CSF	53.17±58.06	205.7±490.7	0.1112
	GM-CSF	30.22±26.62	74.24±152.2	0.1069
	VEGF	1576±1267	760.8±874.2	**0.0197**
	EGF	153.4±76.65	232.6±229.0	0.6303
Chemokines	MIP-1α	38.15±24.18	253.5±705.6	0.2057
	MIP-1β	36.73±29.35	146.5±216.6	**0.036**
	IL-8	138.4±93.94	200±258.4	0.661
	MCP-1	534.5±126.4	593.3±246.7	0.4689
	Eotaxin	335.8±244.4	184.1±91.7	0.0642
	IP-10	205.3±62.2	531.0±364.1	**0.0105**
Other	IL-17A	234.1±207.4	10.3.2±159.8	0.0303
	IL-1Ra	104.2±85.84	367.3±1040	0.228
	IL-1α	23.09±23.05	1046±1451	**0.0009**
	IL-1β	11.41±9.47	5.79±17.05	**0.0028**
	IFN-α2	352.5±727.3	167.1±562.4	**0.0166**

Table 3.3. Cytokine and Chemokine profiles of study participants classified as Responders compared to those individuals classified as Non-Responders measured using a Luminex assay.

	Immunological Marker	Responder (conc. pg/ml)	Non-Responder (conc. pg/ml)	p-value
Primarily Th1	IL-12p40	119.2±159.8	39.06±27.71	0.4167
	IL-12p70	202.3±526.1	61.92±154.3	0.6127
	IFN-γ	257.9±440.5	121.6±210.9	0.3913
	TNF-α	29.8±48.67	11.59±3.572	0.6717
	TNF-β	70.46±146	11.4±14.77	0.0697
	IL-10	10.74±11.97	2.263±3.893	**0.0009**
Primarily Th2	IL-4	112.3±90.98	128.1±171.9	0.8935
	IL-5	48.09±139.2	1.579±2.534	0.0652
	IL-6	90.86±162.2	6.684±8.526	**0.0244**
	IL-13	220.2±350.5	82.7±100.3	0.5107
Growth Factors	IL-2	25±54.67	6.15±9.677	0.1326
	IL-3	31.98±68.54	0.735±0.3323	0.1904
	IL-7	29.44±70.23	24.68±14.88	0.6714
	IL-15	8.133±5.531	4.843±4.437	0.3316
	G-CSF	295.9±631.1	86.92±133.5	0.6102
	GM-CSF	83.14±154.8	60.02±152.1	0.6334
	VEGF	878.5±1054	593.4±506.5	0.828
	EGF	278.6±260.8	167.1±158.7	0.0579
Chemokines	MIP-1α	206.9±688.0	329.2±749.8	0.5514
	MIP-1β	191.4±262	82.75±104.2	0.0942
	IL-8	202.4±260.3	196.5±262.7	0.8235
	MCP-1	577.2±248.9	616.2±248.5	0.4892
	Eotaxin	193.3±99.53	171.1±80.05	0.4267
	IP-10	548.3±404.5	506.4±306.8	0.9112
Other	IL-17A	130.4±190.5	64.5±93.48	0.3842
	IL-1Ra	443.4±1088	265.9±993.3	0.2363
	IL-1α	925.6±1309	1529±2207	0.516
	IL-1β	7.953±21.65	2.906±7.362	0.2626
	IFN-α2	272.6±741.4	30.03±57	**0.0493**

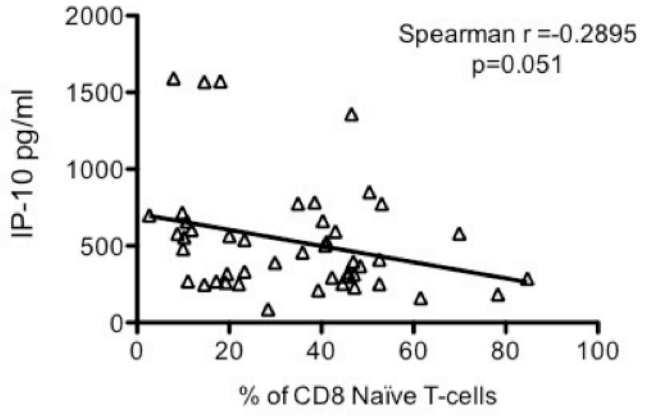


Figure 3.6. Linear regression examining the association between baseline pre-vaccination serum levels of IP-10 (pg/ml) and frequency of naïve (CD27+ CD45RO-) CD8+ T-cells (n=46; Spearman r= -0.2895; p=0.051).

v. Age

Knowing that age[189, 215] can impact the severity of influenza infection and responsiveness to vaccination, we further examined a possible role of age in sero-protection to H1N1 influenza. When examining the relationship between age and the predictors of response, age was negatively associated with the frequency of naïve (CD27+ CD45RO-) CD4+ T-cells (n=46; R^2=-0.182; p=0.003; **figure 3.7**). Even more, we also found that there was a trend towards higher serum IP-10 levels and older age groups (Group 1 [Age 22-34]: n=6, 363.2±180.5 pg/ml; Group 2 [Age: 35-44]: n=12, 420.5±388.0 pg/ml; Group 3 [Age: 45-54]: n=13, 481.4±195.7 pg/ml; Group 4 [Age: 55+]: n= 14, 590.0±293.7; p=0.089; **figure 3.8**). These findings suggest a potential confounding effect of age in the role of sero-protection in HIV-1 infected individuals against influenza vaccination.

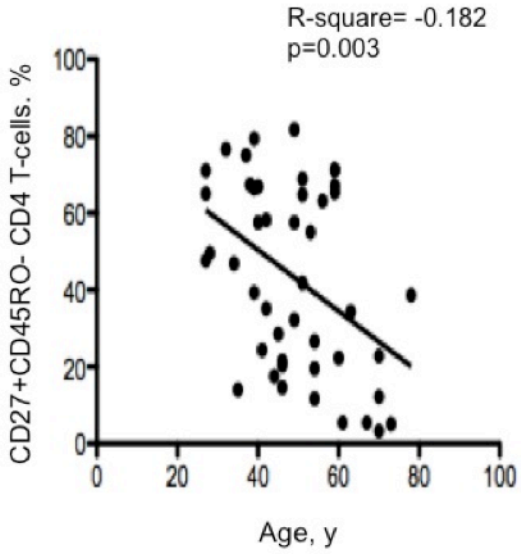


Figure 3.7. Multivariate regression analysis examining the linear regression between age of the study participants and baseline frequency of naïve (CD27+ CD45RO-) CD4+ T-cells (n=46; R^2= -0.182; p=0.003).

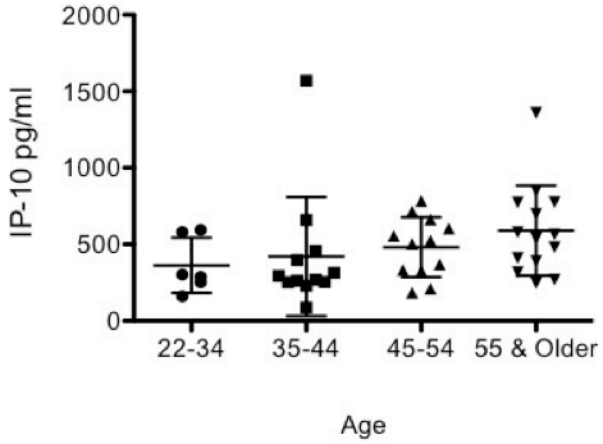


Figure 3.8. Serum IP-10 levels of study participants grouped into cohorts by age (Group 1 [Age 22-34]: n=6, 363.2±180.5 pg/ml; Group 2 [Age: 35-44]: n=12, 420.5±388.0 pg/ml; Group 3 [Age: 45-54]: n=13, 481.4±195.7 pg/ml; Group 4 [Age: 55+]: n= 14, 590.0±293.7; p=0.089). A one way ANOVA was used.

3.4 Discussion

The previous findings by Tebas *et al.*[203], demonstrate a recurring concern in HIV-1 infected individuals. Despite receipt of ART, it appears that not all individuals respond to the standard recommended dose of the H1N1 influenza vaccine. While the definition of Responder in this study is dependent on humoral responses, this study reinforces the importance of CD4+ T-cell help in responses to influenza. Along those lines, the findings show a relationship in the baseline frequency of naïve (direct association) and terminally differentiated (inversely associated) T-cells with sero-protection after immunization with the recommended dose of H1N1 vaccine. Importantly, the findings in this study demonstrate firstly the potential need to preserve the naïve T-cell populations in those infected with HIV-1 and raises the question about the timing for the initiation of antiretroviral therapy. We see here that the amount of immune reconstitution that occurs in those infected with HIV after ART initiation, can possibly still leave these individuals at risk for opportunistic infections if the immune reconstitution is not "sufficient" enough. As shown in this study, the pre-ART CD4+ T-cell nadir was associated with the frequency of naïve CD4+ T-cells post-ART, thereby suggesting the gravity of immune reconstitution in these individuals.

On the other hand, we had hypothesized that the level of T-cell activation would be associated with sero-protection, since levels of T-cell immune activation are associated with negative outcomes in regards to HIV-1 disease progression[9, 216]. However, we saw no significant association with the levels of baseline T-cell immune activation and sero-protection. When examining predictors of vaccine response using a logistic regression model, we found that the independent predictors of response were the baseline frequency of naïve CD4+ T-cells (n=46; p=0.024; R^2=0.111) and terminally

differentiated CD8+ T-cells (n=46; p=0.0004; R^2=0.251). While we saw no direct impact of T-cell activation on vaccine responses, it is still possible that levels of immune activation could be one potential factor contributing to the loss or accumulation of the T-cell populations, CD4+ naïve T-cells and CD8+ terminally differentiated cells, important for vaccine responses. In fact, it has been suggested that immune activation can contribute to the loss of an individual's naïve CD4+ T-cell population[216, 217, 218]. By the same token, loss of the CD4+ T-cell population could contribute to issues in the maintenance of the CD8+ T-cell population, which are important for control of HIV-1 infection[219]. While CD8+ T-cell responses have been shown to play roles in influenza infection[220], studies demonstrate the important roles for CD4+ T-helper cells in promoting B-cell responses and antibody production[39]. Early depletion studies demonstrated that alone CD4 or B-cells were not enough to clear certain influenza infections[220]. Thereby suggesting the need for not only humoral immunity, but also an important role for the help provided by CD4+ helper cells in the humoral response against influenza. As well, as is seen in studies of influenza in HIV-1 infected individuals influenza-specific humoral responses were shown to be associated with CD4 counts[186]. These findings along with the ones in this study reinforce the importance of conserving CD4+ T-cells in HIV-1 infected individuals in order to maintain humoral immunity, which is important in influenza infection. Furthermore, recent studies have demonstrated the important role that T-follicular helper cells play in influenza infection. These cells have been shown to be important in supporting IgG production and expansion of B-cells during the germinal center reaction[221]. In regards to HIV-1 infection, the presence of T-follicular helper cells are shown to help elicit humoral responses similar to healthy individuals responding to H1N1 infection, while, non-responders did not exhibit changes in the expansion of tfh cells. So in addition for the need to understand how to preserve

CD4+ T-cells, including tfh cells, the study in this chapter reinforces the need to also maintain the naïve CD4+ T-cell population, which can play an important role in *de novo* responses to influenza.

In addition as we observed in the study in chapter 4, we found that the cytokine and chemokine environment of the individuals infected with HIV-1 is dysregulated compared to healthy controls. These findings support that despite ART, HIV-1 infected individuals' cytokine levels do not return to normal[14]. The subjects in this study exhibit a reduced serum level of cytokines important in the antiviral response such as IFN-γ, which is important in cellular and humoral immune responses in the respiratory tract of those infected with influenza[222]. On the other hand, we also observed that the serum levels of the pro-inflammatory chemokine, IP-10 were high in these HIV-1 infected individuals. Previous studies have discussed that HIV-1 infected individuals have issues with H1N1 virus clearance related to elevated levels of IP-10[150]. In this study we found an association between higher serum levels of IP-10 and lower baseline frequency of naïve CD8+ T-cells. This association can be concerning, especially, since it has been demonstrated that the maintenance of a healthy T-cell population is important for T-cell mediated help that can aid in avoiding issues with influenza virus infection when antibodies do not provide sterilizing immunity[223].

In regards to sero-protection, the cytokines IFN-α2, IL-10, and IL-6 were found to be elevated in the subjects classified as Responders. Elevated IL-6 levels are shown to be important in the protection against H1N1 infection by promoting survival of innate immune cells[224]. Furthermore, in mice infected with influenza, blockade of the IL-10 receptor was associated with higher rate of death[225, 226]. Therefore, elevated levels of IL-10 in Responders could serve as a mechanism to help control excessive inflammation and increased lung injury observed during severe influenza infection. Additionally,

research by Sandler *et al.*[227] has suggested a role for IFN-α2 in regulating HIV acute infection, antiviral activity, and cell death early in infection as compared to late infection. Thus, low IFN-α2 levels in Non-responders could affect the ability of these individuals to control infection prior to ART initiation and in turn lead to more immune damage. Furthermore, IFN-α2 is shown to enhance B cell responses[263], so higher levels in Responders would suggest a role in enhancing influenza vaccine responsiveness. This damage could then leave these individuals with the inability to properly achieve sero-protection. Hence, examining potential cytokines and chemokines associated with sero-protection and influenza viral clearance can elucidate relevant targets to aid in avoiding more severe infection in those infected with HIV-1.

However, the cytokine and chemokine environment in HIV-1 infected individuals may not be the only confounding factor that could impact sero-protection against influenza infection. HIV-1 infected individuals are shown to exhibit premature immunosenescence and persistent ongoing inflammation characteristic of older HIV-negative individuals. This phenomenon occurs even with effective ART[10]. This premature aging of the immune system is associated with issues in CD4+ T-cell immune reconstitution and development of co-morbidities, such as cancer and cardiovascular disease[10, 228]. Elderly HIV-negative individuals have a reduced number of naïve T-cells[229] and as those infected with HIV-1 continue to survive longer, age will play a role in the ability of medical professionals to protect these individuals from opportunistic infections, i.e. influenza infection. And as has been shown, cell-mediated responses to influenza are important in helping humoral immunity to influenza infection[223]. As we observed in this study, age was negatively associated with the baseline frequency of naïve CD4+ T-cells, therefore elder HIV-1 infected individuals may maintain issues in achieving sero-protection after immunization. Even more, we saw a trending increase in the serum

levels of IP-10 with age, suggesting the possibility of increased issues with influenza viral clearance as HIV-1 infected individuals continue to live longer.

Finally, the CDC already offers immunization recommendations for elderly HIV-negative individuals[230]. Therefore, for HIV-1 infected subjects, the administration of these vaccines at an earlier age may be necessary in order to avoid non-responsiveness due to deterioration of their immune systems. Proactively, it may also be necessary to initiate ART earlier, thereby leading to better preservation of naïve cell subsets and lead to improved immune reconstitution. Additionally, response to vaccination in those infected with HIV-1 can potentially be used as a surrogate marker of immune reconstitution and successful treatment with ART.

Limitations of Study

This study elucidates potential correlates associated with better responsiveness to H1N1 vaccination. However, this study is limited in that it examines only the potential role that the T-cell mediated immunity may play in vaccine responsiveness. While T-cells are shown to also be important in the response against influenza, this study does not investigate possible correlates associated with the humoral arm of immunity that may be involved in better vaccine responses to influenza, especially in HIV-1 infected individuals. This study is also limited in that of the 120 subjects in the original study, only 46 of those subjects had sufficient samples for additional assays, thereby potentially affecting or underestimating additional possible correlates of vaccine responsiveness. Furthermore, while age is a possible counfounding factor in regards to influenza vaccination, our study may be limited in estimating the impact of age on responsiveness since the individuals in this study were younger than most studies examining age.

CHAPTER 4:

High IP-10 Levels Decrease T-cell Function in HIV-1 Infected Individuals on ART.

"Why a chemoattractant seemingly so potent as CXCL10 is elevated in patients who fail to clear HCV has been paradoxical."
–Edgar D. Charles (*J. Clin. Invest.* 2011; Rockefeller University)

4.1 Abstract

Even with effective antiretroviral therapy, it is observed that HIV-1 infected individuals have an imbalanced cytokine and chemokine environment. Changes in systemic cytokines and chemokines can alter immune responses of these individuals. One such chemokine, IP-10 is associated with the pathogenesis of several diseases in addition to HIV-1 infection. Specifically, we found elevated serum IP-10 levels in two cohorts of HIV-1 infected subjects on ART compared to healthy HIV-negative individuals. Using a series of *in vitro* studies we demonstrate that PBMCs exposed to elevated levels of IP-10 exhibit a significant decrease in the number of cells capable of secreting IFN-γ, as well as other cytokines, when stimulated with recall antigens. Furthermore, we found that elevated levels of IP-10 led to decreased calcium signaling and phosphorylation of the MAP Kinase p38. However, we show that production of IFN-γ and other cytokines, cytotoxic capacity, and proliferative capacity can be enhanced using a neutralizing antibody against IP-10. Additionally, our findings demonstrate a potential mechanism of action for elevated levels of IP-10. Specifically, elevated IP-10 levels may exert its effect through blocking of the CXCR3 receptor and demonstrates a role for the amino-peptidase CD26 in processing IP-10 to it's antagonistic for, thereby eliciting an impact on T-cell function. Our findings therefore suggest the need of IP-10 modulating agents for HIV-1 infected subjects on ART in order to enhance T-cell responses to vaccination and HIV-1.

Citation: **Ramirez, LA**, Arango, TA, Thompson, E, Naji, M, Tebas, P, Boyer, JD. High IP-10 Levels Decrease T-cell Function in HIV-1 Infected Individuals on ART. *J. Leukocyte Biol.* 2014

4.2 Introduction

With a lack of an effective vaccine or cure against HIV-1, research continues in the development of a vaccine or immune-therapy that can help boost immune responses in HIV-1 infected individuals. With HIV-1 infection, one of the many changes to the immune system of infected individuals is the obvious change in the production and secretion of cytokines and chemokines[231, 232]. However, these changes are not fully reversed with ART[101, 231, 233]. Findings in this study and others demonstrate specifically an increased level of the pro-inflammatory chemokine IP-10/CXCL10 in both untreated and ART treated HIV-1 infected individuals [133, 134]. While IP-10 is a double-edged sword of sorts depending on the disease, there is evidence that this chemokine's role in both untreated and treated HIV-1 infections is more injurious than helpful.

Aside from IP-10's role in the pathogenesis of several other diseases, its role in HIV-1 infection is not any more positive. During untreated HIV-1 infection, IP-10's interaction with HIV-1 proteins, such as Tat and Nef, show IP-10's role in promoting HIV-1 replication and neuronal cell death[134,140, 143]. Furthermore, IP-10 is associated with HIV-1 disease progression in both progressors and HIV-1 immune controllers[12, 137]. These associations, make sense, IP-10 is involved in the chemotaxis of activated immune cells, this could lead to potential recruitment of more activated lymphocytes and in turn more HIV-1 targets. In relation to ART, IP-10 has also been associated with immunological treatment failure[109]. Too, it is suggested that having lower mucosal IP-10 may be protective against acquisition of HIV-1 infection[138]. Hence, the findings and results presented in the study in this chapter demonstrate the importance of recognizing IP-10's impact on T-cell function in HIV-1 infected individuals, and in particular to this study, the effect of IP-10 specifically in ART treated individuals.

IP-10/CXCL10 is a pro-inflammatory chemokine and a member of the CXCR3 family of ligands, which include MIG/CXCL9 and I-TAC/CXCL11[9]. IP-10's main function is to recruit immune cells to sites of inflammation[126]. These signals have been shown to be dominant over TCR signals[127]. Thereby suggesting a potential role for IP-10 in the regulation of T-cell function. Additionally, recent studies of HCV infection demonstrate a conflicting role for IP-10. It would be expected that a chemokine involved in the recruitment of immune cells to the sites of infection would lead to better prognosis in treatment success for HCV, however, the opposite is shown[130]. Not only, is IP-10 upregulated during chronic HCV infection, it also serves as a negative predictor of response to HCV therapy[130]. Therefore, we hypothesized that elevated levels of IP-10 during treated HIV-1 infection could play a role in affecting normal T-cell function, specifically those functions, IFN-γ production, cytotoxic and proliferative capacity, associated with better control of HIV-1 infection[53]. Finally, the study in this chapter explores a potential mechanism of action through which IP-10 can impact T-cell function in HIV-1 infected individuals on ART.

4.3 Results

i. Study Design

Sera, PBMCs, and isolated CD4+ and CD8+ T-cells from healthy HIV-negative subjects, HIV-1 infected untreated subjects, and HIV-1 infected subjects on stable ART were obtained from the University of Pennsylvania's Human Immunology Core and Center for AIDS research. Subjects ranged in age from 20-55 years of age with and average age of 31 years. HIV-1 infected subjects on ART were well controlled with viral loads less than 50 copies/mL, average current CD4+ T-cell counts over 400 cells/μl and

CD4+ nadirs over 200 cells/µl. HIV-1 infected untreated subjects had a median viral load of 16,511 copies/mL.

Schematic of cell culture treatment is depicted in **figure 4.1**. PBMCs, and isolated CD4+ and CD8+ T-cells were cultured in media alone (RPMI 1640 with L-glutamine + 10% FBS and 1% streptomycin/penicillin) or media with one of the rhIP-10 doses (500, 10,000, or 100,000pg/ml) or Anti-IP-10 neutralizing antibody (1µg/ml) for 24 hours. These cells were then stimulated with viral antigens or anti-CD3 and used in the ELISpot, flow cytometry, Luminex, and Ca^{2+} flux assays. To determine if IP-10 treatment impacted cell viability we determined post IP-10 treatment viability and compared it to cell viability in media alone. Average cell viability post treatment with IP-10 was 95.1±3.3% and 89.2±3.2% for the healthy HIV-negative and HIV-infected on ART samples, respectively (**figure 4.2**).

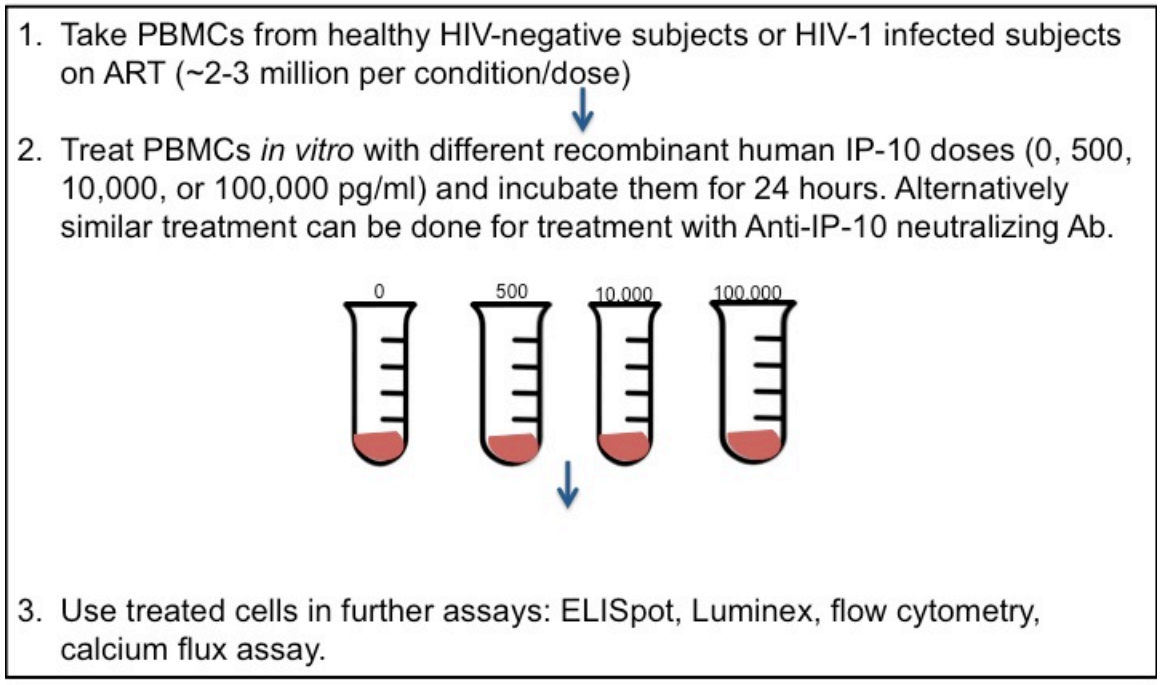
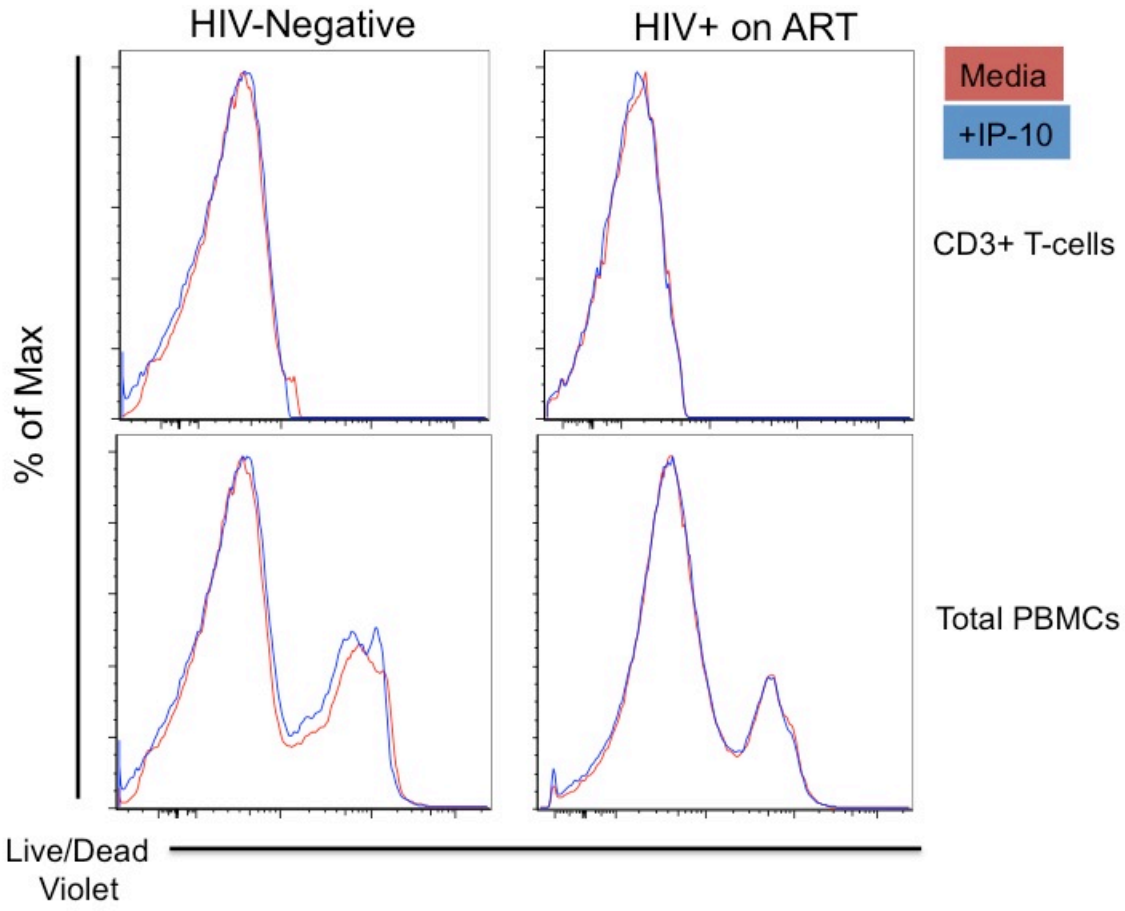


Figure 4.1. Diagram describing the *in vitro* treatment of immune cell cultures with recombinant human IP-10 or anti-IP-10 neutralizing antibody prior to use in immunological assays.



Pre-Treatment		Post-Treatment
93.8±3.9%	HIV-negative: Media	94.5±3.3%
	HIV-negative: +IP-10	95.1±3.3%
89.5±3.4%	HIV+ on ART: Media	89.3±3.3%
	HIV+ on ART: +IP-10	89.2±3.2%

Figure 4.2. **Top panel**: Example of CD3+ lymphocyte Live/Dead Violet mean fluorescence intensity in response to treatment with media alone or 500pg/ml of IP-10. **Middle Panel**: Example of total PBMC Live/Dead Violet mean fluorescent intensity in response to treatment with media alone or 500pg/ml of IP-10. Red line represents treatment with media alone, while the blue line represents treatment with IP-10. **Bottom Panel**: Diagram listing cell viability pre- and post-treatment with media alone or 500pg/ml in HIV-negative subjects and HIV-1 infected subjects on ART.

ii. HIV-1 infected subjects have high serum IP-10 levels

Using a multi-plex Luminex assay we examined the cytokine and chemokine profiles of HIV-1 infected subjects on stable ART and compared it to the profiles of healthy HIV-negative individuals. We found that the HIV-1 infected individuals on ART exhibited significantly lower levels of IL-10, IL-5, IL-13, IL-15, IL-1RA, and IL-1β as compared to healthy HIV-negative controls. While we see that the HIV-1 infected individuals have lower serum levels of Th2 cytokines, specifically, IL-5 and IL-13, we also see that these individuals have significantly lower levels of cytokines important in immune regulation, anti-inflammation, and maintenance and growth of cells, that is IL-10, IL-15, and IL-1 receptor antagonist. What stood out however, was that these individuals exhibited significantly higher serum levels of the pro-inflammatory chemokine IP-10 (**Table 4.1**) Furthermore, we found that untreated HIV-1 infected subjects had significantly higher levels of IP-10 compared to healthy uninfected controls. These results demonstrate that in general HIV-1 infected individuals have high serum IP-10 levels and that despite effective antiretroviral therapy serum levels of IP-10 remain elevated (**figure 4.3**).

Table 4.1. Luminex Assay-Cytokine and Chemokine profiles of the study participants compared to the profiles of healthy HIV-negative controls.

	Immunological Marker	Healthy Control (Conc. pg/ml)	HIV-1 on ART (Conc. pg/ml)	*=p<0.05
Primarily Th1	IL-12p40	54.12 ± 27.13	23.90 ± 31.29	0.073
	IL-12p70	31.88 ± 37.49	7.5 ± 13.90	0.383
	IFN-γ	22.26 ±10.76	9.49 ± 18.61	0.067
	TNF-α	10.96 ± 7.17	9.57 ± 5.63	0.524
	IL-10	10.53 ± 7.07	3.81 ± 2.31	***0.018**
Primarily Th2	IL-4	15.03 ± 20.79	3.99 ± 6.53	0.651
	IL-5	3.61 ± 4.10	1.74 ± 4.75	***0.009**
	IL-6	11.60 ± 5.89	9.81 ± 11.16	0.281
	IL-13	12.54 ± 11.67	7.96 ± 19.66	***0.017**
Growth Factors	IL-2	10.36 ± 12.71	4.23 ± 7.54	0.539
	IL-7	19.69 ± 13.16	12.42 ± 14.18	0.393
	IL-15	12.75 ± 7.02	1.47 ± 0.89	***0.0004**
	G-CSF	53.17 ± 58.06	27.29 ±16.25	0.354
	GM-CSF	30.22 ± 26.62	24.68 ±30.26	0.171
	VEGF	137.8 ± 108.7	124.0 ± 138.4	0.978
	TGF-α	5.46 ± 4.79	3.39 ± 2.09	0.657
	EGF	153.4 ± 76.65	165.2 ± 95.15	0.978
Chemokines	MIP-1α	12.92 ± 4.23	102.2 ± 316.6	0.889
	MIP-1β	36.73 ± 29.35	46.97 ± 33.07	0.279
	IL-8	8.82 ± 4.40	22.01 ± 31.95	0.305
	MCP-1	268.4 ±75.78	269.7 ± 119.8	0.718
	Fractalkine	200.4 ± 247.2	409.9 ± 1039	0.212
	Eotaxin	335.8 ± 244.4	353.4 ± 321.6	0.889
	IP-10	222.4 ± 62.22	364.0 ± 122.0	***00014**
Other	IL-17A	14.29 ± 9.48	9.95 ± 14.69	0.288
	IL-1Ra	104.2 ± 85.84	23.96 ± 41.87	***0.003**
	IL-1α	23.09 ± 23.05	11.97 ± 10.68	0.452
	IL-1β	11.41 ± 9.47	2.94 ± 6.33	***0.006**
	sCD40L	38334 ± 17648	40827 ± 9800	0.978

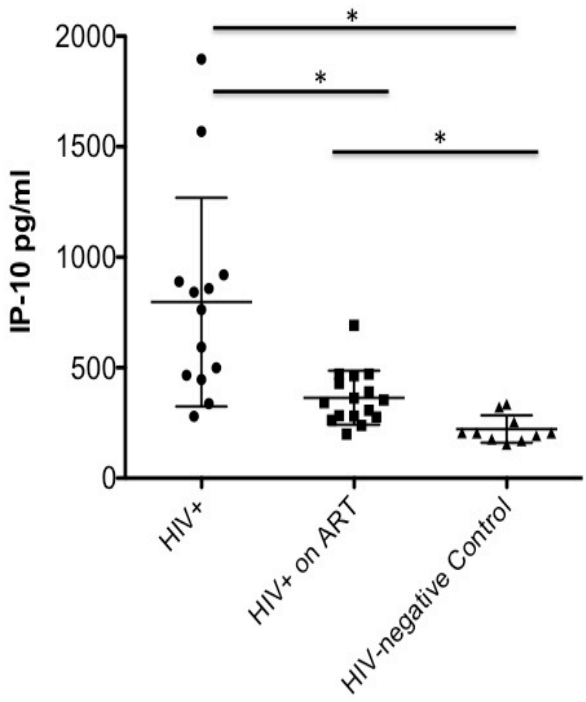


Figure 4.3. IP-10 serum levels isolated from HIV-1 sero-positive, HIV-1 sero-positive on ART, or sero-negative subjects were measured using a standard multi-plex Luminex assay. Graphs represent mean and standard deviations (*represents $p<0.05$; n=13, n=15 and n=10 respectively; $p<0.0001$; 796.8±472.4pg/ml vs. 342.2 pg/ml±88.17pg/ml vs. 222.4 pg/ml±62.22 pg/ml). A Kruskal-Wallis test followed by Dunn's multiple comparison test was used.

iii. Impact of high IP-10 levels on IFN-γ Production

Firstly, we wanted to investigate what effect high levels of IP-10 have on lymphocytes. So we examined the impact of exposure to high IP-10 levels (500, 10,000, 100,000 pg/mL) for 24 hours on PBMCs from healthy HIV-1 negative subjects followed by antigen stimulation with CEF (CMV, EBV, Flu) peptides or influenza proteins. Our findings show that exposure to high levels of IP-10 for 24 hours led to a significant decrease in IFN-γ production after antigen stimulation for both CEF and influenza antigens. Specifically, we observed using a standard ELISpot assay the number of cells capable of secreting IFN-γ after treatment with our observed physiological high IP-10 levels (500pg/ml) decreased from an average of 335.6 ± 336.1 to 70.6 ± 71.9 SFC/10^6 PBMCs for CEF (**figure 4.4A**; p=0.00057) and 977.1 ± 926.5 to 542.9 ± 690.2 SFC/10^6 PBMCs for influenza proteins (**figure 4.4B**; p=0.0115).

Observing the effect of high levels of IP-10 on IFN-γ production on PBMCs from healthy uninfected individuals then led us to examine the impact of high IP-10 levels on PBMCs from HIV-1 infected subjects on stable ART. We found that treatment with IP-10 (500pg/ml) for 24 hours resulted in a significant decrease in the ability of PBMCs from HIV-1 infected subjects on ART to produce IFN-γ in response to CEF peptides (**figure 4.4C**; 3088 ± 232.8 vs. 2329 ± 173.6 SFC/10^6 PBMCs; p=0.0027), influenza proteins (**figure 4.4D**; 211.3 ± 34.2 vs. 0.417 ± 0.83 SFC/10^6 PBMCs; p=0.0011), and HIV-1 consensus sequence subtype B gag peptides (**figure 4.4E**; 510.3 ± 110.1 vs. 145.4 ± 7.6 SFC/10^6 PBMCs; p=0.0071).

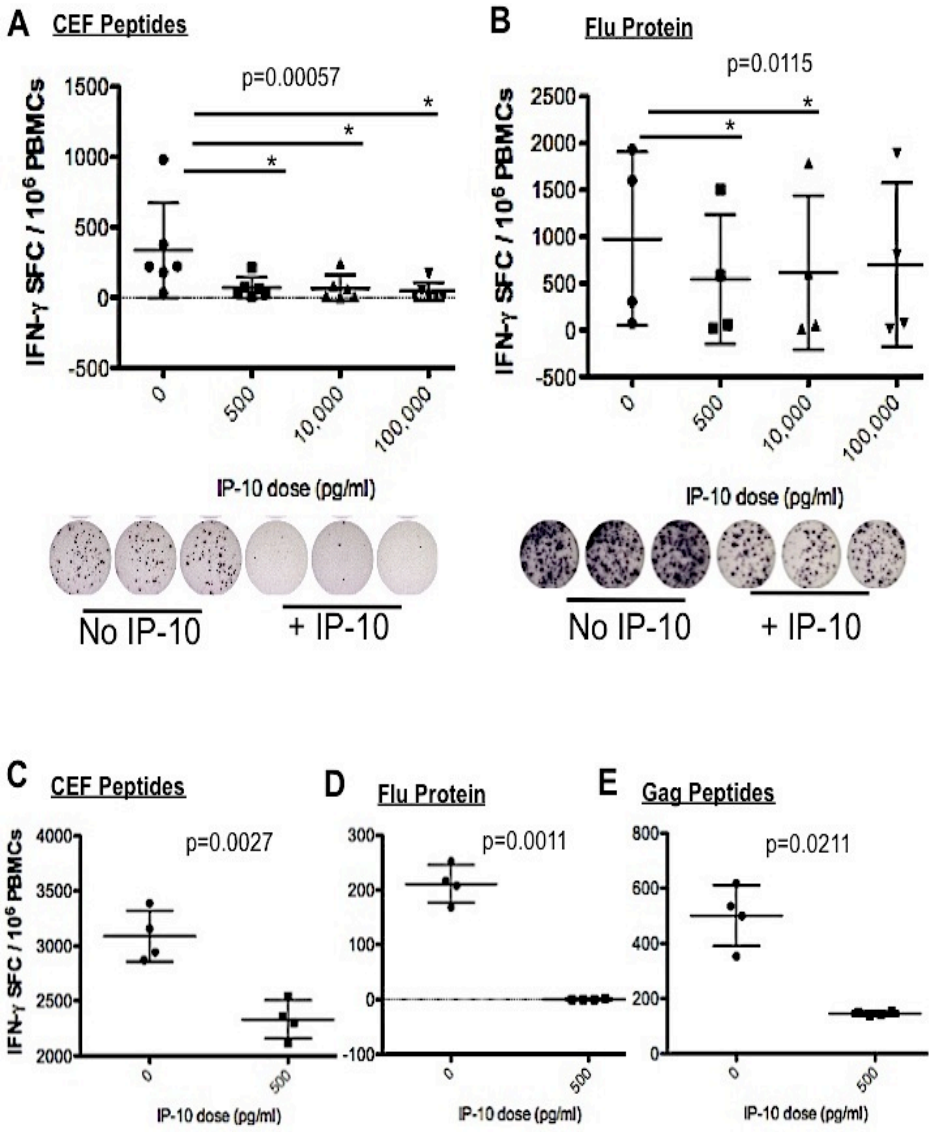


Figure 4.4. PBMCs from HIV-1 sero-negative individuals and HIV-1 infected subjects on ART were exposed to IP-10 for 24 hours. Subsequently, the cells were stimulated with viral antigens. The number of cells capable of secreting IFN-γ was decreased when stimulated with **A)** CEF peptides (n=6; p=0.00057) or **B)** Flu proteins (n=4; p=0.0115); * represents $p<0.05$. The number of cells capable of secreting IFN-γ was decreased in HIV-1 infected subjects on ART when stimulated with **C)** CEF peptides (n=4; p=0.0027); **D)** flu proteins (n=4; p=0.0011) and **E)** gag peptides (n=4; p=0.0211). Graphs represent average IFN-γ production and standard deviations. CEF: is a combination of CMV, EBV, Flu peptides.

iv. Impact of high IP-10 levels on T-cell function

We demonstrated that exposure to high levels of IP-10 could dampen the production of IFN-γ by PBMCs. We next wanted to examine whether high levels of IP-10 impacted the production of other cytokines and chemokines in HIV-1 infected subjects on ART using a Luminex assay. Specifically, we found after CD3 stimulation that the secretion of IL-10 (p=0.0177), GM-CSF (p=0.0012), TNF-α (p=0.0006), IFN-α2 (p=0.002), IL-12p70 (p=0.0009), and IL-13 (p=0.007) decreased significantly with IP-10 treatment (500pg/ml) for 24 hours (**figure 4.5**).

Additionally, Bett's *et al.*[49] demonstrated that HIV-1 non-progressors maintain highly functional CD8+ T-cells compared to progressors. And while the Luminex assay allows us to examine multiple secreted targets, flow cytometry allows us to examine what specific cells are producing those secreted targets. We therefore examined the ability of T-cells from HIV-1 infected subjects on ART to produce IL-2, MIP-1β, and TNF-α after treatment with high levels of IP-10 (500pg/mL) followed by CD3 stimulation. We found that treatment with IP-10 for 24 hours led to no significant impact on the expression of IL-2 by both CD4+ (**figure 4.6A,B**; p=0.813) and CD8+ T-cells (**figure 4.6C,D**; p=0.578). Similarly we found no impact of IP-10 on the expression of MIP-1β in CD4+ (**figure 4.6A,B**; p=0.0625) and CD8+ T-cells (**figure 4.6C,D**; p=0.5). However, we did observe that treatment with IP-10 affected the expression of TNF-α in CD4+ (**figure 4.6A,B**; p=0.0075) and CD8+ T-cells (**figure 4.6C,D**; p=0.0075). Of note, in regards to TNF-α, we see no impact of IP-10 when PBMCs are stimulated with gag peptides via Luminex (p=0.124) or flow cytometry (CD4: p=0.182, CD8: p=0.182), suggesting that IP-10 may have a stronger impact on certain immunological markers depending on the type of stimulation.

Even more, Migueles *et al.*[45] demonstrated the potential importance of proliferative capacity in HIV-1 non-progressors. We examined the effect of treatment with IP-10 for 24 hours on the ability of T-cells from HIV-1 infected subjects on ART to proliferate in response to antigen stimulation for 5 days using a standard CFSE assay. We found that treatment with IP-10 (500pg/ml) led to a trending decrease in the proliferative capacity of T-cells in response to stimulation with CEF peptides (**figure 4.7A,B**; p=0.0625), influenza proteins (**figure 4.7A,B**; p=0.0313), and gag peptide stimulation (**figure 4.7A,B**; p=0.0625).

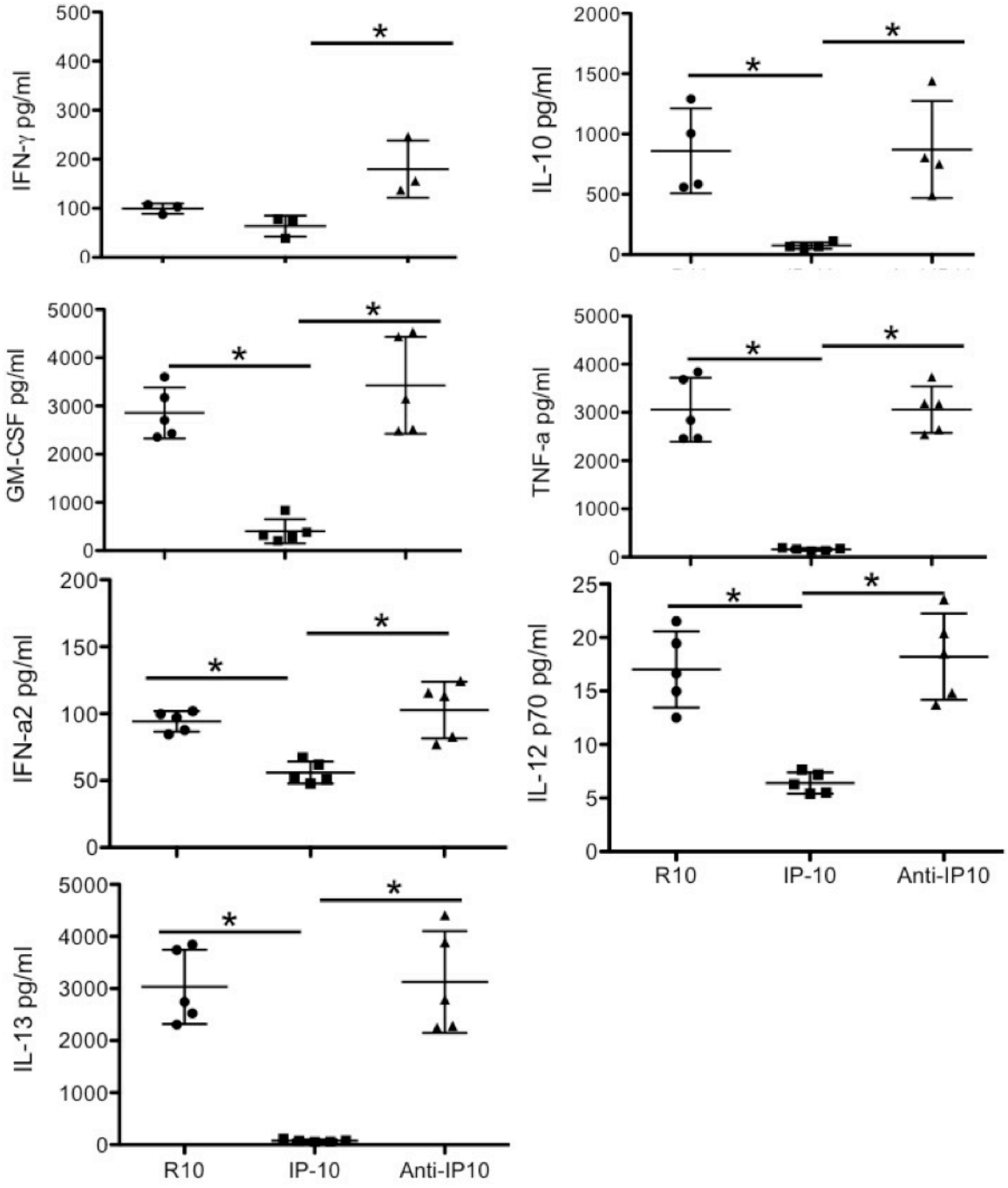
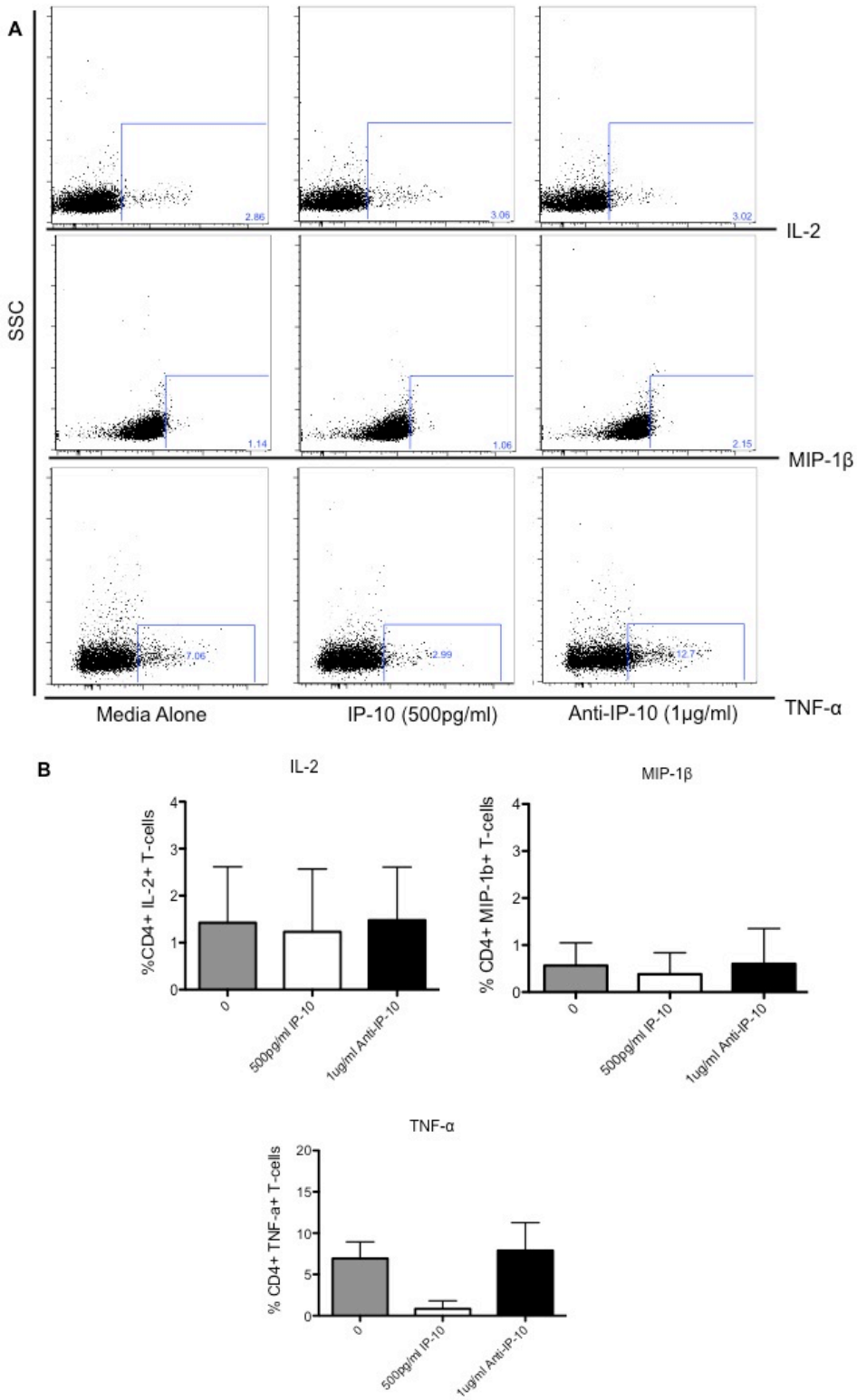


Figure 4.5. Secretion of cytokines and growth factors as measured by a standard Luminex assay. Graphs show the secreted levels of IFN-γ, IL-10, GM-CSF, TNF-α, IFN-α2, IL-12p70, and IL-13 in supernatants after treatment with media alone, IP-10 (500pg/ml), or Anti-IP-10 (1μg/ml) in response to 24 hour stimulation of PBMCs from HIV-1 infected subjects on ART with CD3/CD28/CD49d antibodies. Graphs depict mean and standard deviation. * represents $p<0.05$. A friedman test followed by Dunn's multiple comparison test was used.

Figure 4.6


A
SSC
IL-2
MIP-1β
TNF-α
Media Alone
IP-10 (500pg/ml)
Anti-IP-10 (1µg/ml)
2.86
3.06
3.02
1.14
1.06
2.15
7.06
12.99
12.7
B
IL-2
%CD4+ IL-2+ T-cells
MIP-1β
% CD4+ MIP-1b+ T-cells
TNF-α
% CD4+ TNF-a+ T-cells
0
500pg/ml IP-10
1ug/ml Anti-IP-10

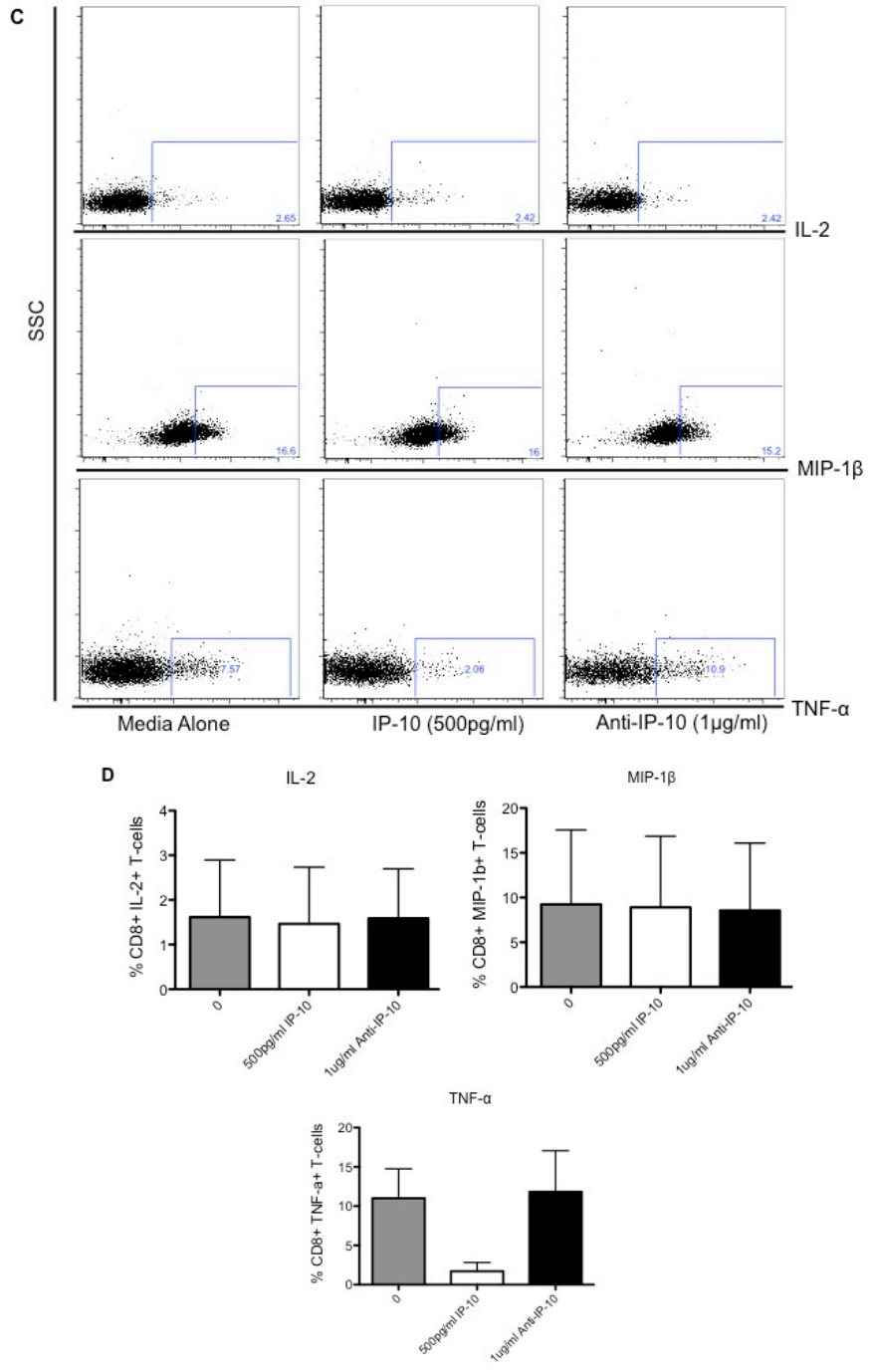


Figure 4.6. Expression of IL-2, MIP-1β and TNF-α after treatment with media alone, IP-10 (500pg/ml), or Anti-IP-10 (1µg/ml) in response to stimulation with CD3/CD28/CD49d antibodies in **A, B)** CD4+ T-cells and **C, D)** CD8+ T-cells from HIV-1 infected subjects on ART as measured by multi-parameter flow cytometry. Graphs in B and D represent mean and standard deviation. A Friedman test followed by Dunn's multiple comparison test was used.

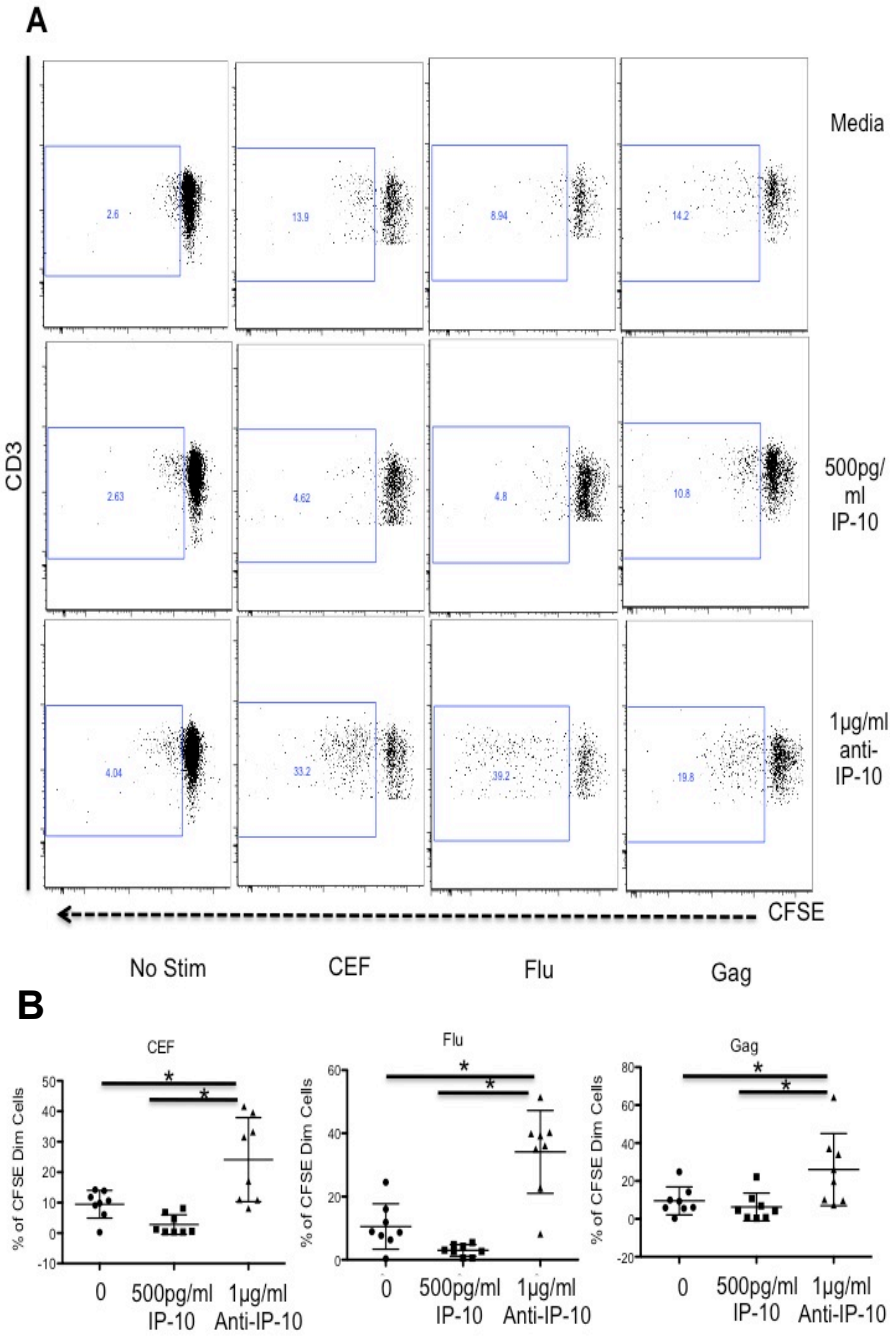


Figure 4.7. **A)** Example of PBMCs from an HIV-1 infected subject on ART treated with media alone, 500pg/ml of IP-10, or 1µg/ml of anti-IP-10 NAb for 24 hours. Following this, cells were incubated with CFSE (2.5µM) for 5 min at room temperature. Cells were washed and incubated with media alone, CEF peptides (0.03µg/ml), Flu proteins (Protein Sciences Corp.: A/Brisbane/59/07, 10ug/ml; A/Brisbane/10/07, 10ug/ml; B/Brisbane/60/08, 10ug/ml), gag peptides (2µg/ml) for 5 days at 37°C in 96-well plates. The mean fluorescence intensity of CFSE was used to determine T-cell proliferative responses of each of the treatment conditions (media alone, 1µg/ml anti-IP10) within each of the antigen stimulation conditions. **B)** Comparison of the percentage of cells that are CFSE dim in the treatment conditions, * represents $p<0.05$. Graphs represent mean and standard deviation.

v. High levels of IP-10 and Calcium Mobilization

Since the IP-10/CXCR3 signaling pathway is involved in calcium mobilization[9], we examined the impact of high levels of IP-10 on the calcium response using a flow-based calcium assay. We found that treatment with high levels of IP-10 (500, 100,000pg/mL) for 24 hours led to a blunting of the calcium response in PBMCs from healthy HIV-uninfected controls after stimulation with a standard CD3 agonist (**figure 4.8A,B**; p=0.0278). Additionally, we observed that the calcium response was dose dependent on the IP-10 dose, with no IP-10 treatment having the highest calcium response, followed by the 500pg/mL dose, and then by the 100,000pg/mL dose.

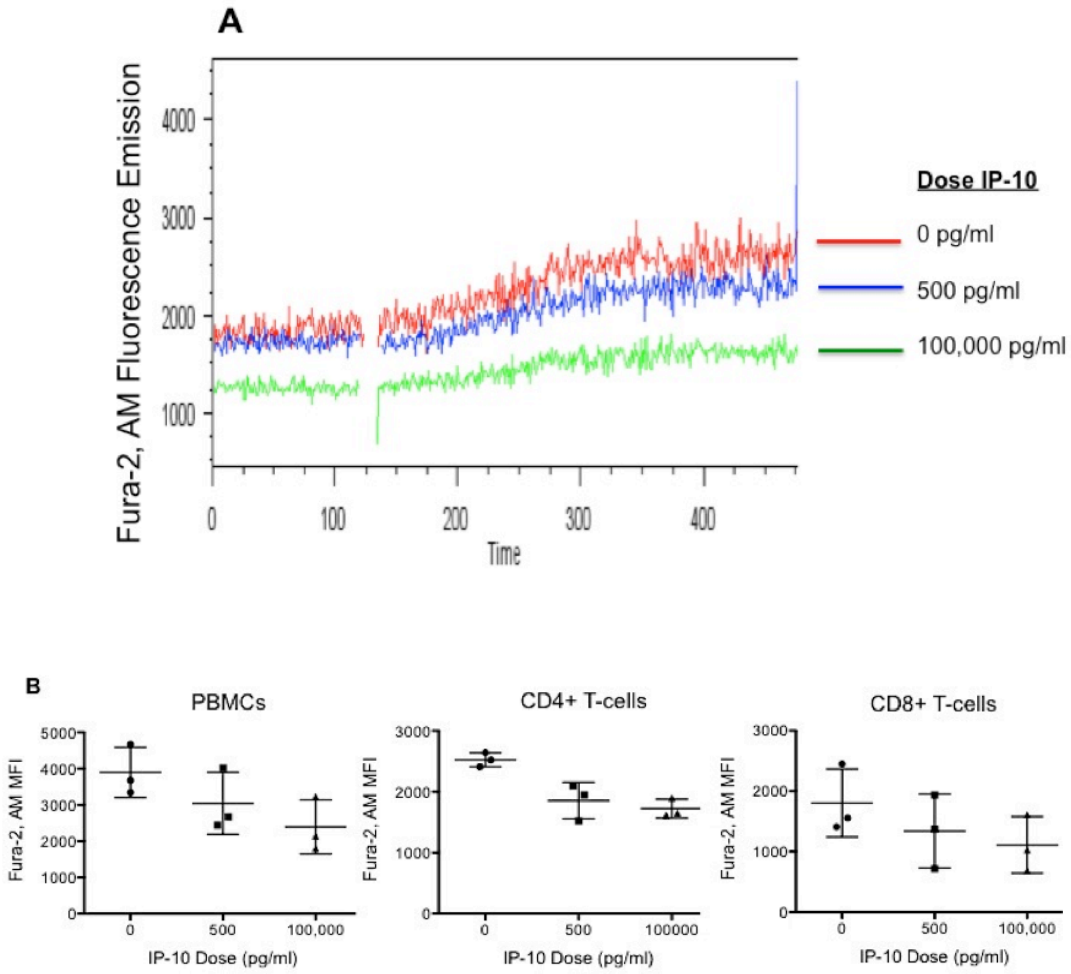
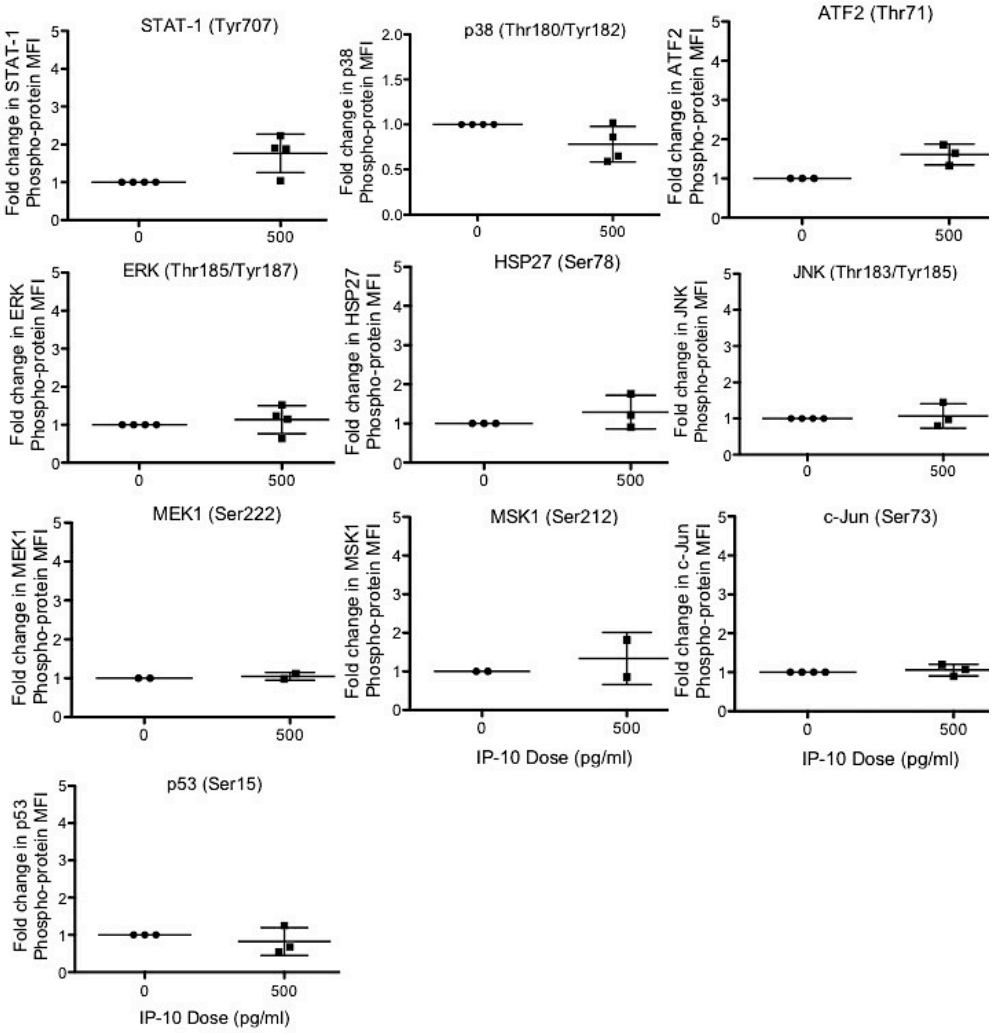


Figure 4.8. **A)** Example time-course Ca^{2+} responses elicited in healthy HIV-negative PBMCs treated with or without IP-10 (500pg/ml or 100,000pg/ml) for 24 hours followed with stimulation with a CD3 OKT3 agonist. Graph represents fluorescence emission of Fura-2, AM cell permeant over the course of 8 minutes. The first-top (Red) line represents no treatment with IP-10, the second (blue) line represents treatment with 0.5ng/ml of IP-10 for 24 hours, and the third (green) line represents treatment with 100ng/ml of IP-10 for 24 hours. **B)** Mean fluorescence intensity of Fura-2, AM in response to treatment with or without IP-10 (500pg/ml or 100,000pg/ml) in total PBMCs (n=3; p=0.0278), CD4+ T-cells alone (n=3; 0.0051, or CD8+ T-cells alone (n=3; p=0.0278). Graphs represent means and standard deviations.

vi. High levels of IP-10 and p38 MAP Kinase phosphorylation

We next wanted to determine whether other signaling pathways were affected by treatment with high levels of IP-10 (500pg/mL) for 24 hours followed by stimulation with CEF peptides or influenza proteins. We found that treatment with IP-10 led to a significant decrease in the expression of phosphorylated p38 (Thr180/Tyr182) MAP Kinase, a member of the IP-10/CXCR3 signaling pathway, after CEF peptide (**figure 4.9A,B**; p=0.021) and influenza protein stimulation (**figure 4.9A,B**; p=0.021). Secondly, we observed that IP-10 treatment followed by CEF peptide (**figure 4.9A,B**; p=0.021) or influenza protein (**figure 4.9A,B**; p=0.021) stimulation led to a significant increase in the expression of STAT-1 (Tyr707) phosphorylation. However, we saw no significant impact of IP-10 treatment on the phosphorylation of other phosphorylated proteins, specifically, ATF2, ERK, HSP27, JNK, MEK1, MSK1, c-Jun, and p53.

A CEF Stimulated



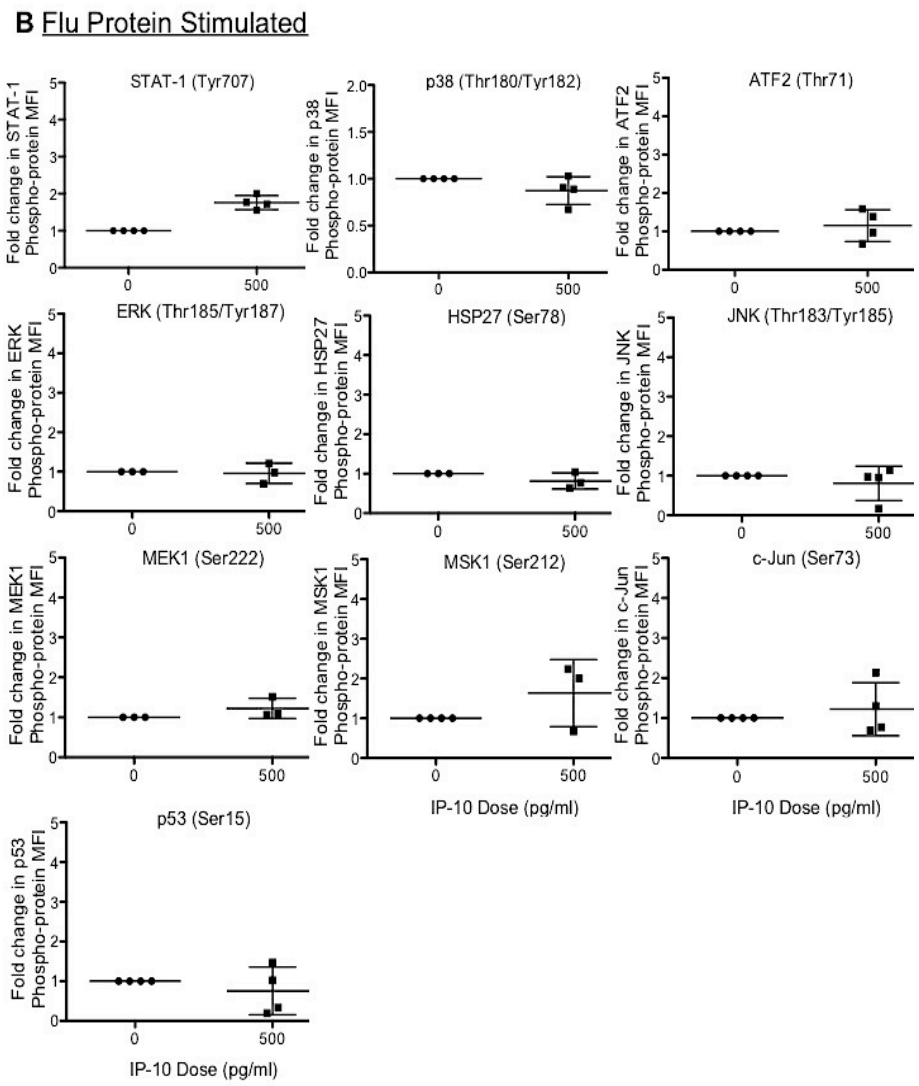


Figure 4.9. Phosphorylated protein fold change in expression due to 24 hour treatment with IP-10 (500pg/ml) followed by CEF or flu protein stimulation respectively. **A)** CEF stimulated phospho-protein expression: STAT-1 (Tyr707; p=0.021), ATF2 (Thr71; p=0.500), ERK (Thr185/Tyr187; p=0.875), HSP27 (Ser78; p=0.250), JNK (Thr183/Tyr185; p=0.625); MEK1 (Ser222; p=0.250), MSK1 (Ser212; p=0.500), c-Jun (Ser73; p=0.375), p53 (Ser15; p=0.875), and p38 (Thr180/Tyr182; p=0.021). **B)** Flu protein stimulated phosphor-protein expression: STAT-1 (Tyr707; p=0.021), ATF2 (Thr71; p=0.250), ERK (Thr185/Tyr187; p=0.581), HSP27 (Ser78; p=0.625), JNK (Thr183/Tyr185; p=1.00); MEK1 (Ser222; p=0.500), MSK1 (Ser212; p=0.750), c-Jun (Ser73; p=0.875), p53 (Ser15; p=0.875), and p38 (Thr180/Tyr182; p=0.021). Expression levels were determined using a multi-plex Luminex assay. Graphs represent means and standard deviations (n=4).

vii. Enhancing T-cell responses with an anti-IP-10 Neutralizing Antibody.

We next wanted to determine whether we could enhance T-cell responses in HIV-1 infected subjects on ART by blocking IP-10 using a neutralizing antibody against IP-10. We observed that after antigen stimulation, the secretion of IP-10 increases (**figure 4.10**; p=0.0043) in addition to the already present levels of IP-10, so examining the impact of blocking IP-10 was of interest. Therefore, we examined the effect of neutralizing IP-10 on the ability of lymphocytes to produce IFN-γ, proliferate in response to antigen stimulation, and degranulate and produce cytotoxins.

When blocking IP-10 using the neutralizing antibody we observed a significant increase in the ability of PBMCs to produce IFN-γ in response to gag peptide stimulation (**figure 4.11**; p=0.0248). We specifically saw an increase in the production of IFN-γ from an average of 735.8±1114 to 1268±1607 SFC/10^6 PBMCs. In addition to IFN-γ, when we examined by flow cytometry the impact of blocking IP-10 with the neutralizing antibody, we found a significant increase in the expression of TNF-α in CD4+ (**figure 4.6A,B**; p=0.0197) and CD8+ T-cells (**figure 4.6C,D**; p=0.0177) after CD3 stimulation compared to IP-10 treatment, while we saw no changes in the expression of IL-2 and MIP-1β by CD4+ (**figure 4.6A,B**; IL-2: p=0.954; MIP-1β: p=0.367) and CD8+ T-cells (**figure 4.6C,D**; IL-2: p=0.522; MIP-1β: p=0.338). Furthermore, when we examined by Luminex the impact of blocking IP-10 on the secretion of other immunological markers, we found that using the neutralizing IP-10 antibody led to increase in the secretion of IFN-γ (p=0.0278), IL-10 (p=0.0286), GM-CSF (p=0.0043), TNF-α (p=0.0002), IFN-α2 (p=0.005), IL-12p70 (p=0.001), and IL-13 (p=0.0023) after CD3 stimulation by PBMCs compared to treatment with IP-10 (**figure 4.5**).

Also, we examined the effect of blocking IP-10 on functions suggested to be associated with the control of HIV-1 infection, that is the ability for CD8+ T-cells to

degranulate and produce cytotoxins, as well as proliferative capacity. Blocking IP-10 led to an increased ability for CD8+ T-cells from HIV-1 infected subjects on ART to degranulate (express CD107a) and produce perforin and granzyme B in response to gag peptide stimulation (**figure 4.12**; p=0.0009).

Similarly, when we examined the impact of using a neutralizing antibody against IP-10, we found a significant increase in the ability of lymphocytes to proliferate in response to stimulation with CEF peptides (p=0.0078), Flu protein (p=0.0078), and gag peptide (p=0.0234) stimulation (**figure 4.7A,B**).

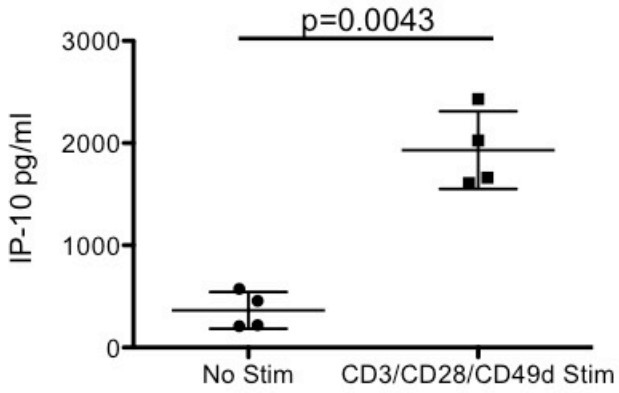


Figure 4.10. Secretion of IP-10 in supernatants after 24 hour stimulation of PBMCs from HIV-1 infected subjects on ART with CD3/CD28/CD49d antibodies. Graph represents mean and standard deviation (n=4; p=0.0043).

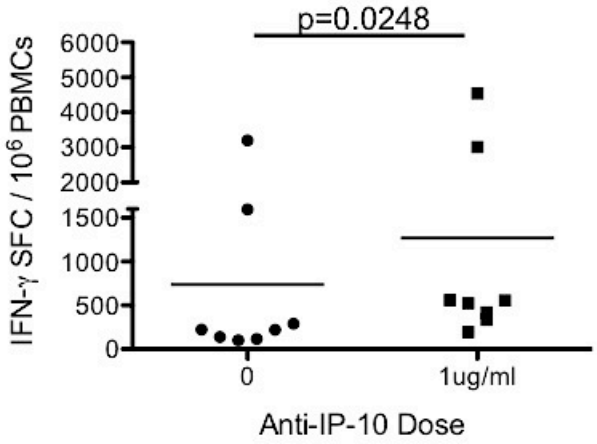


Figure 4.11. PBMCs isolated from HIV-1 infected individuals on ART were stimulated with HIV-1 gag peptides in the presence of anti-IP-10 neutralizing mAb (1µg/ml). Increases were observed in the number of antigen specific cells capable of producing IFN-γ (n=8; p=0.0248). Graph represents mean.

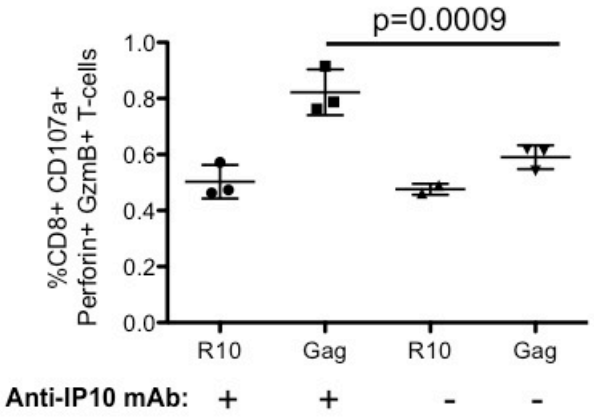


Figure 4.12. PBMCs isolated from HIV-1 sera-positive individuals on ART were stimulated with HIV-1 gag in the presence of anti-IP-10 neutralizing mAb. Increases were observed in the percentage of cells capable of killing or CD8+ CD107a+ Perforin+ Granzyme B+ cells (n=3; p=0.0009). Graphs represent mean and standard deviations. A Friedman test followed by Dunn's multiple comparison test was used.

viii. HIV-1 infected individuals and high CD26 levels/expression

In order to better understand why IP-10 may be affecting T-cell functionality we sought to determine whether processing by CD26 could play a role in IP-10's observed effects. HIV-1 infected subjects on ART had significantly higher serum levels of soluble CD26 as compared to healthy HIV-uninfected subjects (**figure 4.13**; p=0.0084). Furthermore, when examining whether IP-10 treatment (500, 100,000pg/ml) had an impact on the cell surface expression of CD26, treatment with IP-10 led to an increase in the expression of CD26 on CD4+ and CD8+ T-cells from healthy HIV-uninfected subjects (**figure 4.14**; CD4: p=0.012; CD8: p=0.0495) and HIV-1 infected subjects on ART (**figure 4.14**; CD4: p=0.0081; CD8: p=0.0081).

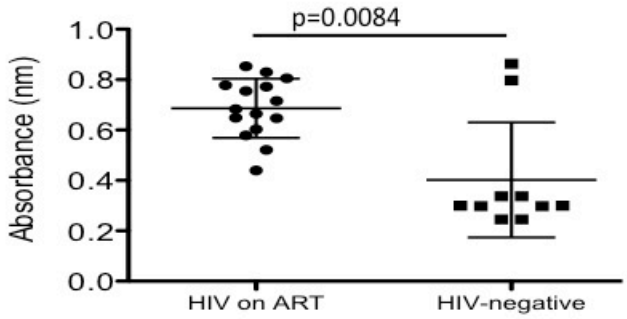


Figure 4.13. Absorbance (nm) values via standard ELISA assay measuring serum soluble levels of the amino-peptidase CD26 (DPPIV) in HIV-1 infected subjects on ART (n=15) and healthy HIV-negative subjects (n=10). Graph represents mean and standard deviation.

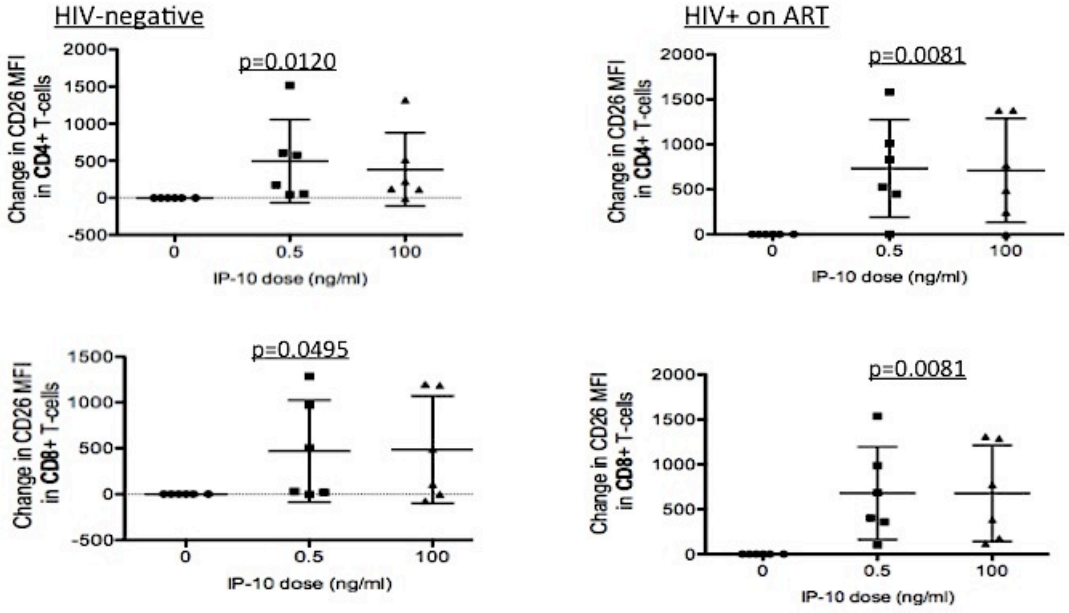


Figure 4.14. Surface expression of CD26 (DPPIV) on CD4+ and CD8+ T-cells from HIV-uninfected subjects (n=6) and HIV-1 infected subjects on ART (n=6) after 24 hour treatment with recombinant human IP-10 (0, 500, or 100,000pg/ml). Graphs represent average change in mean fluorescence intensity and standard deviation. A friedman test was used.

ix. Mechanism of Action

Finally, we wanted to determine whether IP-10 was directly exerting its effects on T-cells or indirectly, such as through antigen presenting cells. Using flow cytometry we found that IP-10 not only impacted the production of IFN-γ by total PBMC CD4+ ($p=0.0286$) and CD8+ T-cells ($p=0.0286$), but also found that there was reduced IFN-γ production when treating isolated CD4+ ($p=0.0026$) and CD8+ (0.0019) T-cells with IP-10 (500pg/ml) followed by CD3 stimulation (**figure 4.15**). Similarly, when examining the calcium response of isolated T-cell subsets, treatment with IP-10 (500, 100,000pg/ml) led to a significant decrease in the calcium response in isolated CD4+ ($p=0.0051$) and CD8+ ($p=0.0278$) T-cells (**figure 4.8B**).

Knowing IP-10 is involved in calcium mobilization through signaling of its receptor, CXCR3, we sought to investigate whether IP-10 was exerting its dampening effects by blocking CXCR3 signaling. When using an antagonist against CXCR3 there was a significant decrease in the production of IFN-γ in response to CEF peptide ($p=0.038$) and influenza protein ($p=0.0212$) stimulation (**figure 4.16**). Even more, as hypothesized, using a CXCR3 antagonist led to a decrease in the calcium response (**figure 4.16**; $p=0.0174$).

Thirdly, we examined whether IP-10 could affect the expression of MHC Class I and II molecules and thereby potentially disrupt normal TCR signaling. We found that treatment with IP-10 (500pg/ml) for 24 hours led to no significant change in the expression of HLA Class IA,B,C and HLA-DR on total CD3+ T-cells (HLA-Class I: $p=0.875$; HLA-DR: $p=0.125$), CD4+ (HLA-Class I: $p=0.875$; HLA-DR: $p=0.875$), and CD8+ T-cells (HLA-Class I: $p=1.0$; HLA-DR: $p=0.375$), and CD3-CD68+CD33+ macrophage (HLA-Class I: $p=0.625$; HLA-DR: $p=0.625$) subsets (**figure 4.17**). Likewise,

it is shown that expression of inhibitory receptors, such as programmed death-1 (PD-1), is associated with blunted T-cell function in those infected with HIV-1[234]. However, we found no change in the expression of PD-1 on CD4+ (p=0.367) and CD8+ (p=0.553) T-cells after treatment with IP-10. Additionally, we saw no effect of blocking IP-10 on the expression of PD-1 (**figure 4.18**). Thereby suggesting other mechanisms of action for IP-10.

Therefore, we hypothesized that IP-10 may be processed by CD26 to its short form and in turn function as an antagonist. So we examined whether using a CD26 inhibitor would lead to improvement in T-cell function in HIV-1 infected subjects on ART. Using a CD26 inhibitor led to an increase in IFN-γ production in response to CEF peptide (p=0.0146), influenza protein (p=0.0387), and gag peptide (p=0.0392) stimulation (**figure 4.19A**). Furthermore, we saw an increase in the calcium response (**figure 4.19B**; p=0.0196). Finally, when we examined the levels of the IP-10 isorforms, HIV-1 infected subjects on ART had Total IP-10 levels at 1062±637.0 pg/ml, IP-10 Long levels at 1330±22.4pg/ml, IP-10 short form levels at 464.8±48.8 pg/ml (**figure 4.20**). When we examined the levels of IP-10 short form relative to Total IP-10 levels we saw that the levels were highest in untreated HIV-1 infected individuals (p=0.002), followed by HIV-1 infected subjects on ART (p=0.064), and then by HIV-negative individuals (**figure 4.20**).

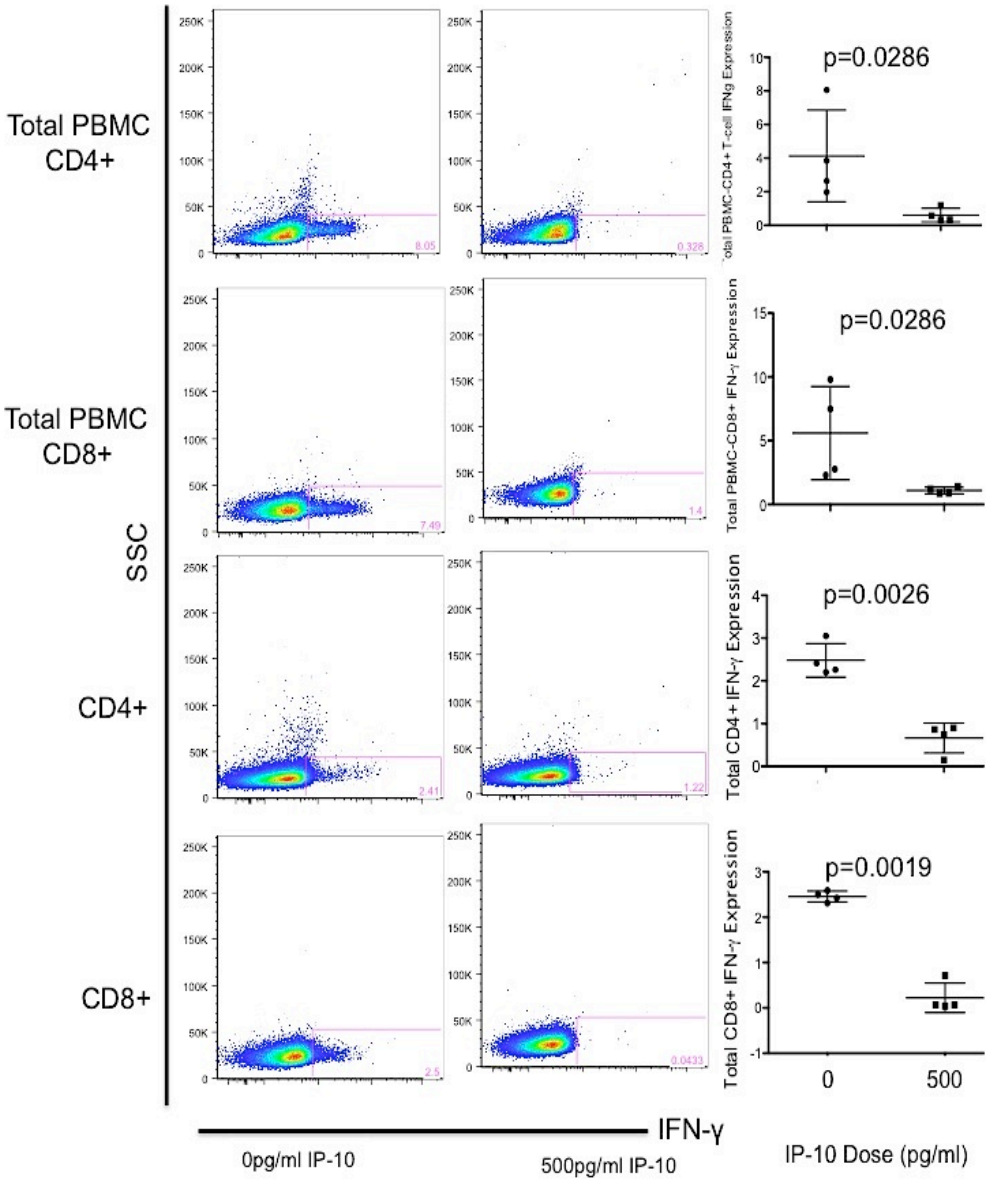


Figure 4.15. PBMCs, CD4+ and CD8+ were obtained from HIV-1 sero-negative individuals. Cells were treated with 500pg/ml IP-10 or not (media alone). Cells were then stimulated with anti-CD3 and costimulatory CD28 and CD49d antibodies and assessed for IFN-**γ** production. Data is presented for Gating strategy and IFN-γ expression in Total PBMC-CD4+ T-cells (n=4, p=0.0286); Total PBMC-CD8+ T-cells (n=4; p=0.0286); isolated CD4+ T-cells (n=4; p=0.0026); and isolated CD8+ T-cells (n=4; p=0.0019) as measured by multi-parameter flow cytometry. Graphs represent means and standard deviations.

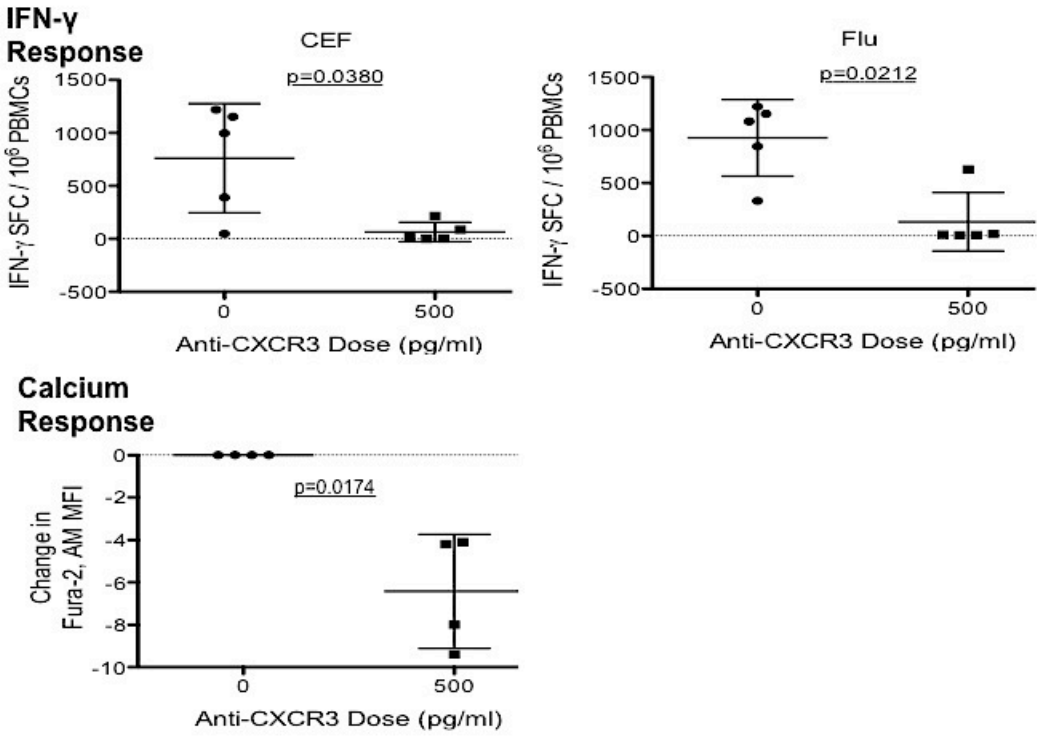
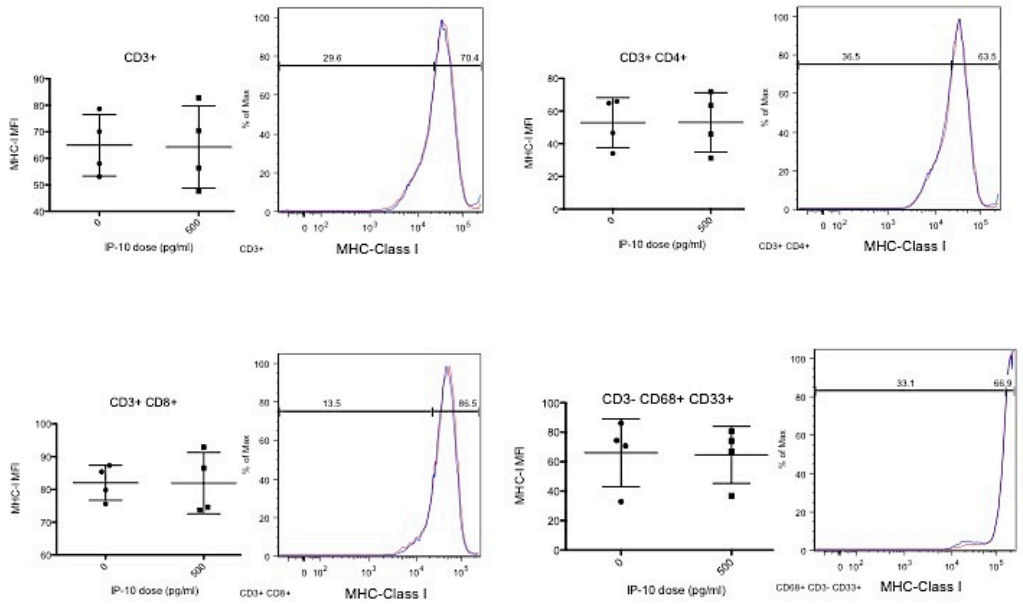


Figure 4.16. PBMCs from healthy HIV-negative subjects were treated with 500pg/ml of a CXCR3 antagonist for 24 hours. This treatment led to a decrease in IFN-γ production in response to CEF (n=5; p=0.0380) and flu protein (n=5; p=0.0212) antigen stimulation and the calcium response (n=4; p=0.0174). Graphs represent mean and standard deviation.

HLA-Class I (A, B, C)



HLA-DR

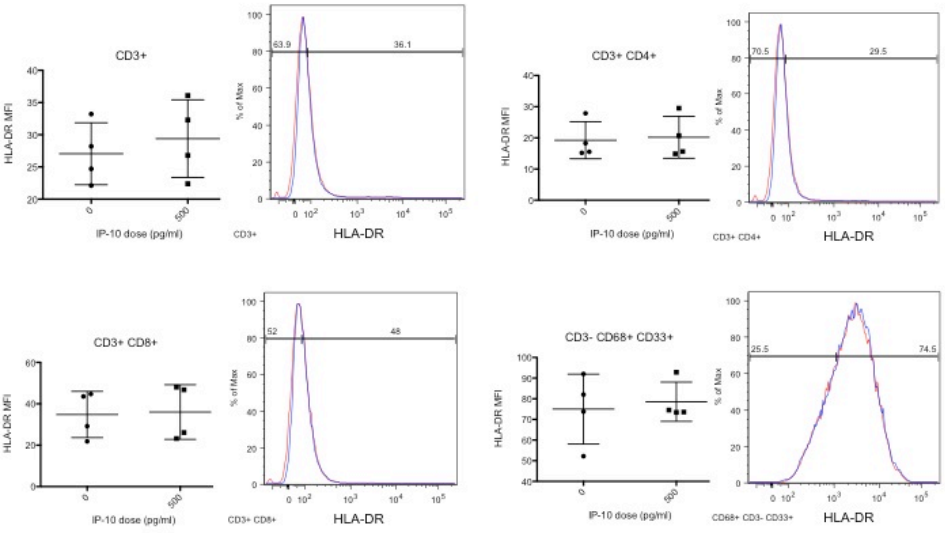


Figure 4.17. HLA-Class I (HLA-A, -B,-C) mean fluorescence intensity in media alone and IP-10 (500pg/ml) treated PBMCs. CD3+ T-cells (p=0.875); CD3+CD4+ T-cells (p=0.875); CD3+CD8+ T-cells (p=1.0); CD3-CD68+CD33+ Macrophages (0.625). HLA-DR mean fluorescence intensity in media alone and IP-10 (500pg/ml) treated PBMCs. CD3+ T-cells (p=0.125); CD3+CD4+ T-cells (p=0.875); CD3+CD8+ T-cells (p=0.375); CD3-CD68+CD33+ Macrophages (0.625). Graphs represent mean and standard deviation.

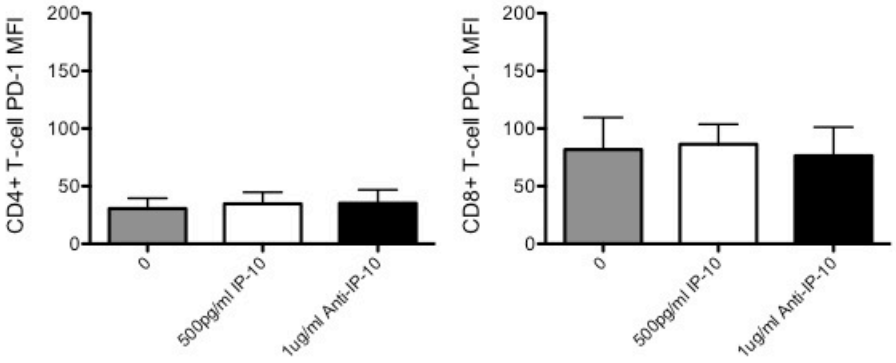


Figure 4.18. PD-1 expression on CD4+ (p=0.367) and CD8+ (p=0.553) T-cells from HIV-1 infected subjects on ART after 24 hour treatment with media alone, IP-10 (500pg/ml), or Anti-IP-10 (1µg/ml) followed by stimulation with CD3 and CD28/CD49d co-stimulatory antibodies. Graphs represent mean fluorescence intensity and standard deviation. A Friedman test followed by Dunn's multiple comparison test was used.

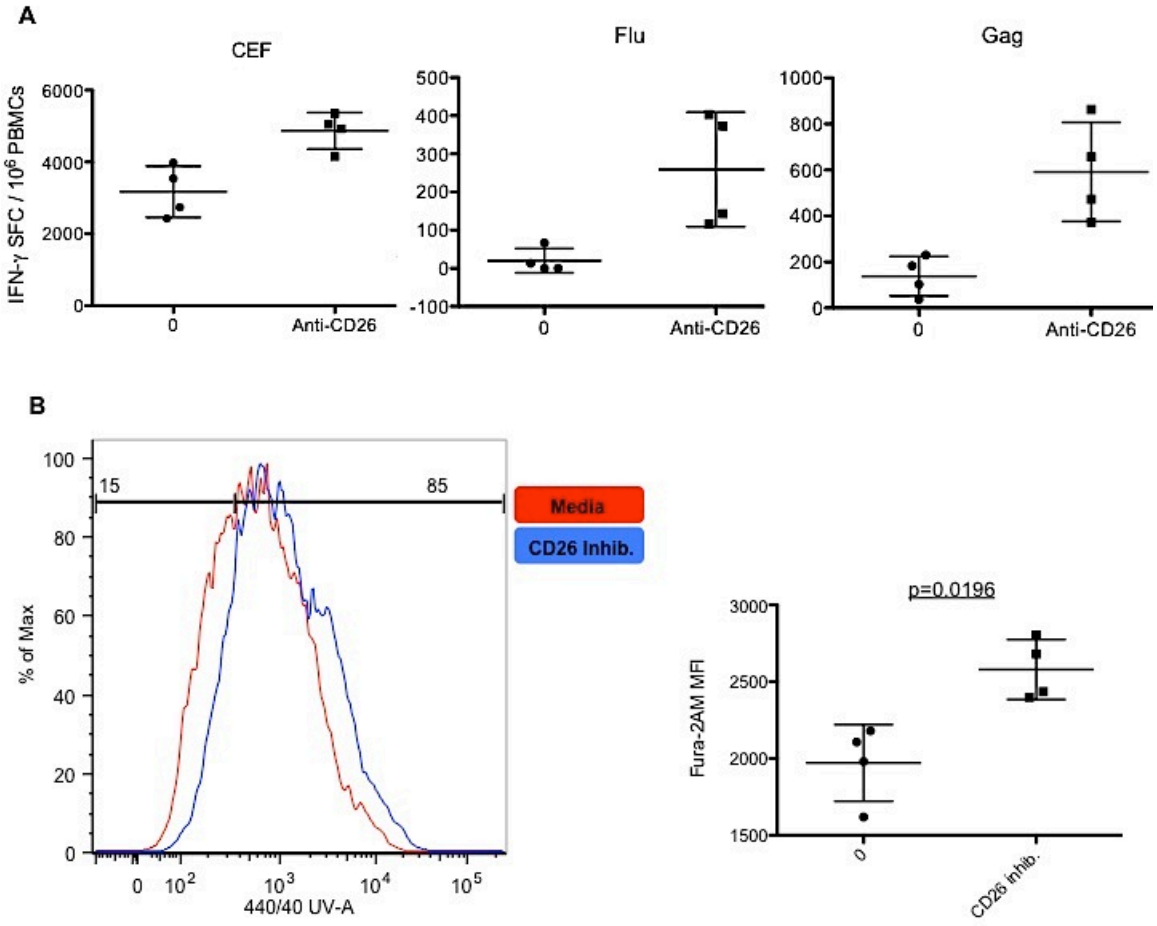


Figure 4.19. PBMCs from HIV-1 infected subjects on ART were treated with 500pg/ml of a CD26 inhibitor for 24 hours. This treatment led to a increase in **A)** IFN-γ production in response to CEF (n=4; p=0.0146) and flu protein (n=4; p=0.0387) and gag (n=4; p=0.0392) antigen stimulation and **B)** the calcium response. (left panel example of histogram illustrating Fura-2, AM mean fluorescence intensity of PBMCs treated with media alone (Red) and CD26 inhibitor [blue]). Right panel compiled calcium responses (n=4; p=0.0196). Graphs represent mean and standard deviation.

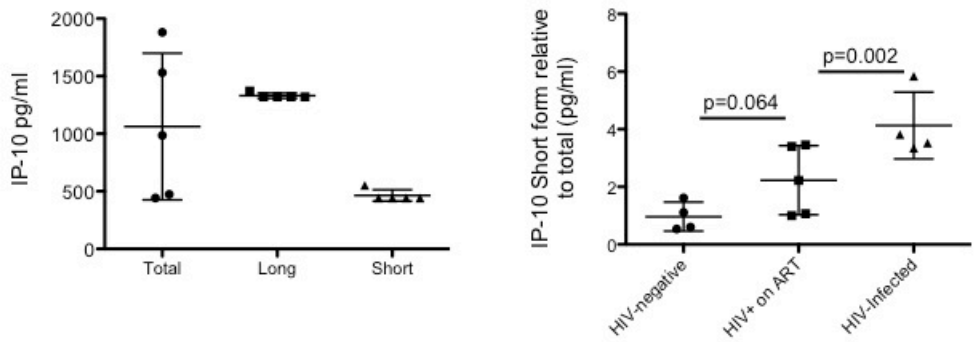


Figure 4.20. Left panel: Plasma levels of Total IP-10 (1062±637.0pg/ml), Long form of IP-10 (1330±22.4pg/ml), and short form of IP-10 (464.8±48.8pg/ml) in HIV-1 infected subjects on stable ART. Right Panel: Plasma IP-10 short form (pg/ml) relative to total in healthy HIV-negative subjects, HIV-1 infected subjects on stable ART, and untreated HIV-1 infected. A one-way ANOVA followed by bonferroni's multiple comparison test was used.

4.4 Discussion

i. IP-10 and T-cell Function

As previously discussed we found that IP-10/CXCL10 was elevated in the sera of HIV-1 infected subjects on ART compared to healthy controls. Additionally, while the serum levels of IP-10 are highest in untreated HIV-1 infected subjects, even with ART, the levels remain elevated compared to healthy uninfected individuals. In this chapter we investigated the impact that these elevated serum levels of the pro-inflammatory chemokine, IP-10, have on HIV-1 infected subjects on stable antiretroviral therapy. The data presented here demonstrates that at elevated levels, IP-10 can blunt T-cell function. Firstly, we found that exposure to elevated IP-10 led to a decrease in the ability of PBMCs from both HIV-uninfected subjects and HIV-1 infected subjects on ART to produce IFN-γ in response to stimulation with recall antigens. In other words, these findings suggest the potential impact elevated IP-10 may have on the ability of these individuals to respond to vaccines. Even more, IP-10's role in other co-morbidities/opportunistic infections, such as type 1 diabetes and hepatitis C virus infection[130, 155], that also affect those infected with HIV-1, marks it as a potential therapeutic target.

In addition to the observed impact of IP-10 on IFN-γ production, we found that IP-10 can also affect the ability of lymphocytes to produce TNF-α, IL-10, somewhat GM-CSF, while we observed no significant effect on the production of other cytokines previously shown to be part of the polyfunctional antiviral response, specifically, IL-2 and MIP-1β. Importantly, we also observed that treatment with IP-10 led to a stunting of the proliferative capacity of lymphocytes from HIV-1 infected subjects on ART in response to stimulation with recall antigens. While definitive correlates of protection are yet to be

established for control of HIV-1 infection, several functions, which include production of IL-2, MIP-1β, IFN-γ, TNF-α, degranulation and production of cytotoxins, and proliferative capacity are suggested to correlate with better control of HIV-1 infection. Likewise, these functions are more prominent in HIV-1 infected non-progressors[40, 45, 49, 53]. Similarly, polyfunctional CD8+ T-cells responses are also seen during other infections, which include influenza, CMV, and EBV[235, 236]. Hence, if elevated levels of IP-10 lead to blunting of these functions, that in turn could impact the ability of ART-treated individuals to elicit protective responses against non-HIV infections.

Elevated serum levels of IP-10 are also associated with more rapid disease progression and HIV-1 viral replication[12, 134]. Conversely, reduced levels of IP-10 are suggested to be protective from acquisition of HIV-1 infection[138]. As shown in this chapter, if elevated levels of IP-10 lead to blunting of important T-cell functions, blocking IP-10 should lead to enhancement of T-cell function. Moreover, we show that not only do HIV-1 infected subjects on ART have elevated serum IP-10 levels, but upon antigen stimulation the levels of IP-10 increase significantly, potentially exacerbating the observed issues further. Indeed, when we block IP-10 using a neutralizing antibody we observe significant improvements in the production of IFN-γ, IL-10, GM-CSF, TNF-α, IL-12p70, IFN-α2, and IL-13. Of these cytokines IFN-γ, TNF-α, IL-12p70, and IFN-α2 are involved in IP-10 signaling[9], so at high levels, IP-10 may serve to regulate its inducers. Studies in mice, show that mice deficient in IP-10, but not MIG, have an increase in effector cells capable of producing IFN-γ[237]. As well, Bromley *et al*.[127] show that signals provided by IP-10 may dominate signals from the TCR. Thus, blocking IP-10 may improve T-cell functionality. Further, when examining functions shown to be important in viral control in HIV non-progressors, we see that blocking IP-10 leads to an enhancement in the ability of lymphocytes from HIV-1 infected subjects on ART to

proliferate in response to recall antigens. However, due to the antigens used in this study, specifically Flu proteins and CEF peptides, could suggest effects of IP-10 on other PBMC subsets, such as antigen-presenting cells since Flu protein has the potential to signal to dendritic cells directly through toll-like receptor stimulation for example. On the other hand CEF peptides could support a direct effect on T-cells due to their mechanism of presentation and signaling. Also, we demonstrate that using a neutralizing antibody against IP-10 can enhance the ability of CD8+ T-cells from HIV-1 infected subjects on ART to degranulate and produce perforin and granzyme B. These results suggest an enhancement in the ability of T-cells to potentially better control HIV-1 infection.

ii. Proposed model of action of elevated IP-10 levels

The results presented in this chapter also led us to propose a potential model for a mechanism of action for elevated serum levels of IP-10. First, we investigated whether IP-10 was acting by exerting its effects directly or indirectly on T-cells. We found that exposure to elevated levels of IP-10 could directly impact IFN-γ in CD4+ and CD8+ T-cells. Interestingly, we did not see an effect of IP-10 on the CCR5 ligand MIP-1β. Previous virological studies in mice suggest that since CXCR3 tends to be expressed on similar cells as CCR5, when one of the receptors is knocked out, infection is not as severe as knocking both out. These findings would suggest a potential redundant role of dual CXCR3/CCR5 expression on T-cells, and thus suggesting a potential reason for the lack of an effect of IP-10[238]. Second, one of IP-10's functions is in the regulation of calcium mobilization[9, 122]. Accordingly, we examined the impact of elevated levels of IP-10 on the calcium response and found that IP-10 blunted the calcium response. It is possible that constant signaling by IP-10 due to its elevation could lead to depletion of calcium reservoirs. Contrarily, previous researchers have demonstrated that IP-10 when in its antagonistic form can blunt the calcium response; we then sought to examine this

further. Doing this, we found again that IP-10 blunted the calcium response of CD4+ and CD8+ T-cells directly. Additionally, these findings point out that high levels of IP-10 affect the calcium responses after CD3 crosslinking. This could be due to the synergistic signaling between CXCR3 and CD3[242]. Third, we explored potential intracellular signaling proteins involved in IP-10/CXCR3 signaling. We specifically found a significant decrease in the phosphorylation of the p38 MAP kinase. Gratton *et al.*[239] illustrate that in order to potentially avoid damage from multiple inflammatory signals, p38 can be down-regulated in order to ensure cyto-protection. Thus, it is possible that at elevated levels IP-10 binding to CXCR3 helps to throttle back the T-cell response upon activation with antigen. Also, as is shown, STAT-1 phosphorylation on tyrosine-707 is increased, but down-regulation of the calcium and p38 signals could impact whether STAT-1 is phosphorylated on its serine-727 site, important for its function and can thereby possibly contribute to the observed dampening of T-cell function[240, 241]. However, further research is needed to understand the mechanisms of IP-10 on these pathways. Fourth, we explored whether at elevated levels, IP-10 was leading to cell death. We found that treatment of PBMCs from both HIV-uninfected and HIV-1 infected individuals on ART with IP-10 did not affect cell viability, suggesting that other potential mechanisms may explain IP-10's role in T-cell function. It is possible that IP-10 could affect the expression of MHC complex molecules, which would affect normal TCR signaling. Furthermore, down-regulation of MHC Class molecules could leave cells vulnerable to NK cell targeting[242]. However, exposure to elevated levels of IP-10 did not affect the expression of HLA Class-I (A, B, and C) and HLA-DR on T-cells or macrophages. Similarly, expression of PD-1 on T-cells, is associated with blunted function in those infected with HIV-1[234]. Nevertheless, we found no change in the expression of PD-1 on CD4+ and

CD8+ T-cells after treatment with IP-10. And we saw no effect of blocking IP-10 on the expression of PD-1. These findings suggest alternative mechanisms for IP-10's effects.

Research by others shows that high levels of IP-10 correlate with non-responsiveness to HCV therapy and suggest a potential mechanism of action[130]. These researchers found that IP-10 is cleaved by the amino-peptidase CD26 (DPPIV) to a short antagonistic form. This antagonistic form can still bind CXCR3, but acts as a competitive antagonist[128]. Our findings suggest that similar to HCV infection, the elevated IP-10 observed in HIV-1 infected subjects on ART may be processed to its short form and in turn block normal signaling via CXCR3. In fact, we found that using an antagonist against CXCR3 led to similar results as the elevated levels of IP-10, that is, we observed a decrease in IFN-γ production and a blunted calcium response. Additionally, blunted signaling via CXCR3 could reduce the synergistic effect between CXCR3 and CD3ε signaling[243]. This could therefore lead to a reduction in T-cell functions such as IFN-γ production and reduction in phosphorylation of p38. Moreover, we examined the serum soluble and surface expression levels of CD26. We demonstrate that HIV-1 infected subjects on ART have higher serum soluble CD26 compared to healthy HIV-uninfected controls. Similarly, we observed that exposure to elevated levels of IP-10 led to an increase in the surface expression of CD26 on CD4+ and CD8+ T-cells. Even more, when we used an inhibitor of CD26, we found an improvement in the ability of HIV-1 infected subjects on ART to produce IFN-γ and elicit a calcium flux response. Finally, when we examined the short form of IP-10, we found that relative to total IP-10 levels, untreated HIV-infected subjects had the highest, followed by HIV-1 infected subjects on ART, and then by healthy HIV-negative subjects, suggesting a potential role for IP-10 short in HIV-1 infection.

With these findings we propose a potential model for IP-10's impact on T-cell function in HIV-1 infected individuals on ART. We propose that at elevated levels, IP-10 is in an environment that has high levels of CD26. By the same token, upon cell activation, either through infection or vaccination, the surface expression of CD26 on cells can potentially increase as well as the production of IP-10. In this environment it is possible that IP-10 can come in contact with CD26 and be processed to its short form. As a way to potentially reduce unnecessary damage from multiple inflammatory signals, the short form of IP-10 provides antagonistic signals that help dampen pathways and functions that can possibly contribute to more immune activation and eventual T-cell turnover. Indeed, it is shown that excessive calcium release can lead to apoptosis[244]. Therefore the short form of IP-10 can stunt the calcium response to avoid apoptosis of T-cells. As well, its been shown that while the antagonistic form of IP-10 reduces calcium signaling and chemotaxis, IP-10's angiostatic functions are maintained[245]. This would allow for IP-10 to limit further recruitment of activated cells and in turn control excessive inflammation to avoid further damage. However, in the context of trying to elicit protective T-cell responses with vaccines, IP-10 short's "protective" role could lead to reduced responsiveness to vaccines. In regards to this, it may be necessary to target IP-10 or its antagonistic form in order to enhance vaccine responses in HIV-1 infected subjects on antiretroviral therapy.

Limitations of Study

While the findings presented in this study elucidate a potential role for IP-10 in affecting T-cell function in HIV-1 infected individuals further investigation is necessary to better understand IP-10's mechanism of regulation. In this study we touch upon potential CXCR3 signaling pathways that could be affected by elevated levels of IP-10. However,

in addition to further investigating signaling pathways affected, such as NFAT and other STAT pathway members, the luminex assay in this study is limited in detecting phosphorylation of STAT-1 at both its tyrosine as well as serine site. Investigating how STAT-1 is affected could help unravel more intracellular mechanisms involved in IP-10 regulation. Similarly, further studies are necessary to understand the effect seen on p38 and its pathway members to see if downregularion is seen in other instances. Furthermore, CXCR3 is expressed primarily on activated and memory T-cells, thereby it may be necessary to investigate whther high levels of IP-10 can impact normal memory responses and differentiation. As well, this study would further benefit from examining CD26 activity during the presence of high levels of IP-10. In regards to the isoforms of IP-10, the 3-plex luminex assay is limited in its ability to account for all total IP-10 levels. This could be due to the need for better capture antibodies, as well as the fact that IP-10 is processed by other aminopeptidases thereby underestimating the levels of total IP-10[130]. Finally, this study is limited in the inaccessibility to obtain the antagonistic form of IP-10 to use in directly investigating its role in T-cell function.

CHAPTER 5:

Discussion

5.1 Significance

Developing an effective prophylactic or therapeutic vaccine against HIV-1 remains an important task in which traditional vaccine approaches have failed. Even more vaccine trials, such as Merck's STEP trial[246], led to disappointing results for the T-cell based vaccine field. Nonetheless, research involving HIV-1 non-progressors and non-pathogenic SIV studies continues to reveal insights into the pathogenesis of HIV-1 infection and potential immune correlates of protection[247]. Additionally, successes in trials like the RV144 trial have shown the potential of eliciting humoral immunity with some efficacy[248]. However, the field of HIV-1 vaccines continues to be a swinging pendulum between T-cell based vaccines and antibody-based vaccines. Rather, these studies demonstrate how crucial it is to understand the role that both arms of the immune system play and it will probably be necessary to elicit both in order to develop and effective vaccine or therapy.

In addition, while HIV-1 infection remains a major cause of morbidity and mortality worldwide, the tremendous progress in the development of antiviral regimens has helped turn HIV-1 infection from a death sentence to a manageable chronic disease. Yet even with these improvements the HIV-1 viral reservoir still poses a barrier to a cure for HIV-1 infection and the immune systems of these infected individuals continue to deteriorate with age as a result of the damage from HIV-1 infection itself[10, 205]. The studies presented in this thesis examine the ability of individuals on stable antiretroviral therapy to respond to a therapeutic vaccine against HIV-1 as well as investigate potential alternative therapeutic targets or factors that affect the ability of these

individuals to respond to vaccination, one such target being the pro-inflammatory chemokine IP-10.

5.2 Therapeutic HIV-1 Vaccine

With traditional vaccine strategies against HIV-1, the results are troubled with safety (e.g. live vaccines) and efficacy issues. However, the field of DNA vaccines has overcome early setbacks of low immunogenicity. As well, DNA vaccines offer a safer strategy to target pathogens for which a vaccine remains elusive. The question also arises whether a vaccine that is effective in a prophylactic setting is effective in those already infected with the virus as a therapeutic vaccine and vice versa. Specifically, unless an HIV-negative individual is already affected with a chronic illness, their immune systems should be apt to respond properly to an effective prophylactic vaccine. On the other hand, even for those on ART, the damage that HIV-1 infection[14] has caused in some if not all these individuals, can blunt their ability to respond to vaccination. Even more, while the RV144 trial showed us the possible need for broadly neutralizing antibodies, the success was not substantial[249]. Therefore, as Walker *et al.*[168] suggest it is likely that a vaccine strategy that in parallel elicits both a strong T-cell response as well as broadly neutralizing antibodies may be the best bet for preventing or treating those individuals already infected.

In the study in chapter 2, we demonstrated that all individuals in the study responded to at least 1 vaccine antigen. In particular, we found that almost all of the subjects showed positive responses to pol antigen, and more than half demonstrated a positive response to gag. However, half of the individuals did not respond to one of the antigens, env, and only 4 of the subjects responded to all three. So in moving forward

with this vaccine, boosting the immunogenicity and breadth of the vaccine antigens could help to elicit antibody responses as well as stronger CD8+ CTL responses in more subjects. Alternatively, this vaccine trial used the same vaccine for the priming and boosting strategies. It is possible that combining this vaccine in a different prime-boost strategy, such as with a viral vector based strategy could enhance responses[171]. Additionally, while examining peripheral immune responses to vaccines against HIV-1 is a good indication of a systemic response, the virus is shown to predominantly affect the mucosa of individuals before it disseminates to other tissues[5]. Therefore researchers may be under- or over-estimating the impact of their vaccine regimens. It is thus necessary to develop minimally invasive methods of examining these mucosal responses as well in order to move the field forward.

Nevertheless, the vaccine in this study demonstrates firstly, that a DNA-based vaccine can elicit potent cellular responses in HIV-1 infected individuals on stable ART against HIV-1 antigens. Secondly, this trial demonstrates that cellular responses elicited by this vaccine were responses shown to be potential correlates of control of HIV-1 infection in elite controllers, specifically, CD8+ CTL responses. However, in order to examine the potential for control of HIV-1 infection, it is necessary to examine a more direct assay of killing that would investigate if the ability to identify and kill virally infected cells is enhanced. As well, not all subjects demonstrated potent CD8+ CTL responses, thus it is necessary to understand how to better elicit these responses if they are in fact important for the control of HIV-1 infection.

Further, we identified pre-vaccination baseline cytokine and chemokine profiles of these individuals. These results demonstrate potential cytokine or chemokine targets that could aid in improving similar vaccine strategies.

5.3 Sero-protection to Influenza in HIV-1 infected individuals

The 2009 H1N1 pandemic demonstrated that being unprepared for such a severe risk could possibly leave many individuals unprotected and in danger. With an already present risk of influenza in the immune-compromised, including those infected with HIV[180, 189], lack of preparation for such an outbreak may lead to a wasting of vaccine doses, leaving those infected with HIV unprotected still. Specifically, Tebas *et al.*[203] demonstrated that despite being well controlled on ART, 39% of vaccinated individuals do not achieve sero-protection against pandemic H1N1 infection. Likewise, due to lack of sero-protection, other studies discuss the need for administration of higher or multiple doses of influenza vaccines in order to improve rates of sero-protection in HIV-1 infected individuals[66, 200]. On the same note, a follow-up study comparing the administration of a higher than standard dose of the seasonal influenza vaccine, showed that immunizing HIV-1 infected subjects with a higher dose of the vaccine improved sero-protection rates[198]. Importantly, our study explored baseline factors that are associated with sero-protection after vaccination with a standard 15µg dose of the H1N1 vaccine (Novartis).

Since levels of T-cell immune activation are shown to be associated with HIV-1 disease progression[216] we expected that baseline levels of T-cell activation would be associated with responsiveness to H1N1 vaccination. However, this was not the case, and instead found a relationship between the baseline frequency of naïve T-cell subsets with responsiveness. It is possible that while the levels of immune activation are not directly associated with sero-protection, the damage caused by residual immune activation can contribute to the turnover and erosion of the naïve T-cell subsets, and thus responsiveness to vaccination. Even more, the association between responsiveness and naïve T-cell subsets may be indicative that the *de novo* flu response may be affected. Additionally, our analysis of baseline cytokine and chemokine profiles

exhibited differences in cytokines between Responders and Non-responders, specifically differences in IL-10 and IL-6. Changes in cytokine secretion between Responders and Non-responders can therein affect the type and immunogenicity of the response and ultimately sero-protection. Furthermore, overall cytokine and chemokine dysregulation present in HIV-1 infected individuals on ART can potentially affect responsiveness and T-cell function.

Nonetheless the findings in our study bring up two potential concerns for those infected with HIV. Firstly, the population of well-controlled HIV-1 infected individuals is living longer and aside from the co-morbidities that are seen to arise in these individuals, age itself is further contributing to dysregulation of the immune system and depletion of naïve cell subsets. Regarding age as a potential confounding factor should inform researchers and clinicians as to investigating better vaccine design and/or dosing for those infected with HIV, especially in the event of a future unforeseen pandemic. Understanding how to better protect these individuals can ensure that limited vaccine doses are administered properly so as to ensure higher rates of sero-protection. Secondly, the damage caused by untreated HIV-1 infection can potentially contribute to immune reconstitution issues after the initiation or antiretroviral therapy. As ART regimens have seen significant improvement and enhanced adherence, issues remain with accessibility to therapy and potential side-effects, nonetheless, the obtained benefits are undeniably welcomed. Therefore, recent research has sought to determine the best timing for the initiation of therapy. If initiating ART at earlier stages of infection can lead to better preservation of cell subsets[250] important in responses to vaccines, then in the case of our study, early ART initiation could better preserve the naïve T-cell subset and improve sero-protection rates. However, for those already being treated for ART it may be necessary to investigate alternate strategies to improve sero-protection

rates. Aside from possible benefits to immune reconstitution, studies examining early initiation of ART in those infected with HIV have also seen reduced rates of transmission in sero-discordant couples[18].

5.4 IP-10 as an immune-therapy target

IP-10, is a chemokine that can be beneficial or detrimental depending on the disease, pathogen, and whether the disease is chronic or acute. Basic research on the CXCR3 family of ligands has elucidated the inter-related roles of IP-10, MIG, and I-TAC and demonstrates that their functions can be independent of each other, redundant, and in some cases antagonistic[120]. Yet, in regards to IP-10, recent research has opened a new door that demonstrates the complexity of this 10kDa chemokine. As the name chemokine implies, IP-10 plays an important role in the trafficking of a wide variety of cell types[9]. Knowing this role, it would be expected that a potent chemokine would be associated with better immune responses, as it would help immune cells traffic to the site of infection. Contrarily, as is the case, excessive recruitment of immune cells can lead to increases in inflammation, which if left uncontrolled can become pathogenic. For example elevated levels of IP-10 contribute to chronic inflammation observed in ulcerative colitis[251], in the spinal cord[148], and arthritis[252].

Sadly, a chemokine, like IP-10 that is beneficial for diseases like breast cancer[160], has become an ominous sign of sorts in regards to HCV and HIV-1 infection. Recent research by Casrouge *et al.*[130] and others[253] has offered insight as to why IP-10 is associated with inability to respond to HCV therapy and clear infection. These researchers demonstrated that IP-10 is processed and truncated (2 amino acid truncation) by CD26 to an antagonistic form. These findings have begun to elucidate on the paradoxical effects of IP-10. In our study we investigated the role of elevated levels

of IP-10 in HIV-1 infected individuals on ART, a novel population in which IP-10's effects is not fully explored. We found that at elevated levels IP-10 could lead to blunting of T-cell functions, specifically IFN-γ production aside from others. Likewise, in HCV infection, Riva *et al.*[254] show that in addition to having lower levels of IP-10's antagonistic form, individuals who clear infection have a higher frequency of HCV-specific IFN-γ producing T-cells. Our findings and those by Riva *et al.*[254] suggest an alternate role for IP-10 in potentially regulating inflammation and immune activation. As seen in our study, at elevated levels, we see a blunting of the calcium response and down-regulation of p38 phosphorylation. Reducing such signals, could serve as a possible safety mechanism to avoid excessive damage from inflammatory stimuli. However, when trying to elicit a response to therapy or a vaccine, blunting T-cell function may lead to the inability to achieve adequate immunogenicity. Apart from HCV infection, IP-10's antagonistic form has been implicated in ovarian cancer[161]. Similarly, reduction of IP-10 has seen benefits during influenza infection, and in mouse models[236, 255].

It is therefore necessary to better understand IP-10 in order to develop better ways to target this chemokine. The development of anti-IP-10 antibodies to treat colitis has seen some success[251]. By the same token, the use of statins that help target IP-10, such as atorvastatin, or indirubin has shown benefits in Chron's disease and highly pathogenic influenza infection, respectively[255, 256]. Yet, in regards to infections like HCV, where the antagonistic form plays a role, research is necessary to understand whether it is better to target IP-10 or the antagonist form. Furthermore, as we show in our study, HIV-1 infected individuals have elevated CD26 levels. So instead of targeting IP-10 altogether, it could be beneficial to target CD26, the culprit that processes IP-10. However, CD26 is involved in the processing of other chemokines, such as RANTES and SDF-1[245], so CD26 as a therapeutic target would have to be investigated further.

5.5 Future Directions

In continuing research on the HIV-1 infected population on stable ART there are certain questions that can be further investigated. Current studies are investigating the potential benefits of initiating ART earlier. As so, it would be interesting to compare vaccine responses, to both HIV-1 and non-HIV vaccines, between early and late/standard ART initiators. Additionally, understanding the impact of early ART to immune reconstitution could provide researchers with a better understanding of potential immune correlates for controlling HIV-1. Furthermore, administration of higher doses of the influenza vaccine appear to improve sero-protection, but there remains a subset that do not achieve protection. Examining the differences in these populations could reveal additional factors impacting vaccine responses in HIV-1 infected individuals on ART.

In regards to the development of a therapeutic HIV-1 vaccine, collaborations between research on reservoir eliciting/targeting agents, and the vaccine field could lead to a better approach at targeting and eliminating the viral reservoir. Likewise, concerning vaccine development, efforts from the humoral and T-cell based fields should be combined so as to better understand the impact of a two-arm vaccine approach. Moreover, advances in vaccine technology, particularly DNA vaccines, can aid in improving the immunogenicity of vaccine antigens in order to elicit better HIV controlling responses.

On the other hand, basic research that helps researchers understand the impact from the dysregulated cytokine and chemokine environment is important. Understanding the roles IP-10 can play in the immune response can aid in the creation of immune-

therapies that help reduce negative effects from this chemokine. However, further research regarding IP-10 in the context of HIV-1 infection on ART is still needed. In addition to understanding the potential role that IP-10's antagonistic form may play in this patient population, better understanding of IP-10's receptor, CXCR3, is also necessary. CXCR3 itself has 3 isoforms that can have normal or inhibitory functions, so exploring their expression and distribution could elucidate on other CXCR3 based mechanisms of control[120]. By the same token, there exist populations of CXCR3 expressing regulatory T-cells that can traffic in response to IP-10, so it is possible they may also play a role in regulating immune responses dependent on IP-10[120, 257, 258]. Yet, we found that high levels of IP-10 affected the secretion of IL-10, an immune-regulatory cytokine. It is possible that even if CXCR3+ T-regs are being recruited, their functionality too may be affected. Alternatively, IP-10 is shown to have alternate binding sites, such as glycosaminoglycans[259], which are shown to potentially affect the proliferation of epithelial cells. So investigating whether this binding plays a role in T-cell function could elucidate on alternate forms of IP-10 regulation. Finally, early studies by Bromley *et al.*[127], demonstrate a possible role for IP-10 and the development of the immunological synapse during antigen presentation. Understanding whether IP-10 or its antagonist form disrupts this interaction could lead to finer targeting of IP-10. In moving forward, our lab is looking to the development of an anti-IP-10 agent that could aid in enhancing T-cell function in those infected with HIV-1 on ART, as well as understanding the role IP-10 plays in other diseases by using *in vivo* mouse models.

Chapter 6:

Materials and Methods

6.1 Therapeutic HIV-1 DNA Vaccine Study

i. Study Participants

This study was an open label, Phase I trial conducted at one center in the United States (clinicaltrials.gov registration NCT01082692). The study protocol was approved by an Institutional Review Board and adhered to the guidelines of Good Clinical Practice and the Declaration of Helsinki. Written informed consent was obtained prior to study enrollment. Adult HIV-1 infected male and female subjects eligible for participation were between 18-55 years of age, currently receiving a highly active antiretroviral therapy (HAART) regimen, undetectable plasma viral loads (<75 copies/mL), $CD4^+$ lymphocyte counts >400 cells/μL, and nadir $CD4^+$ lymphocyte counts >200 cells/μL. These values must have been documented on two separate occasions within 60 days of study enrollment. Female subjects of reproductive potential must not have been pregnant or nursing and have had a negative serum pregnancy test within 30 days of study entry as well as a negative urine pregnancy test on the day of the first immunotherapy dose. Major exclusionary criteria included any past or active AIDS-defining illness, malignancy requiring chemotherapy, autoimmune disease, or receipt of other immunomodulatory therapy within 4 weeks of study entry.

ii. Study Design

Subjects received a four doses of the PENNVAX®-B (gag, pol, env) immunotherapy delivered intramuscularly into the deltoid muscle followed immediately by electroporation (EP) with the CELLECTRA® 2000 Adaptive Constant Current device

(3 pulses of 52ms duration at 0.5A). Each 0.75 mL dose contained 3 mg of DNA encoding Clade B consensus sequence HIV-1 gag, pol, and env expression plasmids in equal proportions. Therapeutic immunization followed by EP occurred on Day 0 (1st dose), Week 4 (2nd dose), Week 8 (3rd dose), and Week 16 (4th dose). Blood collection for immunogenicity and virologic assessments was performed during screening, at Day 0 (prior to dose), and weeks 4 (prior to dose), 8 (prior to dose), 10, 16 (prior to dose), 18, 24, and 48 (discharge visit).

iii. Safety Assessment

Local and systemic injection site reactions, including pain, tenderness, erythema, and edema, were assessed within 30 minutes post-dose on the day of each immunization as well as 2 weeks later. All local injection site reactions were graded according to severity (in accordance with the 2004 AIDS Table for Grading Adult Adverse experiences) where grade 1 = mild (minimal pain and/or tenderness, erythema or edema < 15cm x 15cm); grade 2 = moderate (notable pain and/or tenderness, erythema or edema ≥ 15cm x 15cm); grade 3 = severe (extreme pain and/or tenderness, ulceration, superinfection or phlebitis); and grade 4 = potentially life-threatening (necrosis of the skin). Adverse events were monitored via telephone follow-ups 24 hours after the dose, as well as during in-person visits continuously over the course of the study.

iv. IFN-γ ELISpot

We used ninety-six-well nitrocellulose membrane plates specific for human IFN-*γ* (MABtech, Nacka, Sweden) to examine IFN-*γ* production by PBMCs. PBMCs were either stimulated with media alone, consensus sequence HIV-1 gag, pol, or env

peptides. HIV-1 consensus sequence subtype B peptides (2ug/mL) were combined with $2x10^5$ cells (100ul) per well (triplicate). PBMCs from each time-point (Screening, day 0, week 4, 8, 16, 18, 24, and 48) were assessed. PMA/Ionomycin (0.02ug/mL and 2ug/ml respectively) was done in triplicate as positive controls. Plates were incubated for 18-24 hours. Plates were then washed and detector monoclonal antibody was added for 2 hours followed by washing. Plates then had streptavidin-ALP added for an hour followed by washing and finally substrate was added until reaction was stopped and plates were washed and dried overnight prior to analysis using ImmunoSpot plate reader. The average number of SFC counted in R10 wells was subtracted from the average in individual HIV peptide wells and then adjusted to 1×10^6 PBMCs for each HIV peptide pool. Positive Responses were determined using a one-way ANOVA followed by Dunnett's test, comparing each time-point to baseline.

v. Flow Cytometry

Intracellular cytokine staining was performed as previously described[260]. We measured the potential of cells to express functions shown to lyse HIV-1 infected cells by measuring CD8+ CD107a+ Perforin+ Granzyme B+ responses. Antibody fluors are as follows: BD Biosciences: CD14, CD16 (PacBlue), IFN-γ (FITC), CD3 (APC-Cy7), CD4 (PerCP-Cy5.5), CD8 (APC), CD45RO (AF700), CD107a (PE-Cy7), Abcam: Perforin (PE; clone B-D48), Invitrogen: CD19 (PacBlue), Granzyme B (PETexas-Red), Ebiosciences: CD27 (PE-Cy5). Staining for CD107a+ and stimulation (gag, pol, env, SEB) was done as previously described[260]. Cell surface staining was done at 4°C for 30-45min using anti-CD14, anti-CD16, anti-CD19, anti-CD3, anti-CD4, anti-CD8, anti-CD27, and anti-CD45RO. Intracellular staining was done following permeabilization at 4°C for 45min-1h using anti-perforin, anti-granzyme B, and anti-IFN-γ. Subjects' responses were

considered positive if their CD8+ CD107a+ Perforin+ Granzyme B+ responses were >0.05% after background subtraction. Gating was done as shown in **figure 2.6**. Prepared cells were acquired using the LSR II flow cytometer equipped with BD FACSDiva software (BD Biosciences). Acquired data was analyzed using the FlowJo software.

vi. Luminex

A Luminex cytokine/chemokine assay (Millipore) was used to examine serum from 15 HIV-1 infected subjects on ART and 10 healthy HIV-negative controls. The assay examined the following serum cytokine/chemokine levels: IL-12p40, IL-12p70, IFN-γ, TNF-α, IL-10, IL-4, IL-5, IL-6, IL-13, IL-2, IL-7, IL-15, G-CSF, GM-CSF, VEFG, TGF-α, EGF, IP-10, MIP-1α, MIP-1β, IL-8, MCP-1, Fractalkine, Eotaxin, IL-17A, IL-1ra, IL-1α, IL-1β, sCD40L. Cytokine/chemokine profiles were compared between the HIV-1 infected individuals on ART and the HIV-negative controls using an unpaired t-test.

6.2 Sero-protection after H1N1 Influenza vaccination

i. Vaccine

Subjects received a single 15μg dose of the monovalent, unadjuvanted, inactivated, split virus H1N1 vaccine (Novartis, Basel, Switzerland). Each participant had baseline studies performed at the time of enrollment followed by the intramuscular administration of the 2009 H1N1 influenza vaccine (0.5 mL) to one of the deltoid muscles, followed by 2 phone calls and serological response evaluations completed 21-28 days after vaccination.

ii. Subjects

All subjects provided informed consent and the study was approved by the University of Pennsylvania institutional review board. HIV-1 infected individuals, older than 18 years of age that had an indication to receive the H1N1 vaccine were included in the study. Individuals with a known allergy to eggs or other components of the vaccine, a history of severe reactions to previous immunization with seasonal flu, known cases of H1N1 influenza during the spring of 2009 or previous recipients of the novel H1N1 vaccine were excluded. Additionally, subjects were excluded if they had received other licensed live vaccine within 4 weeks of study entry or inactivated vaccines within 1 week of study entry. Other exclusionary criteria included subjects receiving: experimental treatments (other than phase III antiretroviral trials), systemic chemotherapy for the past 36 months, steroids, immunomodulators, or history of Guillain-Barre syndrome. A total of 120 subjects were included in the study that has been previously presented by Tebas *et al.*[203]. All patients provided informed consent. Forty-six of the 120 subjects had frozen PBMCs available to use for the purpose of this study. These 46 subjects had baseline HAI titers <40, were all on anti-retroviral therapy, their ages ranged from 26-77 with a median age of 48, 69.6% were male, 63% were black, 10.9% Hispanic/Latino, 23.9% Caucasian, and 2.1% Asian/Pacific Islander. Furthermore, their average CD4+ lymphocyte count was 542 cells/μl ± 306.8 cells/μl, average CD4+ lymphocyte nadir of 193 cells/μl ± 187.2 cells/μl, HIV VL were <400 copies/ml in 90% and 85% below limits of quantification. At week 3, 61% of the subjects met the guidelines for protection.

iii. Hemagglutination inhibition assay

Antibody titers of the 120 subjects were measured using a hemagglutination inhibition assay as previously described[214]. The hemaglutination assays were done by McKittrick *et al.*[198] at Bioqual Inc. For the 46 subjects in this study, if their week 3 titer

was greater than 1:40 and had a four-fold increase in their HAI titer, they were classified as sero-protected and responders to vaccination, while those with titers less than 1:40 and/or were less than four-fold increase were classified as non-responders.

iv. Flow Cytometry

Samples from 46 of the 120 subjects were available and were examined for their memory and activation phenotypes using multi-parameter flow cytometry. Antibody fluors are as follows: BD-Pharmigen: CD3 (FITC), CD4 (APC-Cy7), CD8 (AF700), CD27 (APC), HLA-DR (PE-Cy5); Beckman Coulter: CD45RO (ECD); Ebiosciences: CD38 (PE-Cy7); Beckton Dickinson: CD25 (PE); BD Horizon: CD127 (V450); Invitrogen: Viability Dye (Aqua). 46 subjects were analyzed and cellular (CD27, CD45RO) and activation (HLA-DR, CD38) phenotypes were compared using an unpaired t-test between responders and non-responders. Cell surface staining was done at 4°C for 30-45min in the dark and gating was done using FlowJo Software as in **figure 3.2** after sample acquisition on the LSR II running BD FACSDiva software (BD Biosciences).

v. Predictors of response

A multi-variate logistic regression model was used to examine the predictors of response, which included viral load, pre-vaccination CD4+ lymphocyte count, CD4+ lymphocyte nadir, age, naïve and terminally differentiated CD4+ and CD8+ T-cells. A multi-variate logistic regression model was also used to examine the effect of age to frequency of CD4+ naïve T-cells and total activated CD4+ T-cells.

vi. Luminex

A Luminex cytokine/chemokine assay (Millipore) was used to examine serum from the 46 HIV-1 infected subjects on ART and 10 healthy HIV-negative controls. The

assay examined the following serum cytokine/chemokine levels: EGF, Eotaxin, G-CSF, GM-CSF, IFNα2, IFN-γ, IL-1α, IL-1β, IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17A, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, TNF-β, and VEGF. Cytokine/chemokine profiles were compared between the HIV-1 infected individuals on ART and the HIV-negative controls using an unpaired t-test. Cytokine/chemokine profiles were also compared between subjects designated as Responders and Non-Responders using and unpaired t-test.

6.3 Impact of IP-10 on T-cell function in HIV-1 infected subjects on ART

i. Patient Samples

Sera and PBMCs, isolated CD4+, and CD8+ T-cells from Healthy HIV-negative subjects, HIV infected untreated subjects, and HIV-1 infected subjects on ART were obtained from the University of Pennsylvania's Human Immunology Core or the Center for AIDS Research. Healthy controls age ranged from 20-55 years of age, with an average of 31. HIV-1 infected subjects on ART were well controlled with VL<50 copies/ml, current CD4 count over 400 cells/μl and CD4 nadir over 200 cells/μl. HIV-1 infected subjects median VL was 16511 copies/ml.

ii. Cell Culture & IP-10 treatment

Recombinant human IP-10/CXCL10 (Biolegend) treatment doses were determined based on the serum levels of IP-10 found in HIV-1 infected subjects on ART in our and other studies[126,134]. PBMCs, or isolated CD4+ or CD8+ T-cells were cultured in media alone (RPMI 1640 with L-glutamine + 10% FBS and 1% streptomycin/penicillin) or media with one of the rhIP-10 doses (500, 10,000, or 100,000pg/ml) for 24 hours. Additionally, in the cases indicated below, PBMCs from HIV-negative of HIV-1 infected

subjects on ART were treated with 1µg/ml of the anti-IP-10 neutralizing antibody (R&D Systems), or 500pg/ml of a CXCR3 antagonist (EMD Millipore), or 500pg/ml of the CD26 inhibitor (Santa Cruz Biotechnology) for 24 hours. These cells were then stimulated with viral antigens or anti-CD3 (Hit3a clone, BD Pharmigen) and used in the ELISpot, flow cytometry, Luminex, and Ca^{2+} flux assays (**figure 4.1**). Average cell viability post treatment with IP-10 was 95.1±3.3% and 89.2±3.2% for the healthy HIV-negative and HIV-infected on ART samples, respectively (**figure 4.2**).

iii. IFN-γ ELISpot

A standard IFN-*γ* ELISpot (MABtech, Nacka, Sweden) assay as previously described was used[262]. Briefly, PBMCs were treated with or without rhIP-10 for 24 hours prior to this assay and then plated in triplicate at $2x10^5$ cells per well. PBMCs from healthy HIV-negative subjects were also treated for 24 hours with a CXCR3 antagonist (500pg/ml; EMD Millipore). PBMCs from HIV-1 infected subjects on ART were also treated for 24 hours with a CD26 inhibitor (500pg/ml; Santa Cruz Biotechnology). Cells were then either stimulated with media alone, CD8+ specific CEF peptides (0.03µg/ml: CMV, EBV, Flu peptides) or influenza proteins (Protein Sciences Corp.: A/Brisbane/59/07, 10µg/ml; A/Brisbane/10/07, 10µg/ml; B/Brisbane/60/08, 10µg/ml). PBMCs from HIV-1 infected subjects on ART were also stimulated with HIV-1 consensus sequence subtype B gag peptides (2µg/ml), in the presence or absence of 500pg/ml IP-10 or ±1µg/ml of the anti-IP-10 neutralizing monoclonal antibody (R & D Systems). PMA/Ionomycin (0.02µg/mL and 2µg/ml respectively) was done in triplicate as positive controls. BCIP/NBT was used to visualize spots. The spots were counted on an ImmunoSpot plate reader. For the healthy HIV-1 negative subjects, IFN-γ production in the absence of IP-10 was compared to that of each dose treatment with IP-10 using a

Friedman Test followed by Dunn's multiple comparison test. For the HIV-1 infected subjects on ART a Wilcoxon matched-pairs signed rank test was used to examine the effect of IP-10 or effect of blocking IP-10 on IFN-γ production. To examine the effect of the CXCR3 antagonist or the CD26 inhibitor on IFN-γ production a paired t-test was used.

iv. Luminex

a. A Luminex cytokine/chemokine assay (Millipore) was used to examine serum from 15 HIV-1 infected subjects on ART, 13 untreated HIV-1 infected subjects, and 10 healthy HIV-negative controls. The assay examined the following serum cytokine/chemokine levels: IL-12p40, IL-12p70, IFN-γ, TNF-α, IL-10, IL-4, IL-5, IL-6, IL-13, IL-2, IL-7, IL-15, G-CSF, GM-CSF, VEFG, TGF-α, EGF, IP-10, MIP-1α, MIP-1β, IL-8, MCP-1, Fractalkine, Eotaxin, IL-17A, IL-1ra, IL-1α, IL-1β, sCD40L. Cytokine/chemokine profiles were compared between the HIV-1 infected individuals on ART and the HIV-negative controls using an unpaired t-test. A Kruskal-Wallis test followed by Dunn's multiple comparison test was used to compare the levels of IP-10 between untreated HIV-1 positive subjects, HIV-1 infected subjects on ART, and healthy HIV-negative controls.

b. Supernatants from stimulated HIV-1 infected lymphocytes on ART with and without IP-10 (500pg/ml) treatment were isolated. A Luminex assay (Millipore) panel was used to assess cytokine secretion and the impact of IP-10 on CD3 (Hit3a clone, BD Pharmigen, 5μg/ml) and CD28/CD49d (1μg/ml) stimulated lymphocytes. The assay examined secretion of: EGF, Eotaxin, G-CSF, GM-CSF, IFNα2, IFN-γ, IL-1α, IL-1β, IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17A, MCP-1, MIP-1α, MIP-1β, TNF-α, TNF-β, and VEGF. Positive controls included in the

Luminex assay ensured that the assay was able to detect the cytokines/markers analyzed. Immunological molecule secretion in response to stimulation in media alone was compared to the PBMCs treated with 500pg/ml of IP-10 or treated with 1µg/ml of the anti-IP-10 NAb using a repeated-measures ANOVA followed by Bonferroni's multiple comparison test.

c. Cell lysates were isolated from HIV-negative PBMCs treated with or without IP-10 followed by stimulation with CEF peptides or influenza proteins. The assay (Millipore) examined the expression of the phosphorylation sites of the following phospho-proteins: ATF2 (Thr71), Erk (Thr185/Tyr187), HSP27 (Ser78), JNK (Thr183/Tyr185), MEK1 (Ser222), MSK1 (Ser212), STAT1 (Tyr707), c-Jun (Ser73), p38 (Thr180/Tyr182), p53 (Ser15). To examine the fold change in the mean fluorescence intensity (MFI) of phosphorylated proteins, the media alone condition was divided from that of the IP-10 treated condition. Following this, a Wilcoxon matched-pairs signed rank test was used to investigate whether IP-10 treatment led to a significant change in expression of the phosphorylated proteins of interest.

d. *IP-10 3-Plex*: Plasma samples from healthy HIV-negative subjects, HIV-1 infected subjects on ART, and untreated HIV-1 infected subjects were sent to Myriad-RBM. Plasma samples were then run on their IP-10 3-plex assay to asses the levels of Total, Long, and Short form of IP-10. IP-10 short form levels relative to Total IP-10 levels were then compared using a One-way ANOVA followed by Bonferroni's multiple comparison test.

v. Flow Cytometry

a. *IFN-γ expression:* A multi-parameter flow cytometry panel was used to examine the effect of IP-10 treatment (500pg/ml) had on IFN-γ expression in total

PBMCs and isolated CD4+ and CD8+ T-cells. The antibodies are as follows: BD Biosciences: CD14, 16 (PacBlue), CD4 (PerCp-Cy5.5), IFN-γ (FITC), CXCR3 (APC); Biolegend: CD3 (APC-Cy7), CD8 (APC); Invitrogen: CD19 (PacBlue), LIVE/DEAD Violet. To determine the background expression of IFN-γ, a no stimulation control was used. Total PBMCs, CD4+ T-cells alone, and CD8+ T-cells alone were stimulated with anti-CD3 (Hit3a clone) and co-stimulatory antibodies, CD28 and CD49d, as previously discussed by Betts *et al.*[260]. To examine the change in IFN-γ expression, the media alone condition was compared to that of the IP-10 treated condition. Following this a paired t-test was used to examine whether IP-10 treatment led to a significant change in IFN-γ expression.

b. *CD8+ Cytotoxic Panel:* A multi-parameter flow cytometry panel was created to examine the effect of blocking IP-10 using a neutralizing anti-IP-10 antibody (R & D Systems) on the ability of CD8+ T-cells to degranulate and produce perforin and granzyme B. PBMCs from HIV-1 infected subjects on ART received ±1μg/ml anti-IP-10 mAb and ± consensus sequence subtype B gag peptides (2μg/ml) for 24 hours. These PBMCs were then used for the multi-parameter flow cytometry assay as described by Betts *et al.*[260]. The antibodies are as follows: BD Biosciences: CD14, 16 (PacBlue), CD107a (PE-Cy7); Biolegend: CD3 (BV510), CD8 (BV570); Invitrogen: CD19 (PacBlue), Viability Dye (Violet), Granzyme B (PE-Texas Red); Ebiosciences: CD4 (PE-Cy5.5); Abcam: Perforin (PE). A Friedman Test was used to examine the effect of blocking IP-10 on CD8+ triple positive degranulation and perforin/granzyme B production.

c. *Ca^{2+} Flux assay:* 1) PBMCs or CD4+ or CD8+ T-cells alone from HIV-negative individuals were cultured as discussed above in the presence (500 or 100,000pg/ml) or absence of rhIP-10 (Biolegend) for 24 hours.

2) Or PBMCs from healthy HIV-negative subjects were treated for 24 hours with a CXCR3 antagonist (500pg/ml; EMD Millipore).

3) Or PBMCs from HIV-1 infected subjects on ART were treated for 24 hours with a CD26 inhibitor (500pg/ml; Santa Cruz Biotechnology).

Following this, these PBMCs, CD4+ or CD8+ T-cells were washed and placed in a calcium containing solution (140mM NaCl, 4.5 mM KCl, 2 mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES pH=7.4, 10mM D-glucose) and were loaded with Fura-2, AM (3uM, Invitrogen) for 30-45 minutes at room temperature. Fifteen minutes prior to analysis on the LSRB, mouse anti-human CD3 (50ng/ml, OKT3, eBioscience) was added to allow for stimulation via CD3 crosslinking using a purified goat anti-mouse IgG polyclonal antibody (Biolegend) against the anti-CD3 antibody. At the time of analysis on the LSRB, control groups containing cells only, cells in calcium free solution, and cells + fura-2, AM were run to establish background and baseline fluorescence of the fura-2, AM. Change in fluorescence intensity from media alone was examined using a Friedman Test to determine whether treatment with IP-10 led to a change in the calcium response in PBMCs, CD4+ and CD8+ T-cells alone. To examine the change in mean fluorescence intensity from media alone compared to effect of the CXCR3 antagonist or CD26 inhibitor a paired t-test was used.

d. *T-cell proliferation*: frozen PBMCs from HIV-1 infected subjects on ART were treated with media alone, or 1µg/ml of anti-IP-10 NAb for 24 hours. Following this, cells were incubated with CFSE (2.5µM) for 5 min at room temperature. Cells were washed and incubated with media alone, CEF peptides (0.03µg/ml), influenza proteins (Protein Sciences Corp.: A/Brisbane/59/07, 10µg/ml; A/Brisbane/10/07, 10µg/ml; B/Brisbane/60/08, 10µg/ml), gag peptides (2µg/ml), or Concanavalin A (ConA; positive

control) for 5 days at 37°C in 96-well plates. Cultures with medium alone were used to determine background proliferative responses. PBMCs were stained with the following mAbs: BD Biosciences: CD3 (APC-Cy7), CD4 (APC), and CD8 (PE-Cy7). Stained and fixed cells were acquired on the LSRII and analyzed using FlowJo software. The mean fluorescence intensity of CFSE was used to determine T-cell proliferative responses. A Wilcoxon matched-pairs signed rank test was used to examine the percentage of CFSE dim cells in media alone and 1μg/ml anti-IP10 within each of the antigen stimulation conditions.

e. *Multifunctional T-cell Panel*: A multi-parameter flow cytometry panel was used to examine what effect IP-10 (500pg/ml) or anti-IP-10 (1μg/ml) treatment had on IL-2, MIP-1β, TNF-α, and PD-1 expression in total PBMCs from HIV-1 infected subjects on ART. The antibodies are as follows: BD Biosciences: CD14, 16 (PacBlue), CD4 (PerCp-Cy5.5), MIP-1β (FITC), CD3 (APC-Cy7), IL-2 (PE-Cy7); Biolegend: CD8 (BV650), PD-1 (BV711), TNF-α (APC); Invitrogen: CD19 (PacBlue), LIVE/DEAD Violet. To determine background expression, a no stimulation control was used. Total PBMCs, were stimulated with anti-CD3 (Hit3a clone, BD Pharmigen, 5μg/ml) and co-stimulatory antibodies, CD28 and CD49d (1μg/ml), as previously discussed by Betts *et al.*[258**260**]. To examine the change in IL-2, MIP-1β, TNF-α, and PD-1 expression, the media alone condition was compared to that of the IP-10 (500pg/ml) or anti-IP-10 (1μg/ml) treated conditions. A Friedman test followed by Dunn's multiple comparison test was used to examine whether IP-10 or anti-IP-10 treatment led to a significant change in IL-2, MIP-1β, TNF-α, and PD-1 expression.

f. *MHC down-regulation*: frozen PBMCs from healthy HIV-negative controls were treated with media alone or 500pg/ml of IP-10 for 24 hours. Following this PBMCs were

stained with the following mAbs: BD Biosciences: CD3 (APC-Cy7), CD4 (PerCp-Cy5.5); Biolegend: CD33 (BV711), CD8 (BV650), CD68 (APC), HLA-DR (PE); Sigma-Aldrich: HLA Class-I (FITC); Invitrogen: Viability Dye (Violet). Media alone was then compared to IP-10 treated PBMCs using a Wilcoxon matched-pairs signed rank test.

g. *CD26 Surface Expression*: A multi-parameter flow cytometry panel was created to examine the effect of IP-10 treatment on CD26 (DPPIV) surface expression on T-cells. Frozen PBMCs from healthy HIV-negative controls were treated with media alone or IP-10 (500 or 100,000pg/ml) for 24 hours. Following this PBMCs were stained with the following antibodies: BD Biosciences: CD3 (APC-Cy7), CD8 (APC), CD4 (PerCP-Cy5.5), CD26/DPPIV (FITC), CD14, 16 (PacBlue); Invitrogen: CD19 (Pacblue), Live/Dead Violet. Media alone was then compared to IP-10 treated (500 or 100,000pg/ml) PBMCs using a Friedman Test.

vi. ELISA

We used a standard ELISA kit (Millipore) to quantify soluble CD26 levels from sera of 15 HIV-1 infected subjects on ART and 10 healthy HIV-negative individuals. Preparation of reagents and protocol was followed according to manual instructions. Color intensity (absorbance) was then measured at 450nm on an ELISA microwell reader. The absorbance values of the HIV-1 infected subjects on ART were compared to those of the HIV-negative subjects using an unpaired t-test.

CHAPTER 7:

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